Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 25(6): 1 – 16 (2021)
www.ejabf.journals.ekb.eg

Purification and Characterization of a Thermostable β-Mannanase from Halophilic Aspergillus terreus strain ARSA Associated to a Mangrove Plant of Red Sea Coast, Egypt, and its Application in Mannooligosaccharides Production and Juice Clarification

Aida Farag [1], Aliaa El-Borai *[2], Rana Morsy [2], Samy El-Assar [2]

1; Marine Biotechnology and Natural Product Extract Department, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt

2; Botany and Microbiology Department, Faculty of Science, Alexandria University, Egypt.

*Corresponding Author: aliaa_elborai2007@yahoo.com

ARTICLE INFO

Article History:
Received: Aug. 9, 2021
Accepted: Nov. 19, 2021
Online: Dec. 13, 2021

Keywords:
Aspergillus,
β-mannanase,
Mangrove,
Purification,
Mannooligosaccharides,
Juice clarification

ABSTRACT

Mangrove-associated fungi are of great significance in industries due to their various and versatile enzyme production. So, this work studies the purification of β-mannanase produced by a halophilic *Aspergillus terreus* strain ARSA (accession No. MN075514) isolated from marine sediment of mangrove, Safaga, Red Sea coasts, Egypt. The enzyme was precipitated by 50% acetone, followed by purification using gel filtration on Sephadex G-100 yielding an active major protein peak showing 6.77-fold purification. The molecular weight of the purified β-mannanase was approximately 48 kDa, determined by a sodium dodecyl sulfate polyacrylamide gel electrophoresis. K_m and V_{max} values were found to be 3.33 mg/ml and 1666.67 U/ml, respectively. The optimum pH and temperature of the purified enzyme were 6.5 and 60oC, respectively. The enzyme was stable from pH 5.0 to 7.5 and partially stable up to 80oC. The effect of activators and inhibitors was studied providing that EDTA, Hg2+, Tween 80, and SDS (10%) strongly inhibited the enzyme activity, while Mg2+, Mn2+, and Na+, enhanced enzyme activity. Mixtures of mannooligosaccharides are resulted from the hydrolysis of locust bean gum by the enzyme. Crude and purified *A. terreus* ARSA β-mannanase gave promising results on fruit juices clarification as on apple juice extraction with a yield of 128.32 and 167.65%, respectively.

INTRODUCTION

Mannans are considered the main components of the hemicellulose fraction of softwoods, thus showing a great distribution in plant tissues (**Liepman *et al*., 2007)**. Cell walls of most plants contain mannan polysaccharides, they may also found as storage polysaccharides in certain species, e.g., the tubers of konjac or locust seeds **(Bååth *et al*., 2018)**. Multiple enzymes as β-mannanase (EC 3.2.1.78), β-mannosidase, β-glucosidase, and α-galactosidase are involved in mannan hydrolysis. They generally split the β-1,4-glycosidic linkages in the backbone of different mannans, yielding mannooligosaccharides, which are hydrolyzed by β-mannosidase to monosaccharides **(Yang *et al*., 2016).**

Microbial β-mannanase (EC 3.2.1.78) has been widely used in many industries, including clarification of fruit juice, coffee industry, brewing, feed additive, pharmaceutical industries, detergents, in pulp and paper industries, oil recovery and well drilling **(Yang *et al*., 2016; Nadaroglu *et al*., 2017; Favaro *et al*., 2020)**. Mannan-degrading enzymes, along with other glycosyl hydrolases, can be applied in the food industries to macerate and clarify fruit juices (**Nadaroglu *et al*., 2017)**. Moreover, oligosaccharides that are used as prebiotics for improving growth of human intestinal microflora, including mannooligosaccharides, are also used as functional food ingredients **(Shukla and Pletschke, 2013).**

The structural mannan, can be degraded by β-mannanase produced by various species of fungi **(Liu *et al*., 2020)**, some *Bacillus* species **(Kim *et al*., 2018)** and actinomycetes **(Pradeep *et al*., 2016)**. Mangrove forests are worldwide distributed on tropical, subtropical, and sheltered coastlines **(Lin *et al*., 2009)**. They are found at the interface between sea and land, a unique and extreme environment **(Ezawa and Tada, 2009)**. Mangrove areas of Egypt are distributed over small sites along the Red Sea coast and the all area of them is relatively small. The recent calculations indicate that mangroves in Egypt are found in about 525 hectares distributed in 28 different locations along Egyptian Red Sea coasts. Their distribution in the southern Red Sea is more continuous than in the northern Red Sea (Aqaba Gulf), giving the evidence that they are confined to a very restricted favorable habitats **(Afefe *et al*., 2019).** Fungi and bacteria make up about 91 % of the total microbial biomass associated to mangroves, while protozoa and algae account for only 2% and 7%, respectively **(Ghizelini *et al*., 2012)**. Mangrove microbes have influential roles in biogeochemical cycles and provide plants/animals essential nutritional sources (**Shiau and Chiu, 2020**). Marine mangrove fungi are considered as an important source of new many enzymes (**Pointing and Hyde, 2000**) and bioactive compounds (**Lin *et al*., 2001**). Endophytes fungi might play role in decomposition when the tissue becomes senescent or die. Hence, they produce enzymes which are necessary for degradation of lignocellulosic materials (**Basheer *et al*., 2018**).

The characteristics of microbial mannan-degrading enzymes appear to vary according to the producing species of microorganism. So, the aim of this study is to purify β-mannanase isolated from halophilic *Aspergillus terreus* ARSA. Also, some biochemical features and uses of the purified enzyme were explored.

MATERIALS AND METHODS

2.1. β-mannanase production by Aspergillus terreus ARSA

The fungal isolate producing β-mannanase, was isolated from marine sediment of mangrove, Safaga, Red Sea coasts, Egypt. The amplified DNA was partially sequenced using the sequencer (a PRISM BigDye Terminator v3.1 Cycle sequencing Kit). The sequencing data obtained utilizing this strategy was 551 base pair. This sequence was compared with those which gave the highest homology using Blast search computer based program (**Supplementary file A)**. The resulting data indicated that the isolate under study was identified as *Aspergillus terreus*. Sequence alignment between the obtained sequence and the most closely related fungal species (*Aspergillus terreus* isolate SAMP 5) is investigated. The obtained similarity was 100%. The nucleotide sequence was deposited to GenBank sequence database and had accession number MN075514. The phylogenetic relationships of identified strain and the closely related strains were also analyzed as shown in **supplementary file (B)**.

A. terreus ARSA culture was maintained on potato dextrose agar (PDA) slants and used for inoculum preparation, while the modified Czapek,s-Dox medium was used to produce β-mannanase **(Arotupin *et al.*, 2013)**. One ml of a spore suspension containing 2×10^6 spore/ml, which was prepared from 5-days old slants of *A. terreus* ARSA, was used to inoculate 50 ml of sterilized medium dispensed in 250 ml Erlenmeyer flasks. The flasks were then incubated for 7 days at 25°C on a rotary shaker at 160 rpm.

2.2. Preparation of crude β-mannanase, β-mannanase assay, and protein determination

The crude enzyme source was prepared from the clear supernatant. To measure β-mannanase activity, one ml of 0.6% Locust Bean gum (LBG) in 0.05 M phosphate buffer, pH 6.0, was mixed with one ml of diluted enzyme solution. The reaction mixture was incubated at 40°C for 20 min in a shaking water bath. 3,5-dinitrosalicylic acid (DNS) technique was used to determine the released reducing sugars as described by **Miller (1959).** One unit of β-mannanase enzyme was considered as the amount of the enzyme which liberates 1.0 µmole of the reducing mannose per ml under the assay conditions. The protein content preparation was estimated according to the method of **Lowry *et al.* (1951)** using bovine serum albumin as the standard.

2.3. Purification of β-mannanase

A two-step procedure comprising precipitation and gel filtering was used to purify the crude enzyme from *A. terreus* ARSA cells.

2.3.1. Partial purification of β-mannanase

The resulting crude enzyme preparation was obtained after centrifugation at 9000 ×*g* for 20 min and acetone was added to give 35, 50, 65, 75, and 85% saturations with continuous overnight stirring, resulting in proteins precipitation. The precipitate was collected by centrifugation at 9000 ×*g* for 20 min, evaporated, and concentrated; the precipitates were dissolved in 50 mM phosphate buffer (pH 6.0). The enzyme solution was dialyzed against the same buffer, then protein assay and β-mannanase activity were measured as described previously **(El-Borai *et al.*, 2013).**

2.3.2. Gel filtration chromatography

Acetone fraction (50%) obtained after dialysis was loaded to Sephadex G-100 column (1.6 cm × 50 cm) that was pre-equilibrated with 0.05M phosphate buffer, pH 6.0. With the same buffer and a flow rate of 0.5 ml/min, the protein was eluted. The collected fractions of β-mannanase were pooled at 4°C, then protein content and β-mannanase activity were measured **(Beltagy *et al.*, 2016; Farag *et al.*, 2018).**

2.4. Characterization of β-mannanase

2.4.1. Determination of molecular weight of purified β-mannanase

To estimate the molecular weight of pure β-mannanase, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method was used (**Laemmli 1970).**

2.4.2. Substrate specificity

It was done by using various substrates as LBG, xylan, carboxymethyl cellulose (CMC), starch, dextran, and carrageenan, prepared in 50 mM phosphate buffer, pH 6.0, 0.6 % (w/v). Substrates were added individually to identical enzyme portions and incubated for 40 min at 40°C, and then the activity of the enzyme was measured for each.

2.4.3. Determination of kinetic constants

The kinetic parameters of purified β-mannanase were determined using different LBG concentrations (1-10 mg/ml) according to **Lineweaver and Burk (1934)** method. The Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) were calculated by analyzing the data in accordance with the Lineweaver–Burk plot.

2.4.4. Effect of pH on β-mannanase activity and stability

Optimal pH was determined by carrying the enzymatic reaction in the range of pH 3.6–10.6 using 50 mM acetate buffer (pH from 3.6 to 5.0), 50 mM phosphate buffer (pH 5.6–7.5), and 50 mM glycine buffer (pH 8.0–10.6). The pH stability of purified β-mannanase was determined by incubating identical enzyme solutions in different buffers in a pH range of 4–10.6 at room temperature for 2 hrs in absence of the substrate and then the residual β-mannanase activity was calculated under optimal conditions.

2.4.5. Effect of temperature on β-mannanase activity and stability

The optimal enzyme temperature was determined by measuring the activity of the purified β-mannanase toward substrate at optimum pH with varying incubation temperatures between 20^{o}C and 80^{o}C. Enzyme thermal stability was also investigated by preheating identical volumes of each enzyme preparation separately without the substrate for different periods of time (15, 30, and 60 min.) at different temperatures (50, 60, 70, and 80°C). In each case, inactivated enzyme solution was used as a control.

2.4.6. Effect of metal ions, inhibitors, and surfactants on β-mannanase activity

To assay the effect of chemicals on enzyme activity, the purified enzyme solution was preincubated with the tested substance for 2 hours at room temperature (at a concentration of 5 mM and 10mM), $MnCl_2$, NaCl, $MgCl_2$, $MgSO_4.7H_2O$, KCl, $ZnCl_2$, Pb acetate, $HgCl_2$, $CdCl_2$, Tween 80, EDTA, and SDS (10%). The residual β-mannanase activity was determined by adding the substrate, and carrying out standard assay conditions. Control was taken as reaction mixture without adding any metal ion or inhibitor.

2.5. Determination of hydrolytic products of LBG by purified β-mannanase by TLC

Reactions were performed by incubating 6.74 U/ml of purified β-mannanase solution at 60°C in 0.1 M Tris–HCl buffer (pH 8.0) with 0.5% (w/v) LBG. Products of hydrolysis from LBG and mannooligosaccharides were separated on 0.2 mm silica gel 60 aluminum plates using ethyl acetate/acetic acid/water (20:10:10, v/v) as a solvent. Sugars were visualized when TLC plates sprayed with 5% (v/v) sulfuric acid in ethanol, then heating at 150°C until color developed **(Hakamada *et al*., 2014)**.

2.6. Fruit juice clarification

For juice preparation, the fruits (peach, apple, apricot, orange, and grapes) were washed, dried, and homogenised. Enzyme solution (2 ml) was applied to 10 g of fruit sample homogenate (by adding 2 ml distilled water in a controlled atmosphere). Fruit homogenates were treated with enzyme at 60°C for 4 hours at a pH of 6.5.The mixture was filtered and the juice volume was measured. Clarified juice yield (%) was calculated as (volume of clear juice * 100) / (volume of sample). All experiments were performed in triplicate **(Nadaroglu *et al*., 2015).**

2.7. Statistical analysis

Results were expressed as mean ± SD; probability values $P < 0.05$ considered as statistically significant. ANOVA test was used to compare between different studied groups followed by Duncan's method to estimate the significance between each two groups.

RESULTS

3.1. Purification of β-mannanase produced from A. terreus ARSA

Enzyme purification started by fractional precipitation with acetone (35, 50, 65, 75, 85%). The enzyme activity and protein recovery were found to be maximum in the 50 % acetone fraction. After precipitation with 50% acetone fraction and dialysis, enzyme activity increased from 87.932 to 260.8 U/mg protein, with 2.97-fold purification. The partially purified enzyme (50% saturation of acetone) was subjected after dialysis to purification by gel filtration on a Sephadex G-100 column. The elution profiles for protein and β-mannanase from the Sephadex G-100 column are illustrated in **Figure "1"** and show that there are two peaks; the first peak comprises the highest specific activity (684.7 U/mg protein). The highest active pooled fraction reached about 6.77-fold purification. A summary of the purification of β-mannanase from the culture medium of *A. terreus* is shown in **Table "1"**.

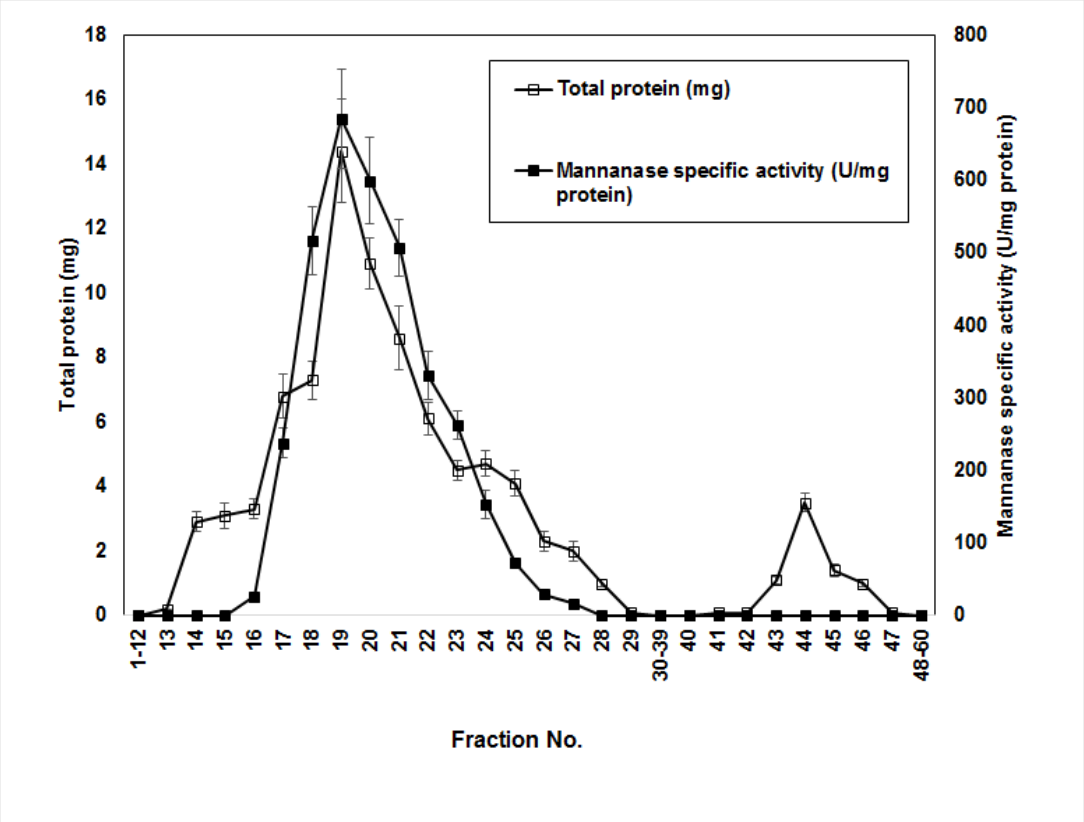

Fig. 1: Gel filtration of the partially purified *A. terreus* ARSA β-mannanase in Sephadex-G100 (two protein peaks; β-mannanase is located in one peak).

Table 1: Summary of purification steps of *A. terreus* ARSA β-mannanase

3.2. Characterization of purified A. terreus ARSA β-mannanase

Purification step	Total protein (mg)	Total activity (U)	β-Mannanase specific activity (U/mg protein)	Purification (Fold)	Recovery (%)
Culture filtrate	1140±142.5	128881± 11716.4	87.932±10.99	1±0.08	100±8.33
Acetone fraction (50%)	130±11.82	33904.1± 2825.3	260.8±32.60	2.97±0.37	26.31±2.39
Sephadex-G100	41.197±3.4	24508.5± 2723.1	594.9±54.08	6.77±0.75	19.02±2.38
P value	0.001*	0.0021*	0.0026*	0.001*	0.001*

3.2.1. Determination of molecular weight of A. terreus ARSA β-mannanase

SDS-PAGE was used for evaluation of purification after each step. The molecular weight of the purified enzyme was estimated after purification steps to be 48 kDa, with a clear improvement in the purification of the enzyme, which was observed by reduction in protein bands to a single protein band **(Figure 2)**.

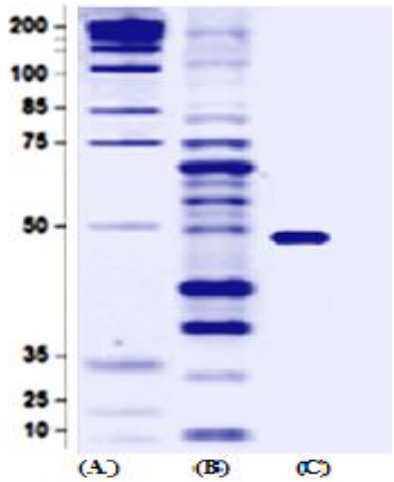

Fig. 2: SDS-PAGE of β-mannanase on 12% gel. Lane A, protein marker; Lane B, fraction from crude β-mannanase; Lane C, fraction from purified β-mannanase from Sephadex-G100. SDS-PAGE standard protein markers are:-Phosphorylase-b, 97.0 kDa; Bovine serum albumin, 67.0 kDa; egg albumin, 45.0 kDa; glyceraldehyde-3-phosphate, 36.0 kDa; Beta-Lactoglobulin, 18.4 kDa; 30 µg proteins, which were loaded in each lane. The molecular weight of *A. terreus* ARSA β-mannanase was found to have a molecular weight of 48.0 kDa.

3.2.2. Determination of kinetic constants of A. terreus ARSA β-mannanase

The results showed that substrate concentration which gave the highest activity was 6.0 mg/ml reaction mixture **(Data not shown)**. Values of K_m and V_{max} of the pure enzyme were calculated from a Lineweaver–Burk plot, and they were 3.33 mg/ml and 1666.67 U/ml, respectively **(Figure 3).**

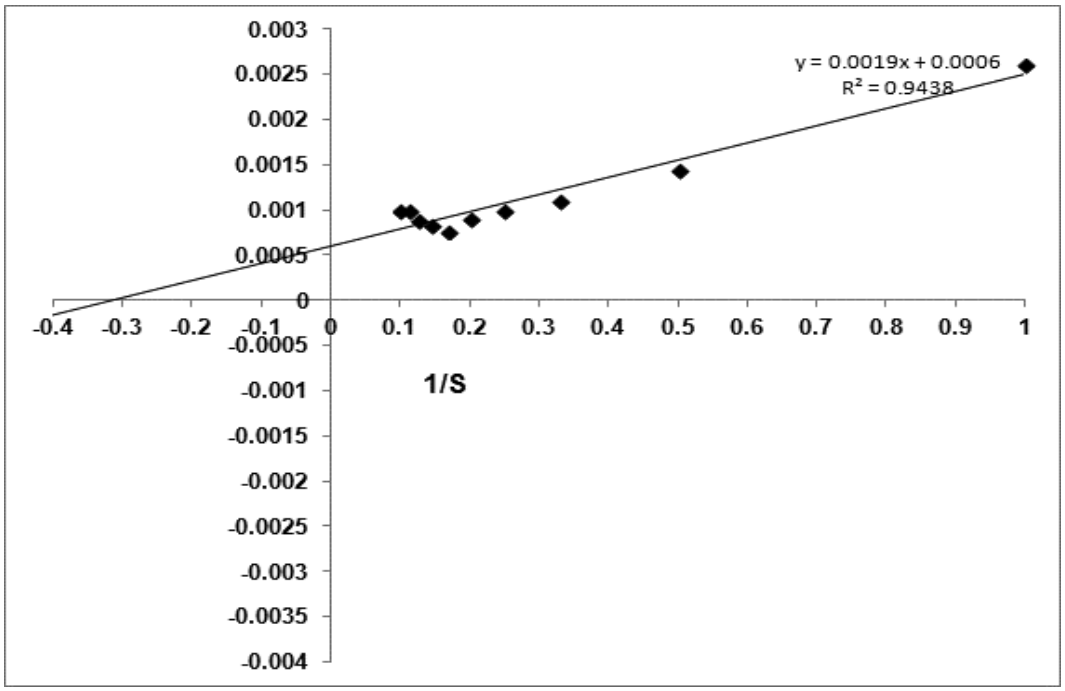

Fig. 3: Lineweaver-Burk plot to determine kinetic constants (K_m and V_{max}) of purified *A. terreus* ARSA β-mannanase. Enzyme activity was measured at different LBG concentrations ranging from 1 till 10 mg/ml.

3.2.3. Substrate specificity of A. terreus ARSA β-mannanase

Among various substrates tested, the enzyme gave high activity (1309.03 U/ml) towards LBG, followed by xylan (363.470 U/ml), and low activity (98.63 U/ml) for CMC, while absence of activity was detected using starch, dextran, or carrageenan **(Data not shown)**.

3.2.4. Effect of pH on A. terreus ARSA β-mannanase activity and stability

The effect of pH on the activity of the purified β-mannanase revealed that the highest specific activity (848.434 U/mg) was detected at pH 6.5 **(Data not shown)**. The pH stability of the purified -mannanase was graphically represented in **Figure "4"**, which demonstrated that it was stable at pH values ranging from 5.0 to 8.0, and its activity decrease with the increasing in pH.

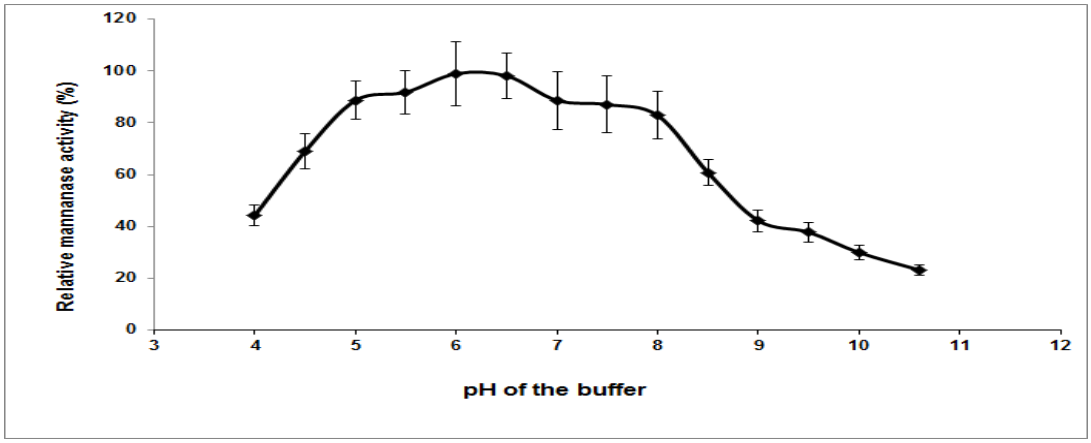

Fig. 4: pH stability of purified *A. terreus* ARSA β-mannanase. In the absence of the substrate, the enzyme was incubated for 2 hours at room temperature in various buffers with pH ranges of 4–10.6, and then the residual activity was evaluated at 40°C for 20 minutes. The relative activity was measured as the percentage of activity evaluated with respect to the maximum mannanase activity.

3.2.3. Effect of temperature on A. terreus ARSA β-mannanase activity and stability

The activity of β-mannanase increased gradually by increasing the reaction temperature, reaching its maximum value at 60°C, which showed the highest activity (874.729 U/mg protein). Higher or lower temperature decreased the enzyme activity **(Data not shown)**. Thermal stability of the enzyme **(Figure 5)** revealed that the purified *A. terreus* ARSA β-mannanase was fairly resistant to heat treatment. At 50°C, the enzyme retained all of its activity after 60 min of exposure. By heating the enzyme at 80°C for 60 minutes, the enzyme retained 54.79% of its initial activity.

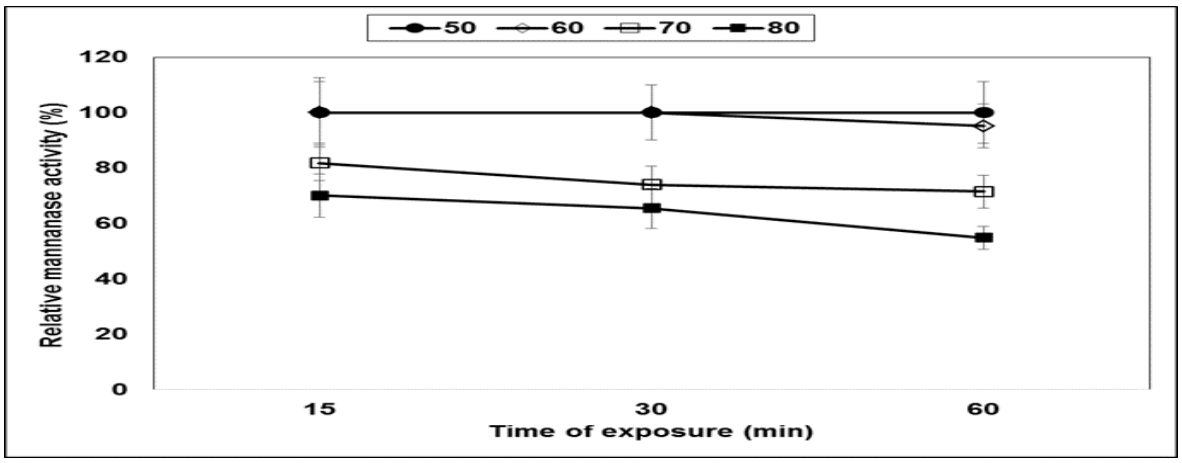

Fig. 5: Thermal stability of purified *A. terreus* ARSA β-mannanase. The enzyme was incubated at 50, 60, 70, and 80°C for different periods of time (15, 30, 60), and then the residual activity toward LBG was measured at 60°C for 20 min. The relative activity was measured as the percentage of activity evaluated with respect to the maximum mannanase activity.

3.2.6. Effect of some chemicals on A. terreus ARSA β-mannanase activity

The effect of some chemicals at 1.0 mM, 10 mM concentrations on the activity of the purified *A. terreus* ARSA β-mannanase **(Figure 6**) indicated that Mg^{2+}, Mn^{2+}, and Na^{+} activated purified enzyme by 1.32, 1.30, and 1.29-fold, respectively. While Zn^{2+} and Cu^{2+} partially inhibited the activity. On the other hand, the enzyme was strongly inhibited by EDTA, Hg^{2+}, Tween 80, SDS (10%) and only retained 9.08, 8.28, 7.75, and 5.79 %, respectively, of its activity compared to untreated enzyme.

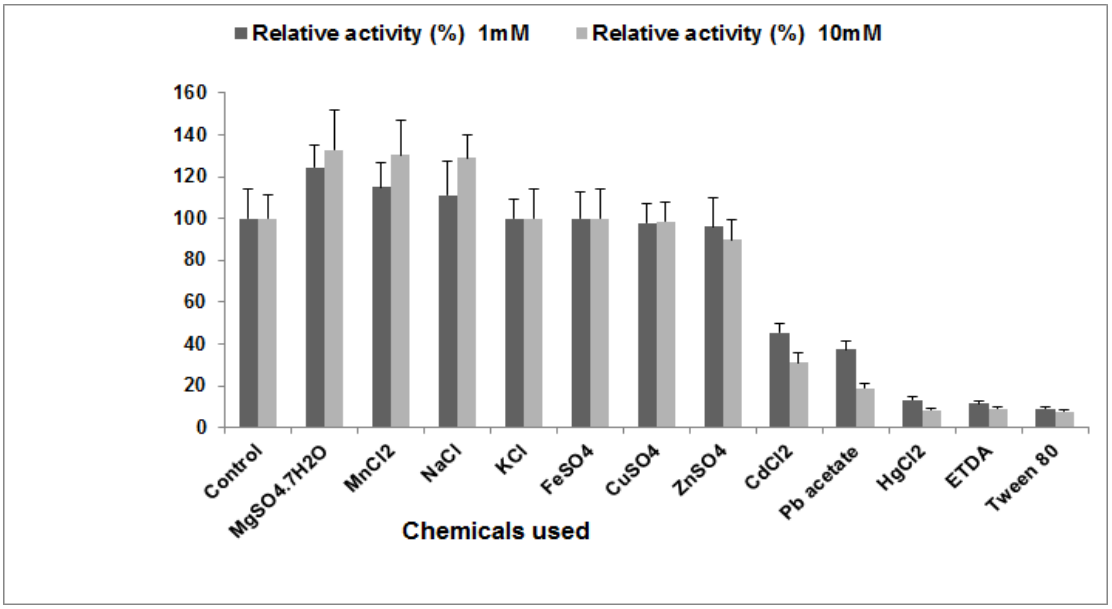

Fig. 6: Effect of chemicals on the activity of the purified *A. terreus* ARSA β-mannanase. At room temperature, 5 mM and 10 mM of each of the activators and inhibitors were incubated with the enzyme, and then the enzyme's activity toward LBG was evaluated. As a control, the enzyme activity was measured without any substances in the reaction mixture.

3.3. Determination of hydrolytic products of LBG by purified A. terreus ARSA β-mannanase by thin layer chromatography (TLC)

Samples of LBG degradation by *A. terreus* ARSA β-mannanase were analyzed for the oligomeric reaction products by TLC. Mannose, mannobiose, mannotriose, mannotetraose, mannopentaose, and mannohexaose were used as standard markers **(Figure 7).** The results of LBG degradation showed that there is a mixture of mannotriose, mannotetraose, mannopentaose, and mannohexaose.

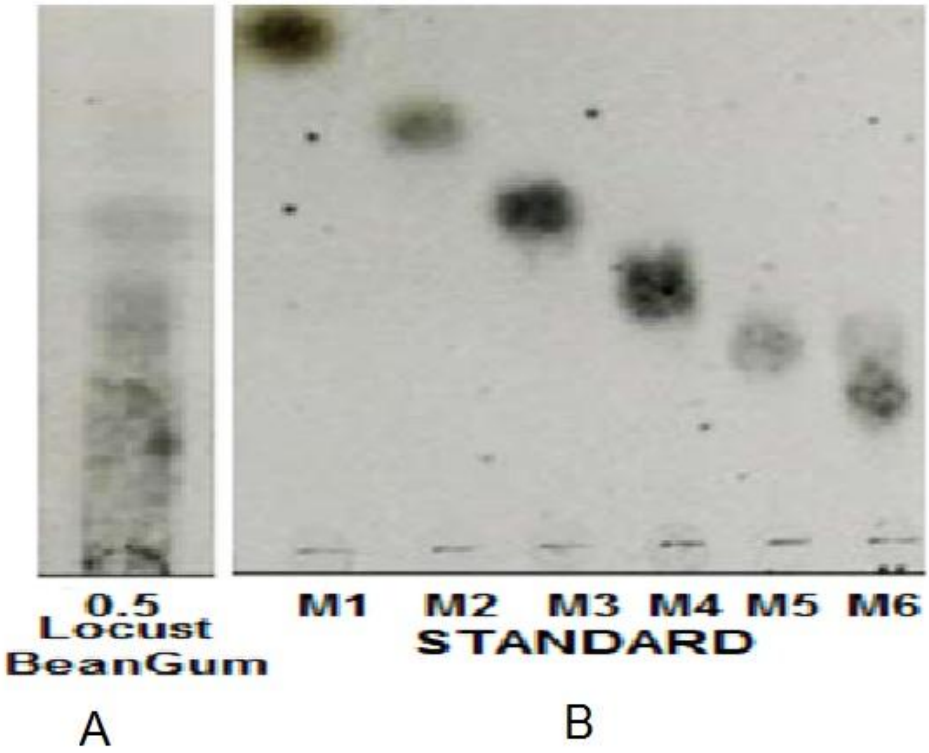

Fig. 7: (A): Hydrolysis products for purified *A. terreus* ARSA β-mannanase enzyme through TLC analysis. **(B):** mannooligosaccharide standards (M1 mannose; M2 mannobiose; M3 mannotriose; M4 mannotetraose; M5 mannopentaose; M6 mannohexaose)

3.4. Clarifying of some fruit juices by A. terreus ARSA β-mannanase

The data recorded in **Table (2**) showed that the enzyme enhanced the yield volume of all fruit juices than crude extract. It was also clear that crude extract and the purified *A. terreus* ARSA β-mannanase were effective on the apple juice with a yield of 128.32 and 167.65%, respectively, at 60°C and pH 6.5. On the other hand, the lowest clarifying yield was occurred in apricot juice using both crude and purified enzyme giving 106.45% and 120% clarification yield, respectively.

Table 2. Clarifying of some fruit juices by purified *A. terreus* ARSA β-mannanase.

Fruits (10 g)	Control	Crude β-mannanase	Purified β-mannanase	P value
Apple **Volume of sample (mL)** **Yield (%)**	17.3±1.730 --	22.2±2.220 128.32±11.665	28.5±2.850 167.65±13.971	0.214 0.046*
Grapes **Volume of sample (mL)** **Yield (%)**	14.6±1.217 --	18.01±1.637 123.97±10.331	22.2±2.018 158.57±14.415	0.106 0.041*
Apricot **Volume of sample (mL)** **Yield (%)**	15.5±1.722 --	16.5±2.063 106.45±11.828	18.6±1.550 120±12.000	0.211 0.092
Peach **Volume of sample (mL)** **Yield (%)**	12.6±1.050 --	13.2±1.320 110.32±9.193	16.4±1.367 130.18±10.848	0.107 0.101
Orange **Volume of sample (mL)** **Yield (%)**	15.6±1.950 --	17.3±2.163 111.61±10.146	22.2±2.775 143.23±11.936	0.078 0.05*

Crude and purified β-mannanase were incubated with homogenates of each fruit for 4 h at 60°C

DISCUSSION

The purification of the β-mannanase enzyme produced by halophilic *A. terreus* ARSA isolated from mangrove sediment was effective and efficient. Ecosystems of mangroves are a suitable habitat for the colonization of fungi and bacteria. Fortunately, providing enzymes with great economic value in various industries, medicine, and sewage treatments **(Saravanakumar *et al.*, 2016).** The crude *A. terreus* ARSA β-mannanase was partially purified by fractional precipitation with acetone. Among all the fractions obtained from partial purification, the 50% acetone fraction gave the maximum enzyme activity and protein

recovery. The partially purified enzyme was subjected to gel filtration on a Sephadex G-100 column chromatography, and the specific activity of the purified enzyme reached 594.90 U/mg protein which was higher than that obtained by purified *A. terreus* FBCC 1369 β-mannanase, which was about 53.75 U/mg as reported by **Soni *et al*. (2016).**

The mannan-degrading enzymes purification was carried out using various combination procedures, such as precipitation, hydrophobic interaction, gel filtration, ultrafiltration, and ion-exchange chromatography. Laboratory-scale experiments were used to test and confirm the method of choice **(Jiang *et al*., 2006; Soni *et al*., 2016).** Comparing our results with the previous separation and purification methods **(Vijayalaxmi *et al*., 2013),** the two-step method has many advantages as high efficiency, high yield, and easy operation.

The purity of β-mannanase was analyzed by SDS-PAGE electrophoresis which revealed a single homogenous band with an approximate molecular weight of 48 kDa. Different studies estimated different molecular weights of β-mannanases purified from different *Aspergillus* species **(Naganagouda *et al*., 2009; Soni *et al*., 2016)**.

K_m and V_{max} values were determined as 3.33 mg/ml and 1666.67 U/ml, respectively, using LBG as a substrate. **Haq *et al*. (2020)** reported that K_m and V_{max} are 11.07 mM and 19.08 μM min-1 respectively, for β-mannanase produced from *Aspergillus awamori* using LBG.

The substrate specificity has a crucial role in enzyme applications. Among different substrates, purified *A. terreus* ARSA β-mannanase can hydrolyze LBG most efficiently. These results are similar to those obtained from *A. terreus* FBCC 1369 β-mannanase **(Soni *et al*., 2016)**.

The property of enzyme to maintain a high activity at relatively high temperature and low pH was interesting for many industrial applications. The highest enzyme activity of purified *A. terreus* ARSA β-mannanase was obtained at pH 6.5, and more than 80% maximal activity was retained at pH 5.0–7.5 after 2 hrs of incubation. Interestingly, the optimal pH of *A. terreus* ARSA β-mannanase was similar to that of *B. subtilis* MA139 (pH 6.0), this optimal pH is also in the range of pH of gastric intestinal tract, and this enables the enzyme to be a potential additive in feed industry (**Qiao *et al*., 2010**). Also, these results are in accordance to other fungal β-mannanase **(Soni *et al*., 2016**; **Sakai *et al*., 2017; Haq *et al*., 2020).**

Purified *A. terreus* ARSA β-mannanase was also thermostable in absence of its substrate. At 50^{o}C, the enzyme retained all of its activity after 60 min and retained about 95.09 % of its activity when incubated at 60^{o}C for 60 min. While at a higher temperature (80^{o}C), the enzyme retained about 54.79 % of its original activity by heating for 60 min. Fortunately, this was higher thermostability than that of *Aspergillus terreus* FBCC 1369 β-mannanase which was stable up to 1.0 h at 50^{o}C, retaining 85% activity **(Soni *et al*., 2016).** Furthermore, our findings are consistent with β-mannanase produced from various fungal species **(Shalaby *et al*., 2017; Haq *et al*., 2020)**. The thermostability of the β-mannanase from *A. terreus* ARSA that have found here made the enzyme a good choice as a catalyst for many industrial applications.

Some metal ions as heavy metals are considered as environmental pollutants and may have an effect on the production of the enzymes and their stability. The results indicated that Mg

$^{2+}$, Mn $^{2+}$, and Na^{+} highly increased enzyme activity at 10 mM concentration. On the other hand, Pb^{2+}, Cd^{2+}, and Hg^{2+} at 10 mM strongly inhibited the enzyme. The results are in partial agreement with those obtained by **Chivero *et al.* (2001)** and **Ge *et al*. (2016**) who found that Hg^{2+}, SDS, Tween 80, and EDTA, all had a partial inhibitory effect. The enhanced activity in the presence of Mg^{2+} suggests that the cation is involved in the regulation of enzyme active conformation, increasing its activity **(Yang *et al*., 2016)**. Also, Mn^{2+} may also play a role in maintaining the conformational stability of enzymes **(Sakai *et al*., 2017)**.

The products of hydrolysis of *A. terreus* ARSA β-mannanase was in line to the mannanase produced from *Penicillium oxalicum* GZ-2 and *Reinekea* sp. KITYO10 **(Hakamada *et al*., 2014).** Our findings are in good agreement with **Kim *et al.* (2018**) who found that degradation of LBG yielded a mixture of mannobiose, mannotriose, mannopentaose, and other unidentified manno-oligosaccharides. Oligosaccharides produced from mannans hydrolysis can be widely used as prebiotics and can promote growth of chickens and prevent them from diseases **(Soni *et al*., 2016).** Thus, the production of mannoligosaccharides by β-mannanase from *A. terreus* ARSA makes it a good candidate for potential application in the feed industry.

The use of enzymes enhances the output of juices, decreases its viscosity, and plays an important role in the fruit juice clarification. The process of juice clarification is mainly performed by the hydrolysis of carbohydrates polymers, which causes turbidity **(Nadaroglu *et al*., 2017)**. Seasonal fruits such as apples, oranges, pomegranates, strawberries, and kiwis contain mannans **(Adiguzel *et al*., 2016**). The use of mechanical methods, as filtration and centrifugation are easily managed, but leads to poor yield. Clarification with purified mannan hydrolyzing enzyme has advantages, but expensive **(Zhao *et al*., 2019)**. With regards to juice yields which were represented by volumes, purified *A. terreus* ARSA β-mannanase was efficient on the juices from apple, grapes, orange, peach, and apricot with a yield ratio of 167.65, 158.57, 143.23, 130.18, and 120.00%, respectively. The juice yields in this research were comparatively slightly higher than those obtained by purified *Pediococcus acidilactici* (M17) β-mannanase and *Weissella viridescens* LB37 β-mannanase **(Adiguzel *et al*., 2016; Nadaroglu *et al*., 2017; Zhao *et al*., 2019)**. The use of crude β-mannanase can be more preferred because enzyme purification procedures need time and cost. However, when purified enzyme is used, the yield and clarity of juice will be enhanced much more (**Zhao *et al*., 2020**). Many researchers carried out fruit juice clarification process by β-mannanase, and their results are in good agreement with our findings **(Adiguzel *et al*., 2016; Zhao *et al*., 2019)**.

CONCLUSION

β-mannanase enzyme has been extensively used in industries including detergents, feed, textile, and biorefinery. In this study a thermostable β-mannanase was produced from a local halophilic fungal isolate, which was identified as *Aspergillus terreus* strain ARSA. The enzyme was purified/characterized and its molecular weight was determined as 48 kDa. The obtained enzyme is capable of hydrolysis of mannan. Also, was found to be with a unique property of fruit juice clarification, as a result, it may be useful in lowering the cost of juice production, as well as in the food industry and biotechnological applications.

REFERENCES

Adiguzel, G.; Sonmez, Z.; Adiguzel, A. and Nadaroglu, H. (2016). Purification and characterization of a thermostable endobeta-1,4 mannanase from *Weissella viridescens* LB37 and its application in fruit juice clarification. Eur. Food Res. Technol., 242(5): 769–776.

Afefe, A. A.; Abbas, M. S.; Soliman A. S.; Khedr, A. A. and Hatab, E. E. (2019). Physical and chemical characteristics of mangrove soil under marine influence. A case study on the mangrove forests at Egyptian – African red sea coast. Egypt. J. Aquat. Biol. Fish., 23 (3): 385–399.

Arotupin, D. J. and **Olaniyi, O. O.** (2013). Screening and identification of mannanase–producing fungi isolated from selected agricultural wastes. *British Microbiol. Res. J.*, 3(4): 635–644.

Bååth, J. A.; Martínez-Abad, A.; Berglund, J.; Larsbrink, J.; Vilaplana, F. and Olsson, L. (2018). Mannanase hydrolysis of spruce galactoglucomannan focusing on the influence of acetylation on enzymatic mannan degradation. Biotechnol. Biofuels., 11: 114.

Basheer, M.A.; Mekawey, A.A.; El-Kafrawy, S.B. and Abouzeid, M.A. (2018). Antimicrobial Activity of Endophytic Fungi of Red Sea Aquatic plant *Avicennia marina*. Egy. J. Microbiol., 53(1): 231-240.

Beltagy, E. A.; El-Borai, A. M.; Lewiz, M. M. and ElAssar, S. A. (2016). Purification and characterization of alginate lyase from locally isolated marine *Pseudomonas stutzeri* MSEA04. Acta Biol. Hung., 67(3): 305-317.

Chivero, E. T.; Mutukumira, A. N. and Zvauya, R. (2001). Partial purification and characterization of a xylanase enzyme produced by a microorganism isolated from selected indigenous fruits of Zimbabwe. Food Chem., 72: 179–185.

El-Borai, A. M.; Abedin, R. M.; Shall, M. A.; El-Assar, S. A. (2013)**.** Purification and Immobilization of Dextransucrase from Immobilized *Lactobacillus acidophilus* ST76480.01. J. Pure Appl. Microbiol., 7(4): 2915-2923.

Ezawa, S. and Tada, Y. (2009)**.** Identification of salt tolerance genes from the mangrove plant *Bruguiera gymnorhiza* using *Agrobacterium* functional screening. Plant Sci., 176(2): 272-278.

Farag, A. M.; Hassan, S. W.; El-Says, A. M. and Ghanem, K. M. (2018). Purification, characterization and application of tannase enzyme isolated from marine *Aspergillus nomius* GWA5. J. Pure Appl. Microbiol., 12(4): 1939-1949.

Favaro, C. P.; Baraldi, I. J.; Casciatori, F. P. and Farinas, C. S. (2020). β-Mannanase Production Using Coffee Industry Waste for Application in Soluble Coffee Processing. Biomolecules., 10: 227.

Ge, J. P.; Du, R. P.; Zhao, D.; Song, G.; Jin, M. and Ping, W. X. (2016 a). Bio-chemical characterization of a β-mannanase from the *Bacillus licheniformis* HDYM-04 isolated from flax water-retting liquid and its decolarization ability of dyes. RSC Advances., 6**:** 23612–23621.

Ghizelini, A. M.; Mendonça-Hagler, L. C. S. and Macrae, A. (2012). Microbial diversity in Brazilian 434 mangrove sediments: a mini review. Braz. J. Microbiol., 43:1242-1254.

Hakamada, Y.; Ohkubo, Y. and Ohashi, S. (2014). Purification and characterization of β-Mannanase from *Reinekea sp.* KIT-YO10 with transglycosylation activity. Biosci. Biotechnol. Biochem., 78(4): 722-728.

Haq, I. U.; Shakoor, S.; Nawaz, A.; Arshad, Y. and Mukhtar, H. (2020). Purification and characterization of mannanase from *Aspergillus awamori* for fruit juice clarification. Prot. Pep. Lett., 27: 1.

Jiang, Z.; Wei, Y.; Li, D.; Chai, P. and Kusakabe, I. (2006). High-level production, purification and characterization of a thermostable β-mannanase from the newly isolated *Bacillus subtilis* WY34. Carbohydr. Polym., 66(1): 88–96.

Kim, S.; Hwa Lee, M.; Lee, E. S.; Do Nam, Y. and Ho Seo, D. (2018). Characterization of mannanase from *Bacillus* sp., a novel *Codium fragile* cell wall-degrading bacterium. Food Sci. Biotechnol., 27(1): 115–122.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature., 227: 680–5.

Liepman, A. H.; Nairn, C. J.; Willats, W. G. T.; Sørensen, I.; Roberts, A. W. and Keegstra, K. (2007). Functional genomic analysis supports conservation of function among cellulose synthase-like A gene family members and suggest diverse roles of mannans in plants. J. Plant Physiol., 143: 1881–1893.

Lin, X. Y.; Lu, C. and Ye, Y. (2009)**.** Toxicity of crude extracts from several terrestrial plants to barnacle larvae on mangrove seedlings. Ecol. Eng., 35(4): 502-510.

Lin, Y.; Wu, X.; Feng, S.; Jiang, G.; Luo, J.; Zhou, S.; Vrijmoed, L. L. P.; Jones, E. B. G.; Krohn, K.; Steongröver, K. and Zsila, F. (2001). Five unique compounds: xyloketales from mangrove fungus *Xylaria sp*. from the South China Sea Coast. J. Org. Chem., 66(19): 6252- 6256.

Lineweaver, H. and Burke, D. (1934). The determination of enzyme dissociation constants, J. Am. Chem., 56: 658–66.

Liu, Z.; Ning, C.; Yuan, M.; Yangl S.; Wei, X.; Xiao, M. et al. (2020). High-level expression of a thermophilic and acidophilic β-mannanase from *Aspergillus kawachii* IFO 4308 with significant potential in mannooligosaccharide preparation. *Bioresour. Technol.* 295:122257.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L. and Randall, R. J. (1951). Protein measurement with Folin-phenol reagent. J. Biol. Chem., 48: 17-25.

Miller, G. L. (1959). Use of dinitrosalisilic acid reagent for determination of reducing sugar. J. Anal. Chem., 31: 426-428.

Nadaroglu, H.; Adiguzel, A. and Adiguzel, G. (2015). Purification and characterization of β-mannanase from *Lactobacillus plantarum* (M24) and its applications in some fruit juices. Int. J. Food Sci. Technol., 50: 1158–1165.

Nadaroglu, H.; Adiguzel, G.; Adiguzel, A. and Sonmez, Z. (2017). A thermostable-endo-β-(1,4)-mannanase from *Pediococcus acidilactici* (M17): Purification, characterization and its application in fruit juice clarification. Eur. Food Res. Technol., 243: 193–201.

Naganagouda, K.; Salimath, P. V. and Mulimani, V. H. (2009). Purification and Characterization of Endo-β-1,4 Mannanase from *Aspergillus niger* gr for Application in Food Processing Industry. J. Microbiol. Biotechnol., 19(10): 1184–1190.

Pointing, S. B. and Hyde, K. D. (2000). Lignocellose-degrading marine fungi. Biofouling., 15 (1-3): 221- 229.

Pradeep, G.; Cho, S. S.; Choi. Y. H.; Choi, Y. S.; Jee, J. P.; Seong, C. N. et al. (2016). An extremely alkaline mannanase from *Streptomyces sp*. CS428 hydrolyzes galactomannan producing series of mannooligosaccharides. World J. Microbiol. Biotechnol., 32:84.

Qiao, J.; Rao, Z.; Dong, B. and Cao, Y. (2010). Expression of *Bacillus subtilis* MA139 *β*-mannanase in *Pichia pastoris* and the enzyme characterization. Appl. Biochem. Biotechnol., 160(5) : 1362–1370.

Sakai, K.; Mochizuki, M.; Yamada, M.; Shinzawa, Y.; Minezawa, M.; Kimoto, S.; Murata, S.; Kaneko, Y.; Ishihara, S. and Jindou, S. (2017). Biochemical characterization of thermostable β-1,4-mannanase belonging to the glycoside hydrolase family 134 from *Aspergillus oryzae*. Appl. Microbiol. Biotechnol., 101: 3237–3245.

Saravanakumar, K.; Rajendran, N.; Kathiresan, K. and Chen J. (2016). Chapter Five-Bioprospects of microbial enzymes from mangrove-associated fungi and bacteria, Advances in Food and Nutrition Research, Academic Press, Volume 79, pp. 99-115.

Shalaby, A. G.; Esawy, M. A. and Hussein, M. M. (2017). Comparative study between free and immobilized *Penicillium chrysogenum* mannanase: a local fungal isolate. J. Appl. Pharm. Sci., 7 (6): 97-104.

Shiau, Y. and Chiu, C. (2020). Biogeochemical processes of C and N in the soil of mangrove forest ecosystems. Forests, 11:492.

Shukla, P. and Pletschke, B. I. (2013). Advances in Enzyme Biotechnology. Springer Science & Business Media. 50-53.

Soni, H.; Rawat, H. K.; Pletschke, B. I. and Kango, N. (2016). Purification and characterization of β-mannanase from *Aspergillus terreus* and its applicability in depolymerization of mannans and saccharification of lignocellulosic biomass. 3 Biotech., 6(2): 136.

Vijayalaxmi, S.; Prakash, P.; Jayalakshmi, S. K.; Mulimani, V. H. and Sreeramulu, K. (2013). Production of extremely alkaliphilic, halotolerent, detergent, and thermostable mannanase by the free and immobilized cells of *Bacillus halodurans* PPKS-2. Purification and characterization. Appl. Biochem. Biotechnol., 171(2): 382–395.

Yang, Y. M.; Cai, J.; Wang, C. G.; Du, X. and Lin, J. G. (2016). Characterization of endo-β-mannanase from *Enterobacter ludwigii* MY271 and application in pulp industry. Bioprocess Biosyst. Eng., 40:35.

Zhao, D.; Wang, Y.; Na, J.; Ping, W. and Ge, J. (2019). The response surface optimization of β-mannanase produced by *Lactobacillus casei* HDS-01 and its potential in juice clarification, Prep. Biochem. Biotechnol., 49: (2) 202-207.

Zhao, D.; Zhang, X.; Wang, Y.; Na, J.; Ping, W. and Ge, J. (2020). Purification, biochemical and secondary structural characterization of β-mannanase from *Lactobacillus casei* HDS-01 and juice clarification potential. Int. J. Biol. Macromol., 154: 826–834.