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Purification and physicochemical characterisation of *Aspergillus niger* USM F4 β-mannanase

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ABSTRACT

Aims: This present study focused on purification of fungal β-mannanase produced by *Aspergillus niger* USM F4 and also physicochemical characterisation of the purified enzyme.
Methodology and results: The purified β-mannanase with a molecular mass of ~47.4 kDa was demonstrated on SDS-PAGE gel. The enzyme signified a purification degree of 4-fold, with final specific activity of 196.42 U/mg. It reached an optimum catalytic activity at pH 4.0 and 60 °C. The thermal stability of the enzyme was up to 70 °C and maintained the 50% activity after 30 min at 80 °C. Meanwhile, the pH stability was in the range of pH 3.0-9.0 and a 30 min half-life at pH 10.0. All chemical substances manifested an inhibitory effect on purified β-mannanase, with SDS (28.16 ± 0.05% residual activity) as the strongest inhibitor, followed by cupric ion (Cu^{2+}) (49.51 ± 0.09% residual activity). As a whole, the enzyme displayed a substrate specificity in the order of locust bean gum (LBG) > carboxymethylcellulose > soluble starch > xylan from oat spelt > α-cellulose. Its preference for LBG has generated the K_m and V_{max} values of 0.20 mg/mL and 9.82 U/mL, respectively.
Conclusion, significance and impact of study: The outcomes of our study offer potential for use at industrial scales, particularly in the oligosaccharides production that involve acid-related activity, wide-ranging temperature and pH stability.

Keywords: Mannanase, palm kernel cake, physicochemical characterisation, protein purification

INTRODUCTION

Indonesia and Malaysia are recognised as the global supreme palm oil producers other than Nigeria, Colombia, and Thailand. In general, the palm oil is originated from the pressing process of fresh fruits and seeds of oil palm which later produced the residual wastes known as empty fruit bunch (EFB) and palm kernel cake (PKC). Malaysia spawned a massive amount of PKC with 2.59 million tonnes of production in 2018 (MPOB, 2019). The statistic has indirectly made PKC resources as cheap and accessible leftover. According to the nutrients profile, PKC owns a relatively high protein content (14-16%), essential amino acids, minerals (Marini *et al.*, 2005), and vitamin E (Krishnaiah *et al.*, 2012). PKC is a latent source of mannan oligosaccharide since its crude fibre impregnated hemicelluloses with superficially predominated by 57.8% mannan, 11.6% cellulose, and 3.7% xylan (Abd-Aziz *et al.*, 2009; Nurhayati *et al.*, 2018). Hence, PKC can stimulate the production of mannan-degrading enzymes by analysing the end-product of reducing sugar such as mannose (Syarifah *et al.*, 2012). Other than monomer mannose, the execution of enzymatic mannan conversion will also tag along a small amount of galactose and cellulose (Illuyemi *et al.*, 2006).

Fungi species including *Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp., *Sclerotium* sp., *Bispora* sp., and *Scopulariopsis* sp. are identified as potential producers for fungal mannan-degrading enzymes (Regalado *et al.*, 2000; Mudau and Setati, 2008; Luo *et al.*, 2009; Blibech *et al.*, 2011; Wu *et al.*, 2011). Of that, *Aspergillus* is the most actively reported fungal genus due to the high yield of extracellular enzymes production and its natural characteristic in degrading a plethora of organic substances. The acclimatising capability of the genus *Aspergillus*, especially in solid-state fermentation (SSF)

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system, is irrefutable because the fungi grow *via* polarised elongation using the hyphal tip (Takeshita, 2016). This tip can deeply penetrate the solid substrate, prior to colonisation and nutrients absorption. As a surplus, most of the species can endure an extreme temperature, low water activity and high osmosis pressure. These characteristics have provided a competence benefit for the genus *Aspergillus* against other natural microflora in adapting the SSF system. SSF is a heterogeneous bioprocess of solid, liquid and gaseous phases (Costa *et al*., 2018).

β-mannanase (E.C. 3.2.1.78) hydrolyses the β-1,4-linkages in mannan backbone and sequentially releasing mannooligosaccharides. The biological and chemical properties of this enzyme have been employed for over extensive years and vastly engaged with agriculture including animal feed, fruit ripening, pulp bleaching, and detergent (Carrington *et al*., 2002; Benech *et al*., 2007; Srivastava and Kapoor, 2013; Tewoldebrhan *et al*., 2017). These events have driven to the purification development of fungal and bacterial enzymes. Thus far, pure β-mannanase can be produced by fungi and bacteria, with a tendency for acidic to neutral pH conditions as well as a range of moderate thermophilic temperatures (Naganagouda *et al*., 2009; El-Sharouny *et al*., 2015; Cheng *et al*., 2016). In this study, we discovered the efficient physicochemical properties of *A. niger* β-mannanase through its purification development and enzymatic characterisation.

MATERIALS AND METHODS

Chemicals

All chemicals were procured from Sigma-Aldrich (USA), Hi-Media Lab. LTD (India) and Fluka (USA). Solid substrate or PKC in the SSF system was provided by a local palm oil mill factory located at Seberang Perai district area, Pulau Pinang, Malaysia.

Filamentous fungus and solid-state fermentation system

An isolate identified as *A. niger* USM F4 was originated from fungal culture stock of Industrial Biotechnology Research Laboratory (IBRL), Universiti Sains Malaysia, Pulau Pinang, Malaysia. The fermentation cycle was performed in 250 mL flask according to the method of Syarifah (2010). A complete SSF system comprised 80% (v/w) water content, 10 g PKC (≤ 0.5 mm particle size), 1 × 10^7 spore/mL fungal spore suspension, 2% (w/w) molasses and 2% (w/w) ammonium nitrate (NH_4NO_3). All flasks were incubated at 30 ± 2 °C and harvested after 5 days of cultivation period.

β-mannanase extraction and assay

Fermented PKC was extracted using Tween 80 and filtered through the Whatman filter paper No.1. Then, the crude enzyme filtrate (0.5 mL) was mixed with 0.5% (w/v) locust bean gum (LBG), which was pre-dissolved in 50 mM citric acid-trisodium citrate buffer pH 4.0. Next, the test tubes containing the enzyme-substrate mixture were incubated at 60 °C for 30 min. Dinitrosalicylic acid (1.5 mL) was added into the mixture and boiled for 5 min. The enzyme activity was verified at 575 nm by using spectrophotometer (Lin and Chen, 2004). One unit of β-mannanase activity was described as the sum of enzyme needed to release one µmol of mannose equivalents per minute according to the assay conditions.

Protein content measurement

Protein content was measured spectrophotometrically at 280 nm and bovine serum albumin was set as a standard (Lowry *et al*., 1951).

Purification of *A. niger* USM F4 β-mannanase

The enzyme was purified through three-step purification processes: ultrafiltration, molecular sieve chromatography and anion exchange chromatography. Initially, a total of 100 mL of crude enzyme filtrate was submitted to Vivaflow Masterflex C/S (Cole Parmer, USA) equipped with a 10 kDa cut-off membrane threshold, before molecular sieve chromatographic step. Then, the sample was further purified on a Superdex 75 gel column size of 30 × 1 cm which previously equilibrated with citric acid-trisodium citrate buffer (50 mM, pH 4.0). Elution was attained at a rate of 0.12 mL/min. The fractions with high enzyme activity were pooled and proceeded with anion exchange chromatography. The pooled samples were further filtrated using Amicon Ultra-15 (3 kDa cut-off membrane; Millipore, USA). Then, the filtrates were streamed to Q-sepharose fast flow column and eluted with 50 mM citric acid-trisodium citrate buffer (pH 5.0) containing 0.8 M sodium hydroxide. At a flow rate of 0.2 mL/min, the fractions were collected and further analysed for the β-mannanase activity (A_{575}) and total protein content (A_{280}).

Electrophoresis and β-mannanase molecular mass

The purity of chromatographic enzyme and its molecular mass were verified through electrophoretic separation in SDS-PAGE (Bio-Rad, USA) (Laemmli, 1970). Under non-denaturing conditions of 12.5% separating gel and 2.5% stacking gel, all samples including the crude and purified enzyme were loaded into the gel lane and compared against a standard marker (low molecular weight protein marker, Pharmacia, Sweden). The electrophoresis process was performed approximately 3 h in a 1× solution of running buffer at 4 °C using a constant voltage of 80 volts. For protein bands visualisation, SDS-PAGE gel was stained with the silver nitrate (Bollag *et al*., 1996). The molecular mass of the purified β-mannanase was estimated according to the plot from a pair-wise combination of the molecular mass of the standard and the relative mobility (Rf). The range of protein sizes varied from 18.4 kDa (β-lactoglobulin) to 116 kDa (β-galactosidase).

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Physicochemical characterisation of β-Mannanase

The influence of pH conditions on enzyme activity and stability

The ideal pH of enzyme activity was analysed by introducing the purified β-mannanase to acid-trisodium citrate buffer (pH 3.0, 4.0 and 5.0), phosphate buffer (pH 6.0 and 7.0), Tris-HCl buffer (pH 8.0 and 9.0) and glycine-NaOH (pH 10.0). Each buffer was set at 50 mM. The enzyme was mixed with 0.5% (w/v) LBG which was pre-dissolved in different buffers, prior to the standard assay procedure. A few of pH conditions that exhibited high enzyme activity were selected for the stability test. Without LBG, the purified enzyme was pre-incubated with the pH buffers for 30 min, with 5 min intervals. Then, the residual enzyme activity was promptly analysed according to the enzyme assay.

The influence of temperature conditions on enzyme activity and stability

The thermal effect on activity was done by introducing the purified enzyme to temperatures ranging from 50 to 80 °C for 30 min in citric acid-trisodium citrate buffer (50 mM, pH 4.0). After that, the stability test was done by pre-incubating the enzyme (without LBG) at different temperatures for 30 min. For every 5 min, the remaining residual activity was immediately approximated by the enzyme assay.

The influence of chemical substances on purified β-mannanase

Purified enzyme liquid was incubated for 30 min at 60 °C in the presence of 1.0 mM 2-mercaptoethanol, sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA), strontium chloride ($SrCl_2$), potassium chloride (KCl), zinc chloride ($ZnCl_2$), iron (III) chloride ($FeCl_3$), zinc sulphate ($ZnSO_4$), copper (II) sulphate ($CuSO_4$), sodium chloride (NaCl), calcium chloride ($CaCl_2$), manganese (II) chloride ($MnCl_2$), aluminium chloride ($AlCl_3$), and magnesium chloride ($MgCl_2$). The purified enzyme (without any additive) was set as a control in this experimental study and the relative activity of β-mannanase was measured under the optimised assay conditions (LBG, pH 4.0 and temperature of 60 °C).

Substrate specificity and kinetic study

The specificity of β-mannanase was evaluated by assaying the activity against 0.5% (w/v) LBG, carboxymethyl cellulose, soluble starch, α-cellulose, and xylan from oat spelts, The substrate that gave the best enzyme activity was analysed at concentrations of 0.25 to 1.5% (w/v) and was determined under the optimised assay conditions. To scrutinise the preference of the purified enzyme on the selected substrate, the K_m and V_{max} were evaluated from Lineweaver-Burk plots (Lineweaver and Burk, 1934).

RESULTS AND DISCUSSION

Cultivation of *A. niger* USM F4 with PKC as a mannan-based substrate has successfully generated an extracellular β-mannanase. The design of isolation and purification of the enzyme is summarised in Table 1. The first ultrafiltration step using Vivaflow Masterflex C/S has eliminated ≤ 10 kDa nano-particles protein, while the lower protein cut-off (≤ 3 kDa) was removed by Amicon Ultra 15. The efficiency of both processes was proven since the multiple purification reached 1.42-fold and 2.58-fold, respectively.

Table 1: Purification steps of the the β-mannanase from *Aspergillus niger* USM F4.

Purification step	Activity (U/mL)	Total volume (mL)	β-mannanase activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude culture filtrate	29.73	100	2973	60.5	49.14	100	1
Ultrafiltration using Vivaflow Masterflex C/S	138.89	20	2777.8	39.67	70.02	93.43	1.42
Superdex 75	72.42	2	144.84	1.44	100.58	4.87	2.05
Ultrafiltration using Amicon Ultra 15	26.01	2	52.02	0.41	126.88	1.75	2.58
Q-sepharose fast flow	8.25	2	16.5	0.084	196.42	0.55	4

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According to the elution of the Superdex 75 column, fractions 7 to 9 indicated an ascending pattern of β-mannanase activity together with the first peak protein (Figure 1). Fraction 8 was recorded as the highest enzyme activity specifically 72.42 U/mL and 0.72 mg/mL protein content. The same graph also showed the second and third peak proteins between fractions 10 to 19. β-mannanase was further purified to homogeneity *via* anion exchanger Q-sepharose fast flow (Figure 2). The enzyme activity gradually increased from fractions 11 to 13, with fraction 12 recorded the highest activity. This event represented 196.42 U/mg final specific activity, 0.55% recovery yield and 4-fold purification factor of β-mannanase. SDS-PAGE analysis revealed a single band of β-mannanase with the molecular mass of ~47.4 kDa (Figure 3).

The specific activity of fungal β-mannanase was reported approximately from 19.09 to 27864.6 U/mg (Mudau and Setati, 2008; Adesina *et al.*, 2013). The recovery yields varied from 0.53 to 71.2% which was in consensus with studies by Puchart *et al.* (2004) and Kim *et al.* (2011). In most purification schemes, there are one to five general steps of microbial β-mannanase purification and each step can affect the purification fold. The degree of purification which involved one or two purification steps can range from 1.14 to 64.73-fold (Mudau and Setati, 2008; Harnpicharnchai *et al.*, 2016). In comparison to its counterpart, the extension steps could generate a much higher fold approximately 13.6 to 810-fold (Zakaria *et al.*, 1998; Naganagouda *et al.*, 2009; El-Sharouny *et al.*, 2015). The purification fold in our study was much lower than the previously reported findings, albeit we engaged with four purification steps. It is widely known that fungal enzymes are more tedious to be purified. The ultrafiltration process may also contribute to the low purification fold. The concept for protein filtration or concentration using ultrafiltration is based on pressurising the liquid through a setting membrane while maintaining the protein of interest. However, this process could disassemble the protein arrangement and leads to its denaturation. This study supported previous findings of which the molecular mass of purified mannanase from the genus *Aspergillus* was in the range of 40-110 kDa (Ademark *et al.*, 1998; Regalado *et al.*, 2000; Puchart *et al.*, 2004; Naganagouda *et al.*, 2009; Wu *et al.*, 2011).

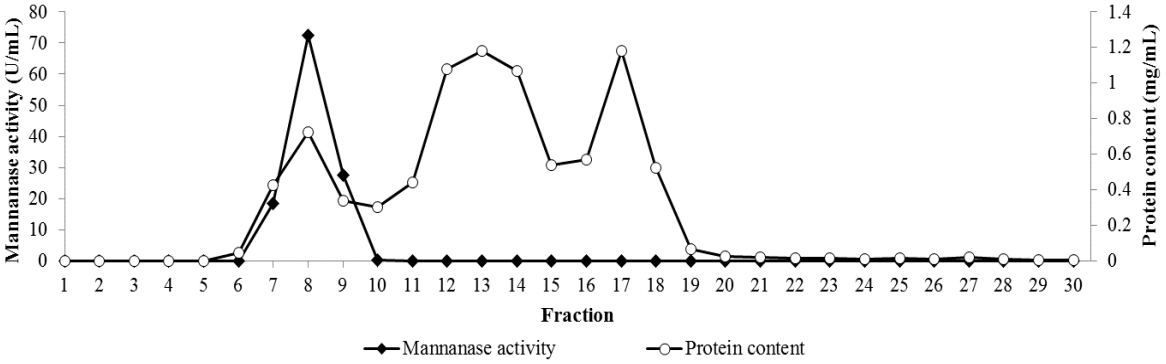

Figure 1: Purification of β-mannanase using Superdex 75 column chromatography with 50 mM citric acid-trisodium citrate buffer (pH 4.0).

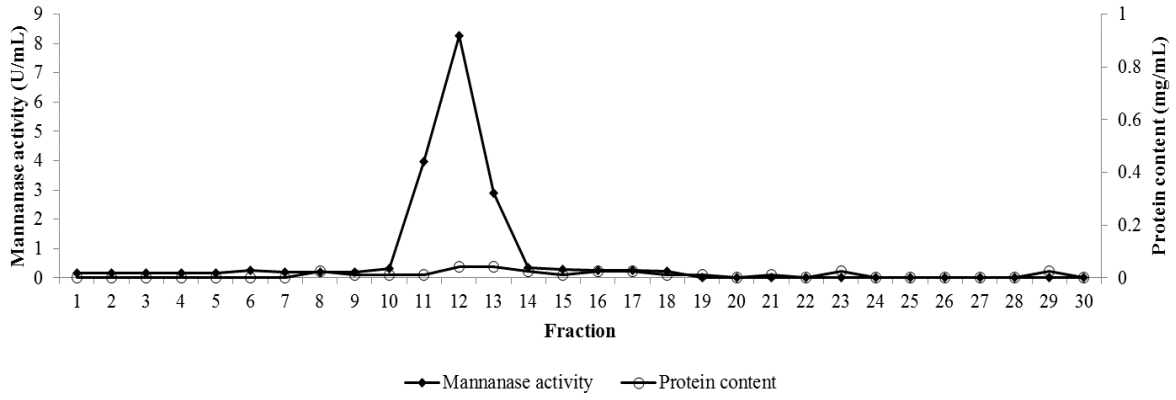

Figure 2: Purification of β-mannanase using Q-sepharose fast flow column with 50 mM citric acid-trisodium citrate buffer (pH 5.0) containing 0.8 M NaCl.

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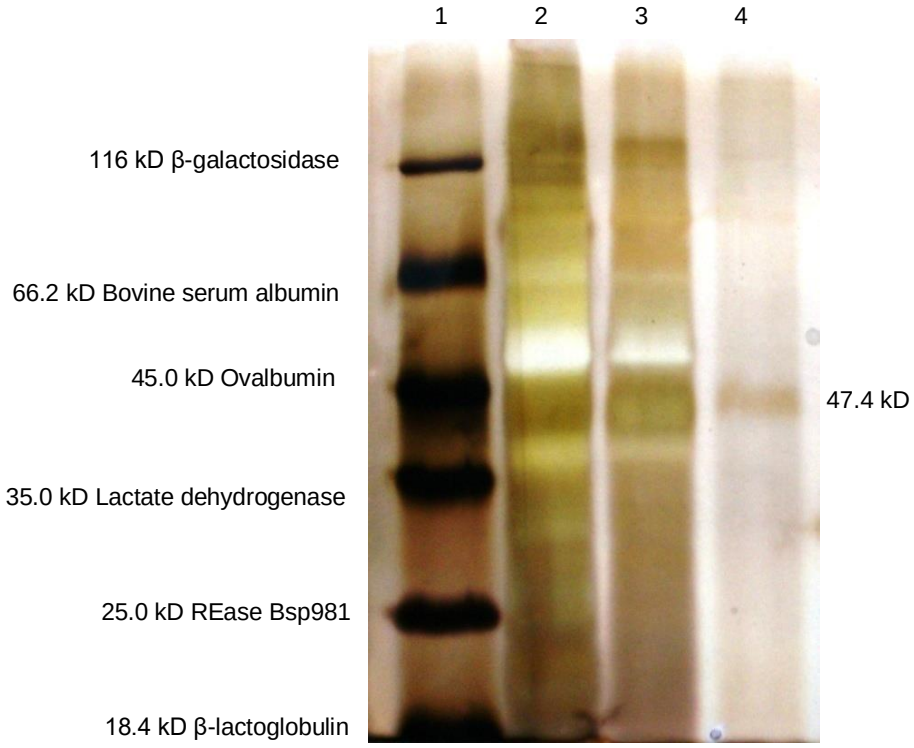

Figure 3: SDS-PAGE (12.5%) of purified β-mannanase from *A. niger* USM F4. Lane 1: low molecular protein standard, lane 2: crude culture filtrate, lane 3: Superdex 75 column sample and lane 4: purified sample from Q-sepharose fast flow column.

The optimum pH condition for the purified *A. niger* β-mannanase to degrade the LBG was at pH 4.0 (50 mM citric acid-trisodium citrate buffer) (Figure 4). The relative activity was 93% at pH 3.0 and the value gradually increased to its peak at 100% at pH 4.0. The activity decreased from pH 5.0 onwards. During the stability test, purified β-mannanase showed a remarkable endurance towards pH 3.0 to 9.0 thresholds until 180 min. A 30 min half-life of the residual activity was discovered at pH 10.0 (Figure 5). According to the graph, the enzyme showed higher stability at pH 4.0 than pH 5.0 and 6.0.

The purified fungal β-mannanase is known to have a tendency on the acidic to neutral pH condition. It has been reported that the enzyme secreted from *Aspergillus* species, particularly *A. niger*, *A. fumigatus*, *A. terreus* and *A. oryzae,* are more stable at pH conditions of 3.5 to 7.0 (Ademark *et al.*, 1998; El-Refai *et al.*, 2014; Soni *et al.*, 2016). In general, the purified fungal enzymes are well-adapted to a broad range of pH values viz. pH 3.0 to 8.0 (Mudau and Setati, 2008; Naganagouda *et al.*, 2009; Soni *et al.*, 2016). However, the current pH stability was 1.0 unit higher than the above-mentioned reports, suggesting that the purified β-mannanase from *A. niger* USM F4 was still active in alkaline conditions.

Temperature is another *de facto* aspect that influenced the catalytic activity of proteins and both physical factors (pH and temperature) in determining the application of proteins in industrial settings. In this study, the relative activity of the purified β-mannanase started with 89% at 50 °C and optimally active (100%) at 60 °C (Figure 6). The activity exhibited a declining trend at 70 °C (97%) and 80 °C (92%). At temperatures of 50 °C to 70 °C, the enzyme activities were stable up to 180 min. However, at 80 °C, there was a 50% shortfall detected right after 30 min of the thermal exposure (Figure 7).

In term of characterisation of temperature, the result was aligned to Wu *et al.* (2011), Soni *et al.* (2016) and Karahalil *et al.* (2019). They recorded 60 °C to 70 °C as optimum temperature ranges for mannan degradation by the genus *Aspergillus* β-mannanase. Adesina *et al.* (2013) contrarily evident the β-mannanase required a moderate temperature of 30 °C to trigger and maximise the process. In terms of thermal stability, the recent experiment indicated β-mannanase from *A. niger* could endure high temperature up to 70 °C for 180 min. The result was better than previously reported by Wu *et al.* (2011) and Adesina *et al.* (2013) in which the enzyme stability only resists at temperatures of 60 °C and below.

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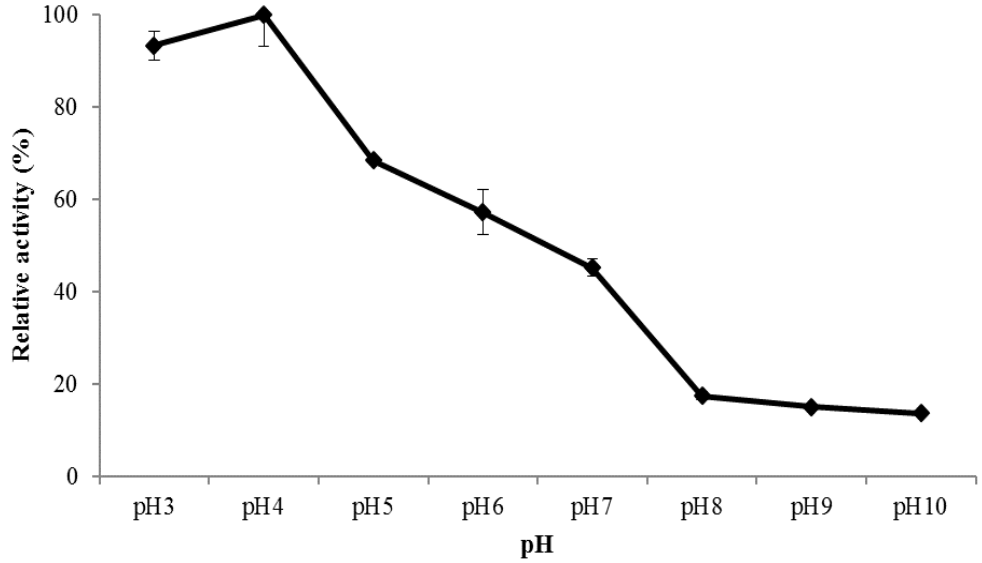

Figure 4: Influence of pH on the activity of *A. niger* β-mannanase. Conditions: the enzyme was blended with 0.5% (w/v) LBG in 50 mM citric acid-trisodium citrate buffer, phosphate buffer, Tris-HCl buffer and glycine-NaOH buffer, 60 °C.

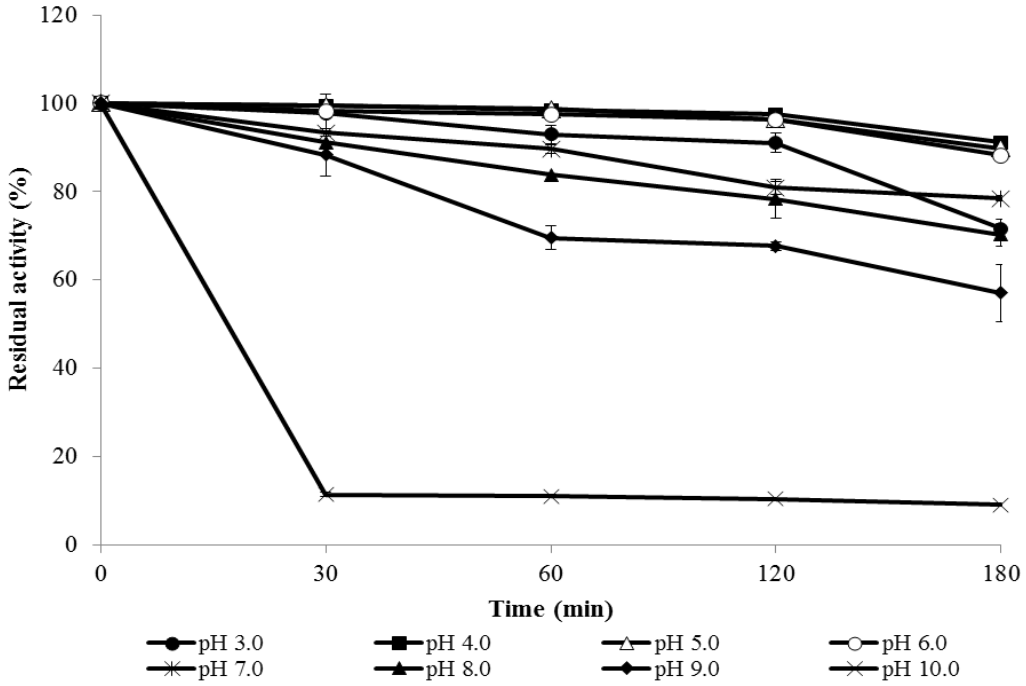

Figure 5: Stability of purified β-mannanase from *A. niger* USM F4 at various pHs. Conditions: the enzyme was pre-incubated without LBG in 50 mM citric acid-trisodium citrate buffer (pH 3.0-5.0), phosphate buffer (pH 6.0-7.0), Tris-HCl buffer (pH 8.0-9.0) and glycine-NaOH buffer (pH 10.0) and the activity was determined using 0.5% (w/v) LBG in 50 mM citric acid-trisodium citrate buffer at 60 °C.

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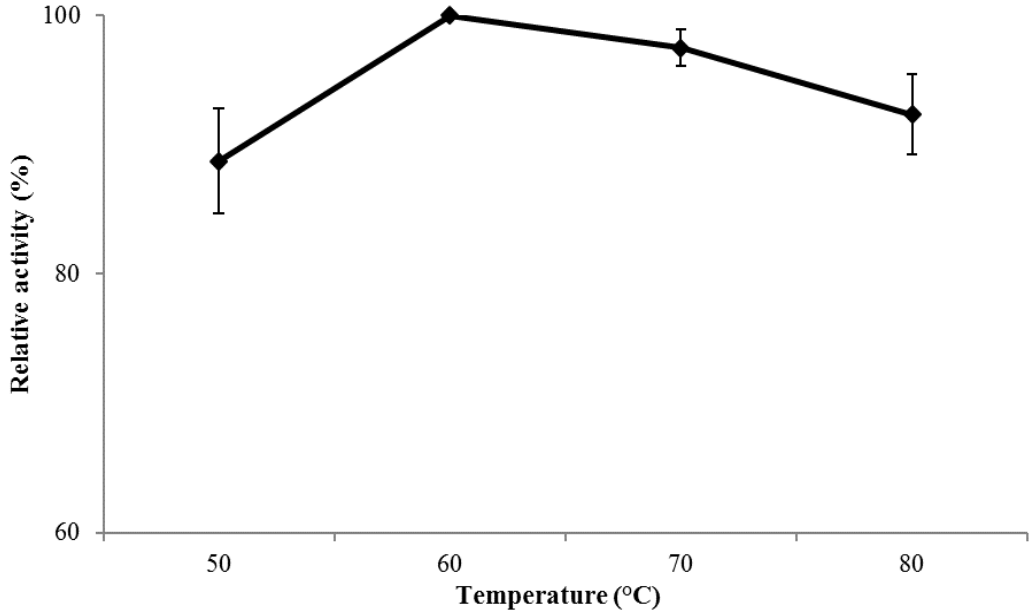

Figure 6: Influence of temperature on the activity of purified β-mannanase from *A. niger* USM F4. Conditions: the enzyme was mixed with 0.5% (w/v) LBG in 50 mM citric acid-trisodium citrate buffer (pH 4.0) and incubated at 50 °C to 80 °C.

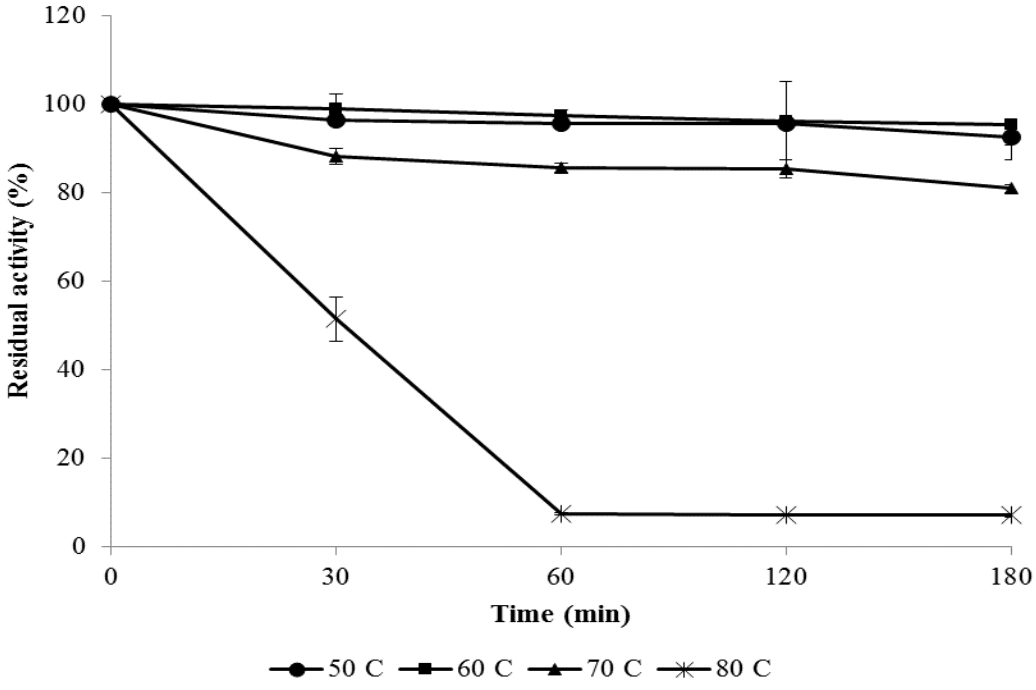

Figure 7: The thermal stability of purified β-mannanase at different temperatures. Conditions: purified enzyme was pre-incubated without substrate in 50 mM citric acid-trisodium citrate buffer (pH 4.0) at temperatures of 50 °C to 80 °C. The assay was estimated using 0.5% (w/v) LBG in 50 mM citric acid-trisodium citrate buffer at 60 °C.

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The influence of several substances including organic compounds, metal ions and chelating agents on purified β-mannanase activity is depicted in Table 2. All substances inhibited the enzyme activity, with SDS pronounced as the strongest inhibitor at 1.0 mM. It led to a decrease in purified enzyme activity to 28.16 ± 0.05%. Cupric ion (Cu^{2+}) also exhibited a strong β-mannanase inhibitor after SDS, resulting in 49.51 ± 0.09% of residual activity. SDS was recognised as an inhibitor for bacterial mannanase especially *Bacillus* sp. The exposure of purified bacterial mannanase and SDS at 60 °C ended up with the activity reduction (Yoon *et al.*, 2008). It is worth to mention the inhibitory effect of SDS on another hemicellulose such as *Trichoderma inhamatum* xylanase where its activity for type-1 and type-2 enzymes was demolished in 10 mM concentration (Silva *et al.*, 2015). In general, the increase in SDS concentration has caused a decrease to the enzyme activity. The incident indicates that the SDS may interrupt the enzyme activity by invading its hydrophobic part and modified the three dimensional structures of the enzyme protein.

Table 2: The effect of chemical substances on purified β-mannanase activity.

Substance	Relative activity[a] (%)
Control	100.00
$SrCl_2$	62.06 ± 0.12
$ZnCl_2$	57.04 ± 0.11
$FeCl_3$	54.95 ± 0.10
$ZnSO_4$	54.11 ± 0.10
$CuSO_4$	49.51 ± 0.09
$CaCl_2$	54.11 ± 0.10
$MnCl_2$	72.52 ± 0.14
$AlCl_3$	66.66 ± 0.12
NaCl	67.92 ± 0.13
KCl	69.59 ± 0.13
$MgCl_2$	58.71 ± 0.11
EDTA	56.20 ± 0.10
2-mercaptoethanol	67.08 ± 0.12
SDS	28.16 ± 0.05

[a]The experiment was done in triplicate in separate occasions and data was expressed as in mean ± standard deviation.

The metal ions could connect with proteins *via* multiple networks linked to the amine or carboxylic acid group. According to Naganagouda *et al.* (2009), Cu^{2+} was among the top four inhibitors, besides mercury ions (Hg^{+}), silver ions (Ag^{+}), and *N*-bromosuccinimide (NBS). To date, information on the influence of divalent ions on mannanase and its catalytic site is scarcely researched and poorly explained. The ions occurrence may probably be associated with redox reaction on the amino acids which indirectly contributed to the enzyme activity fluctuation (Tejirian and Xu, 2010). The inhibition may also associate with the ionic charge and radius size of the enzymes. Amino acids have more intensive affinity on smaller radius sizes than the large sizes. Usually, the attachment between small radius sizes and amino acids cause numerous damages of the catalytic site (Zeng *et al.*, 2014).

As reported by Naganagouda *et al*. (2009), Soni *et al.* (2016) and Karahalil *et al.* (2019), purified β-mannanase has a higher affinity towards LBG, a mannan polysaccharide containing-1,4-mannosidic linkages. The filamentous fungus has actively degraded the LBG and synthesised 100 ± 0.50% enzyme activity (Table 3). According to the kinetic study of LBG, the K_m and V_{max} values were evidenced to be 0.20 mg/mL and 9.82 U/mL, respectively. The assayed activities produced from other substrates were 17.00 ± 0.22% (carboxymethyl cellulose, CMC), 16.59 ± 0.17% (soluble starch), 16.17 ± 0.22% (xylan from oat spelt) and 15.61±0.03% (α-cellulose). The result was in line with that of β-mannanase produced from *A. niger* (Naganagouda *et al.*, 2009), *A. terreus* (Soni *et al.*, 2016) and *A. fumigatus* (Karahalil *et al.*, 2019). The presence of different sugar units such as glucose, xylose and amylose may construct a steric interference to the accessibility of *A. niger* β-mannanase on CMC, soluble starch, xylan from oat spelt and α-cellulose. The mechanism concerning mannan-degrading enzymes capacity on LBG and other heterogeneous substrates, mainly in selecting the mannose or glucose at distal subsites, is remained unclear (Tailford *et al.*, 2009). Thus far, the majority of fungal mannanase with glycoside hydrolase (GH) families 5 and 26 have a greater affinity towards glucomannan LBG (Xia *et al.*, 2016).

Table 3: Substrate specificities of purified β-mannanase assayed at 60 °C, pH 4.0.

Substrate type	Relative activity[a] (%)
LBG	100.00 ± 0.50
Xylan from oat spelt	16.17 ± 0.22
CMC	17.00 ± 0.22
α-cellulose	15.61 ± 0.03
Soluble starch	16.59 ± 0.17

[a]The experiment was done in triplicate in separate occasions and data was expressed in mean ± standard deviation.

We have done a comparison to evaluate the enzyme affinity on LBG in Table 4. In general, enzyme with low K_m has a high affinity for its substrate and commonly necessitates a lower concentration of substrate to attain V_{max}. From the table, the K_m generated by both *A. niger* β-mannanase [USM F4 = 0.20 mg/mL, Naganagouda *et al.* (2009) = 0.11 mg/mL] were lower than *A. terreus* [Soni *et al.* (2016) = 5.9 mg/mL] and *A. fumigatus* [Karahalil *et al.* (2019) = 10.48 mg/mL]. The application of Lineweaver-Burk plot or double reciprocal plot in evaluating the K_m and V_{max} values has statistical inherent limitation. Our linear plot was more enunciated at low substrate concentration (graph not shown) and caused the occurrence of imbalance weighting of errors. This event has generated long lever arm effect which may influence the estimation of K_m value due to extrapolation (Johnson, 2013).

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Table 4: The affinity of β-mannanase produced from different filamentous fungi on natural LBG.

Fungus	K_m (mg/mL)	V_{max} (U/mL)	References
A. niger USM F4	0.20	9.82	-
A. niger	0.11	14.13	Naganagouda *et al.* (2009)
A. terreus	5.9	39.42	Soni *et al.* (2016)
A. fumigatus	10.48	948.4	Karahalil *et al.* (2019)

CONCLUSION

As a summary, the purified β-mannanase from *A. niger* USM F4 has a molecular mass of ~47.4 kDa and reached its maximum activity under physical conditions of pH 4.0 and a temperature of 60 °C. These conditions were similar to the crude enzyme assay conditions. The enzyme was stable over an extensive range of pH values and temperatures, required no extra chemical substances to manifold the activity and had an affinity on the natural mannan source (LBG) that inexpensively available. Besides, *A. niger* does not produced toxin and generally recognised as safe (GRAS). These characteristics are appropriately relevant for application in the industrial league.

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