

Evaluation of the activities of concentrated crude mannan-degrading enzymes produced by *Aspergillus niger*

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ABSTRACT

The mannan-degrading enzymes produced by *Aspergillus niger* were concentrated and the activities were evaluated. The optimum pH for β -mannanase, endoglucanase and α -galactosidase were obtained at pH 3.5 while pH optimum for β -mannosidase was occurred at pH 3.0. The β -mannanase, endoglucanase, β -mannosidase and α -galactosidase was stable at pH 3.5 to 7, pH 3.5 to 6.5, pH 4 to 7 and pH 3.5 to 5.0, respectively. The enzymes obtained in this study were characterized and defined as acidic proteins. The β -mannanases from *A. niger* had two optimum temperatures (at 50 °C and 60 °C) and its half-life was 6 h and 4 h at 60 °C and 70 °C, respectively. The β -mannosidase, endoglucanase and α -galactosidase displayed optimal activity at 70 °C, 60 °C and 50 – 60 °C, respectively. The β -mannosidase had half-life of 1.5 h at 70 °C, while α -galactosidase had a half-life of 2.5 h at 60 °C and endoglucanase had a half-life of 6 h at 60 °C and 45 min at 70 °C.

Keywords: *Aspergillus niger*, mannan-degrading enzymes, β -mannanase, endoglucanase, β -mannosidase, α -galactosidase

INTRODUCTION

The biotechnological potential of mannan-hydrolysing enzymes, in particular the β -mannanases, has been demonstrated within various industrial areas. β -mannanases have potential application in animal feed production (Wu *et al.*, 2005; Lee *et al.*, 2005; Sae-Lee, 2007), paper bleaching (Talbot and Sygusch, 1990), coffee production (Araujo and Ward, 1990) and laundry detergents (Schafer *et al.*, 2002). Bioconversion of agriculture waste containing mannan-based polysaccharides into valuable products such as animal feeds also required microorganisms capable to produce mannan degrading enzymes. Mannan-based polysaccharides are complex hetero-polymers. These types of polymers exist in the form of galactomannan and galactoglucomannan (de Vries, 2003). In the seed of leguminous plants, they also exist as storage polymers in the form of galactomannan (Lee *et al.*, 2005). Many microorganisms such as *Aspergillus niger* (Ademark *et al.*, 1998), *Trichoderma reesei* (Arisan-Atac *et al.*, 1993) and *Sclerotium rolfsii* (Gubitz *et al.*, 1996) are capable of decomposing mannans. Agriculture waste containing mannan-based polysaccharides is not suitable as animal feed because high mannan content impairs the digestibility and utilization of nutrients either by direct nutrients encapsulation or by increase the viscosity of the intestinal contents (Lyayi *et al.*, 2005). This will reduce the rate of hydrolysis and the absorption of nutrients in the diet. Inoculation of mannan degrading enzymes into such

wastes will help the digestion of the mannans to release digestible sugars, which can then be fully absorbed and metabolised by monogastric animals.

For a complete hydrolysis of heteromannan, the enzyme cocktail containing endo-1,4- β -mannanase (EC 3.2.1.78), β -mannosidase (EC 3.2.1.25), endoglucanase (EC 3.2.1.4) and α -galactosidase (EC 3.2.1.22) is required. Endo-1,4- β -D-mannanases initiates depolymerisation by random hydrolysis of the β -D-1,4-mannopyranosyl linkages within the main chain of mannan backbone releasing manno-oligosaccharides of various length (Franco *et al.*, 2004). The β -mannosidase and α -galactosidase remove terminal mannose and galactose residues from the mannan backbone, respectively (Stalbrand *et al.*, 1993). The β -mannosidase is a type of glycosidases that cleaves the β (1,4)-mannobiose and mannotriose as well as the trisaccharides, Man-GlcNAc-GlcNAc and disaccharides Man-(β 1-4)GlcNAc and Man-(β 1-4)ManNAc (Elbein *et al.*, 1977). It catalyzes the removal of β -D-mannose residues from the non-reducing ends of oligosaccharides. The α -galactosidases hydrolyse terminal non-reducing α -galactose residues from α -galactosides including galactose containing oligosaccharides, galactoglucomannans and galactolipids and removes α -1,6-linked galactose residues from galactomannan polymers (Stoll *et al.*, 1999). Endoglucanase is required to hydrolyse, in random manner, soluble derivatives of cellulose and amorphous cellulose (Eriksson and Wood, 1985).

For the selection of appropriate microorganism to be used in direct conversion of agriculture waste containing mannan-based polysaccharides to valuable feed, knowledge on the characteristics of mannan degrading enzymes produce by the target microorganism is greatly needed. Little is known about the potential and characteristics of mannan-degrading enzymes produced by *Aspergillus niger*, which is easily grown fungus in solid substrates. The ability of *A. niger* to grow in palm kernel cake, waste containing mannan-based polysaccharides, for mannanase production has been reported (Abd-Aziz *et al.*, 2008). The objective of this study was to evaluate the activities of the concentrated crude mannan-degrading enzymes produced by *A. niger*. The enzymes evaluated include β -mannanase, endoglucanase, β -mannosidase and α -galactosidase. The characteristics of the enzymes studied include pH and temperature optimum, as well as pH and temperature stability.

MATERIALS AND METHODS

Microorganisms and fermentation

The mould, *Aspergillus niger* ATCC 20114, was purchased from American Type Culture Collection (ATCC). Stock culture was maintained on potato dextrose agar (PDA) and routinely sub-cultured at every 4 weeks. The mould was cultivated in 1 L Erlenmeyer flask consisted of 400 mL medium and autoclaved at 121 °C, 15 psi for 20 min prior to inoculation. The fermentations for enzyme production were carried out using basal medium containing (g/L): guar gum, 21.3; bacteriological peptone, 57; NH_4NO_3 , 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5; KH_2PO_4 , 1.2; KCl, 0.6, and trace element solution, 0.3 (mL/L) and pH of the medium was adjusted to 5.47 with phosphoric acid prior to sterilization. The flasks were incubated for 10 days at 30 °C under continuous agitation (150 rpm) on rotary orbital shaker.

Preparation of concentrated crude enzymes

The culture broth was centrifuged at 10,000 g for 10 min to remove the cells. The proteins in supernatant were precipitated using 80% (w/v) $(\text{NH}_4)_2\text{SO}_4$, which was optimal for the precipitation of all the concerned enzymes (data not shown). The precipitate was recovered by centrifugation at 10000 g (10 min) and then dissolved in a small volume of 50 mM sodium citrate buffer at pH 4.5. The protein solution was filtered through tangential flow membrane filtration system (Millipore Pellicon XL) employing membrane with molecular cut-off (MWCO) of 8 kDa to remove the salts. The concentrated protein solution was buffered with 20 mM Tris-HCl buffer, pH 7.0.

Determination of temperature and pH stability

In the experiment to study the thermal stability of the enzymes, the buffered enzyme preparations, β -mannanase (30 nkat/mL), endoglucanase (5 nkat/mL), β -mannosidase (0.1 nkat/mL), α -galactosidase (0.3 nkat/mL)

(50 mM sodium citrate buffer, pH 4.5) were incubated in a water bath at different temperatures (30 to 80 °C) for 6 h. Similar enzyme preparations were also used for the experiment to determine the pH stability of the enzymes, but the pH was adjusted to different values. The following buffers were used to adjust the pH to the required value; pH 2.0 to 2.5 – 50 mM potassium chloride; pH 3.0 to 5.5 – 50 mM sodium citrate; pH 6 to 8.5 – 50 mM phosphate buffer. The enzyme solution was incubated at 50 °C for 24 h. During the incubation, aliquots were withdrawn at different time intervals, cooled on ice immediately to stop the reaction, and stored at 4 °C prior to enzyme assay.

Determination of temperature and pH optimum

The optimum temperatures for enzyme activities were determined by performing standard assay at 30 °C to 90 °C in sodium citrate buffer (50 mM, pH 4.5). Bovine serum albumin (BSA, Sigma) was added at a concentration of 100 $\mu\text{g/mL}$. Samples were withdrawn at every 24 h intervals, for the determination of enzymes activity. The residual activity was calculated as percentage of the original activity. The optimum pH value was evaluated by assaying the activity at 50 °C using substrates dissolved in different types of buffer at pH values ranged from pH 2.0 to 8.5. Different buffers were used to adjust the different pH; 2.0 – 2.5 (50 mM potassium chloride buffer), pH 3.0 – 5.5 (50 mM sodium citrate buffer) and pH 6 – 8.5 (50 mM phosphate buffer). Relative activity was calculated as percentage of maximal activity under standard assay conditions.

Analytical procedure

Mannanase and endoglucanase activity were assayed using 0.5% (w/v) locust bean gum (LBG) and 1% (w/v) carboxymethylcellulose solution in 50 mM sodium citrate buffer, pH 4.0 as substrate (Ghose, 1987; Sachslehner and Haltrich, 1999). The enzyme preparation (200 μL) was added into 1800 μL of substrate. The reaction mixtures were incubated at 50 °C in a water bath for 5 min and 30 min for mannanase and endoglucanase, respectively. The quantity of reducing sugar released was measured using dinitrosalicylic acid (DNS) method (Miller, 1959). One unit of enzyme activity is defined as the amount of enzyme producing 1 nmol of mannose or glucose per sec under the assay condition.

On the other hand, β -mannosidase and α -galactosidase were assayed using *p*-nitrophenyl- β -D-mannoside (PNP- β Man) and *p*-nitrophenyl- α -D-galactoside (PNP- α Gal) as substrates, respectively (Grosswindhager *et al.*, 1999). In this method, 250 μL of substrates *p*-nitrophenyl- β -D-glycosides (PNP- α Gal and PNP- β Man) were added to 500 μL of 50 mM sodium citrate buffer (pH 4.5). To start the reaction, 25 μL of enzyme preparation was added to the mixture and then incubated at 50 °C for 10 min. To stop the reaction, 2.0 mL of 1 M Na_2CO_3 solution was added to the reaction mixture. The absorbance of liberated *p*-nitrophenol was measured

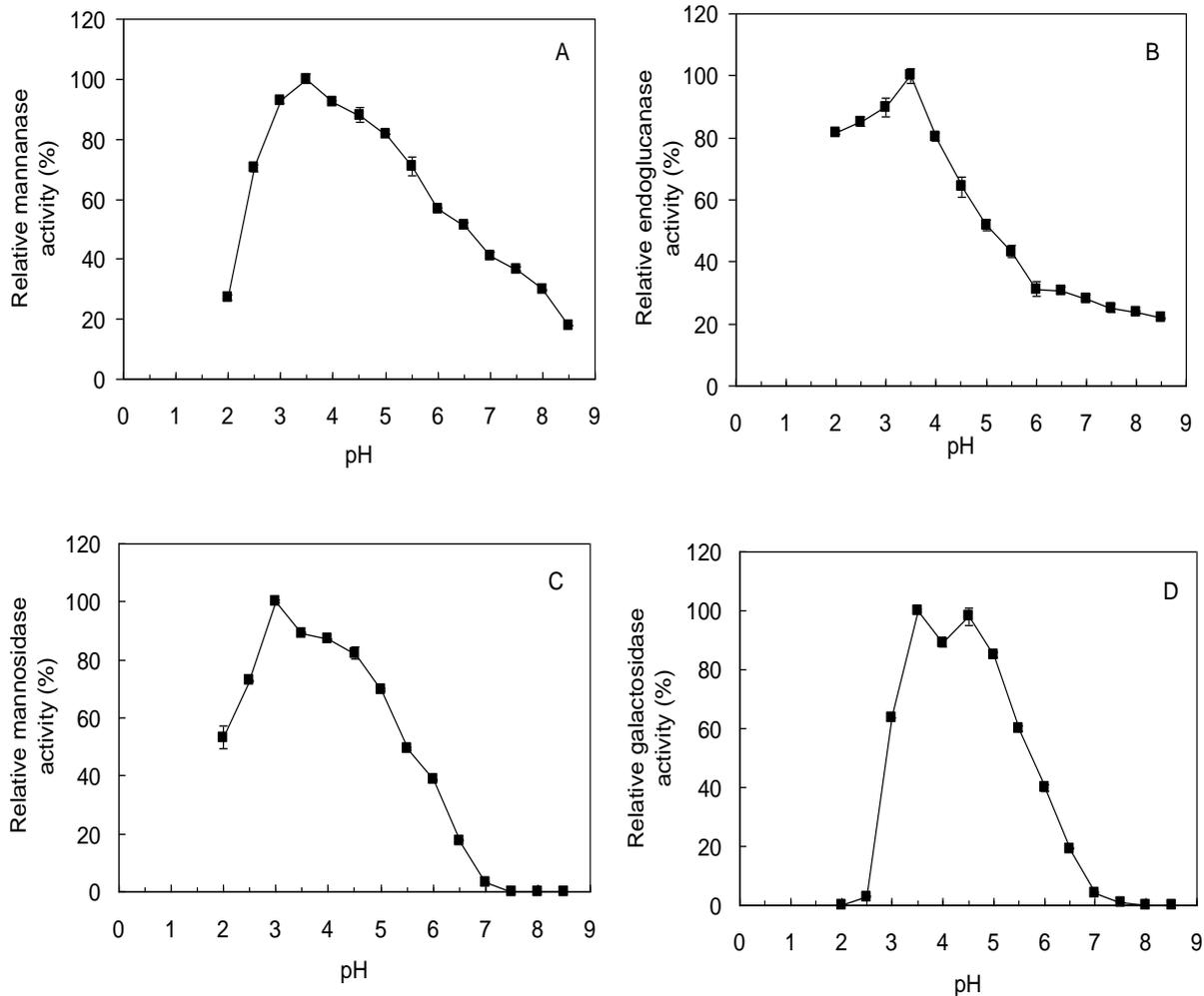


Figure 1: Effect of pH on the activity of (A) β -mannanase, (B) endoglucanase, (C) β -mannosidase and (D) α -galactosidase from *A. niger*. Incubation times for β -mannanase, endoglucanase, β -mannosidase and α -galactosidase assays were 5, 30, 10 and 10 min, respectively. Relative activity is calculated as percentage of maximal activity. Error bars are mean \pm S.D. of triplicate determinations

using spectrophotometer at 405 nm. One unit of enzyme activity is defined as the amount of enzymes producing 1 nmol of *p*-nitrophenol per sec under the assay condition.

For all enzymes assays, one unit of enzyme activity is expressed in SI units, Katals (1 kat = 1 mol/s). This unit of enzyme activity was recommended by the Commission on Biochemical Nomenclature (Florkin and Stotz, 1973) to replace the earlier International Unit (1 IU = 1 μ mol/min = 16.67 nkat). Recently, the unit in Katals was widely used by many researchers to determine the mannan-degrading enzymes activities (Singh *et al.*, 2000; Ademark *et al.*, 2001; Reddy *et al.*, 2002).

RESULTS AND DISCUSSION

pH optimum

The β -mannanase activity was the highest at pH 3.5 (Figure 1A). The enzyme was active in the acidic region,

retaining more than 65% of its maximal activity at pH 2.5. On the other hand, the relative activity was reduced to 30% at pH 8.0. The acidic pH optimum (pH 3.5) for enzymes produced by *A. niger* was similar to, or lower than, those of fungal β -mannanases that usually lie between pH 3 – 5.5 (Stalbrand *et al.*, 1993; Christgau *et al.*, 1994; Ademark *et al.*, 1998; Sachslehner and Haltrich, 1999). However, it was lower than those reported for bacterial β -mannanases which have pH optima close to neutral pH (Viikari *et al.*, 1993). Recently, β -mannanase produced by *Scopulariopsis candida* most active at pH 6 has been reported (Mudau and Setati, 2008).

The optimal pH of endoglucanase was at pH 3.5 (Figure 1B), similar to that reported for thermophilic fungus, *Thermoascus aurantiacus*, which exhibited its maximum activity at pH 3.5 and 4 (Kalogeris *et al.* 2003). The optimal activity for endoglucanase at higher pH has also been reported. For example, the optimal pH for

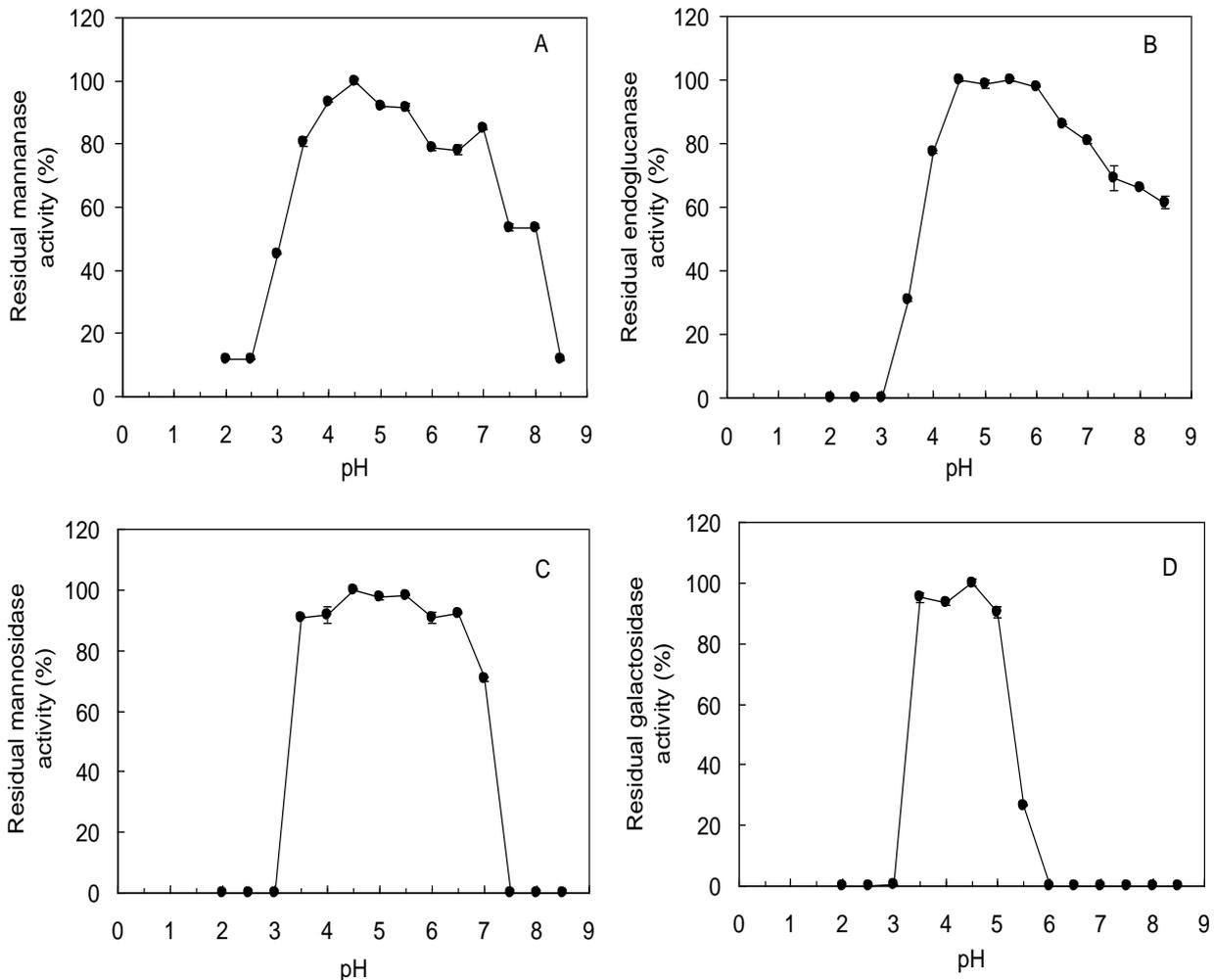


Figure 2: Effect of pH on the stability of partially purified (A) β -mannanase, (B) endoglucanase, (C) β -mannosidase and (D) α -galactosidase from *A. niger*. The residual activity is shown as percentage of the original activity. Error bars are mean \pm S.D. of triplicate determinations

endoglucanase of *Mytilus edulis* isolated from blue mussel was 4.6 (Xu *et al.*, 2000), while *Talamromyces emersonii* showed optimum activity at pH 4.8 (Murray *et al.*, 2001). In general, fungal cellulases were optimally active at pH ranging from 4.0 to 6.0, whereas bacterial cellulases show a pH optimum at pH ranging from 6.0 to 7.0 (Wood, 1985).

The pH optimum for β -mannosidase activity was obtained at pH 3.0 in 50 mM sodium citrate buffer at 50 °C (Figure 1C). This optimal pH values was closed to the values for β -mannosidase from *T. reesei* (Kulminskaya *et al.*, 1999), *A. niger* (Bouquetet *et al.*, 1978) and *A. awamori* (Neustroev *et al.*, 1991) which was obtained at pH 3.5. The optimum pH for this concentrated crude enzyme preparation was very acidic as compared to β -mannosidase from *Rhizopus niveus* (pH 5.0) (Hashimoto and Fukumoto, 1969) and *Bacillus* sp. (pH 6) (Akino *et al.*, 1988). The pH optima for the activity of partially purified α -galactosidase was observed at pH 3.5 and pH 4.5 (Figure 1D). The existence of these two peaks indicates that this

strain produced more than one form of α -galactosidase enzyme. Some fungi have been proven to produce more than one form of α -galactosidase. For example, *A. tamari* (Civas *et al.*, 1984), *P. simplicissimum* (Luonteri *et al.*, 1998) and *T. reesei* (Margolles-Clark *et al.*, 1996). Manzanares *et al.* (1998); Knap *et al.* (1994) and den Herder *et al.* (1992) found that *A. niger* produced more than one form of α -galactosidase. Similar pH optima have also been described for different fungi, *P. simplicissimum* (pH 3.5 – 4.5) (Luonteri *et al.*, 1998), *Thermomyces lanuginosus* (pH 4.5 – 5.0) (Puchart *et al.*, 2000) and *P. purpurogenum* (pH 4.5) (Shibuya *et al.*, 1995).

pH stability

The mannanase was stable at pH 3.5 to 7.0 where 80% of its activity was retained (Figure 2A). The β -mannanase was quite stable at its optimal pH which retained more than 80% of its original activity at pH 3.5 for 24 h at 50 °C.

From the stability point of view the β -mannanase was more stable in the acidic pH region than in alkaline; where at pH 8.5 its relative activity was only 10% at 50 °C. McCleary (1988) reported that β -mannanase of *A. niger* was stable at pH ranged from 3.8 – 8.0. The endoglucanase was stable at pH 4.0 to 7.0 as more than 80% of the maximal activity was still remained after 24 h of incubation at 50 °C (Figure 2B). β -glucanase *T. emersonii* displayed activity over a broad pH, ranging from pH 2.5 – 8.0 (Murray *et al.*, 2001). The endoglucanase was relatively less stable at low pH than at higher pH tested. Even though the optimal pH for endoglucanase was at pH 3.5, but about 70% of the activity was loss at this pH value.

The β -mannosidase enzyme has broad pH stability, as more than 80% of the maximal activity remained following the 24 h incubation at 50 °C in buffers at pH ranging from 3.5 to 6.5 (Figure 2C). However, it was more

stable in acidic region than alkaline region. Similar report was given by Kulminskaya *et al.* (1999) which stated that the β -mannosidase from *T. reesei* was stable at pH ranging from 3.5 to 6. Akino *et al.* (1988) found that β -mannosidase from *Bacillus* sp. was stable at more neutral pH, from pH 6.5 to 8.0 after 30 min incubation at 40 °C. The α -galactosidase produced in this study was stable in a very narrow pH range, retained its activity more than 80% at pH 3.5 – 5.0 (Figure 2D). The pH optimum of α -galactosidase was in the same range as its pH stability. Incubation for 24 h at 50 °C in sodium citrate buffer at pH 3.0 and 6.0 resulted to total lost of the activity. The endoglucanase from *T. lanuginosus* at 50 °C was stable at a wide pH range (pH 3.0 to 7.5) (Puchart *et al.*, 2000)

Temperature optimum

Optimal temperature for mannan-degrading enzymes

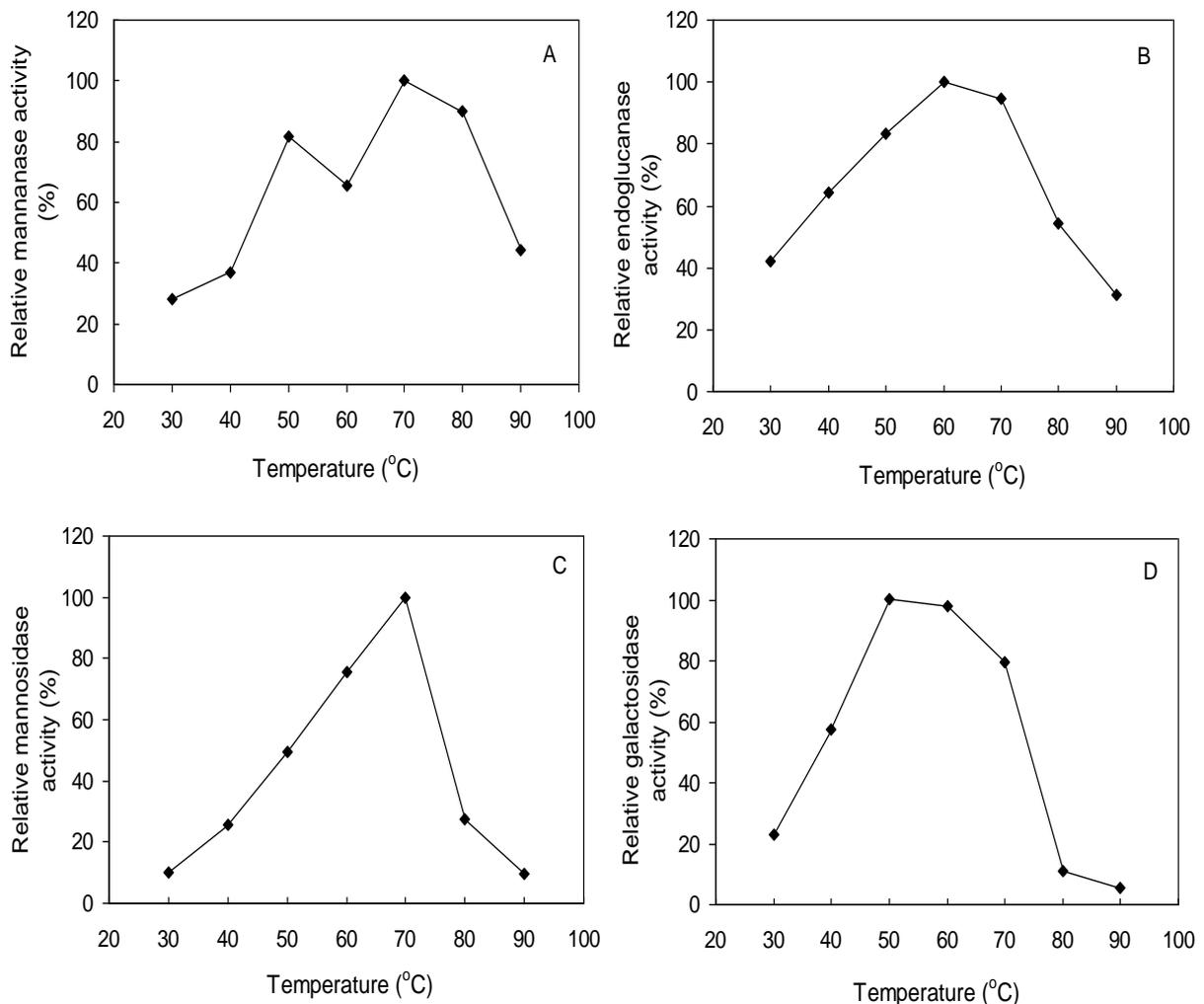


Figure 3: Effect of temperature on the activity of (A) β -mannanase, (B) endoglucanase, (C) β -mannosidase and (D) α -galactosidase from *A. niger*. Thermostability studies were conducted at temperature ranged from 30 to 90 °C. Relative activity was calculated as percentage of maximal activity. Values are mean of triplicate experiments

was varied depending on the sources. The β -mannanase obtained in this study has two separate and distinct maxima activity at 50 °C and 70 °C as shown in Figure 3A. These peaks may indicate that these species secreted two or more distinct β -mannanase into their environment. Viikari *et al.* (1993) reported that mannanases were usually secreted into the culture fluid as multiple enzyme form. Gūbitz *et al.* (1996) also found two mannanases which had optimum temperature between 72 °C and 74 °C from *S. rolfisii*. β -mannanase obtained in this study was in the range of those previously reported in the literature. For example, 57 °C for *Streptomyces* sp. (Takahashi *et al.*, 1984), 75 °C for *Thielavia terrestris* (Araujo and Ward, 1990), and 65 °C for *Aspergillus* sp. (Eriksson and Winell,

1968). It was also notable that the enzyme exhibited a high level of activity over a broad temperature range, with levels of activity in excess of 50% of the maximum at 45 °C – 85 °C after 5 min of incubation.

The effect of temperature on the activity of endoglucanase is shown in Figure 3B. The maximum activity of endoglucanase was occurred at 60 °C. The optimal temperature for endoglucanase of *Rhizopus oryzae* (Murashima *et al.*, 2002), *Bacillus polymyxa* (Gosalbes *et al.*, 1991) and *B. brevis* (Louw *et al.*, 1993) was 55 °C – 55 °C, 60 °C and 65 °C, respectively. The endoglucanase exhibited 50% of its maximal activity at 35 °C – 80 °C after 30 min of incubation.

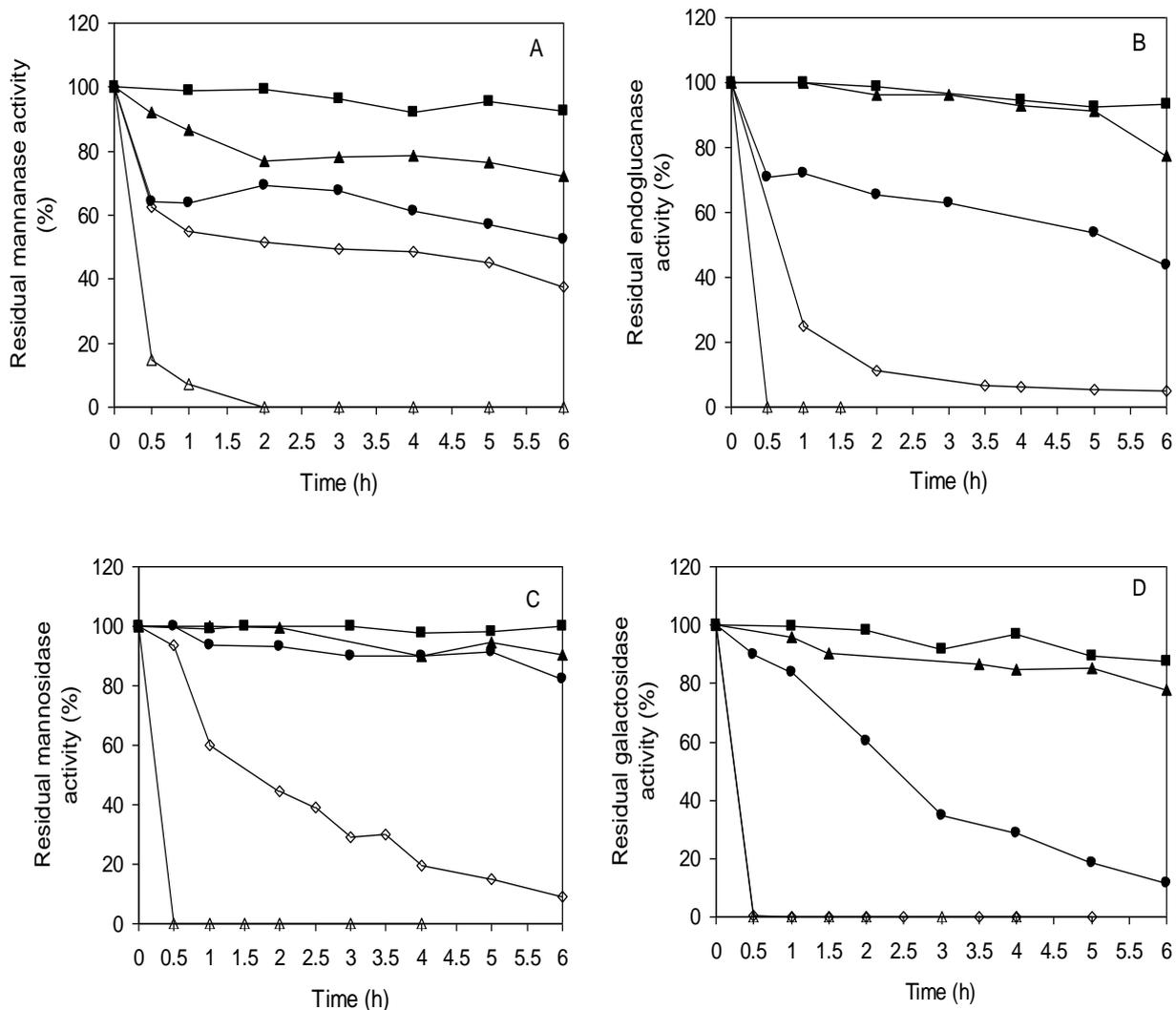


Figure 4: Effect of temperature on the stability of (A) β -mannanase, (B) endoglucanase, (C) β -mannosidase and (D) α -galactosidase from *A. niger*. The residual activity is shown as percentage of the original activity and was measured after incubation at different temperatures (40 – 80 °C) at 50 mM sodium citrate buffer, pH 4.5. Values are mean of triplicate experiments. Symbols represent: (■) 40 °C; (▲) 50 °C; (●) 60 °C; (◇) 70 °C; (Δ) 80 °C

The β -mannosidase activity was optimal at 70 °C under assayed conditions (Figure 3C) indicating that the enzyme was thermoactive. Bouquelet *et al.* (1978) reported that β -mannosidase from *A. niger*, was active at 55 °C while *A. awamori* was found to have maximum activity at 66 °C with a half life of less than 30 min at the optimum temperature (Neustroev *et al.*, 1991). Interestingly, β -mannosidase was the most active at 70 °C, which was about the same as the optimum temperature measured for the β -mannanases obtained in this study. This is important characteristic from the view of possible industrial applications. The presence of β -mannosidase and β -mannanase activity at high temperatures suggests that this strain might be useful source of enzymes to degrade mannan polysaccharides.

The optimum temperature for α -galactosidase at pH 4.5 was found at 50 °C – 60 °C (Figure 3D) in the same range for *A. niger* (Somari and Balogh, 1995; Manzanares *et al.*, 1998), *A. awamori* (Neustroev *et al.*, 1991), *P. ochrochloron* (Dey *et al.*, 1993) and *B. stearrowthermophilus* (Talbot and Sygusch, 1990). The α -galactosidase from *Humicola* sp. (Kotwal *et al.*, 1999) and *Thermomyces lanuginosus* (Puchart *et al.*, 2000) have a slightly higher optimum temperature of 65 °C – 70 °C.

Temperature stability

The β -mannanase appeared to be thermostable at 60 °C and 70 °C with half life of 6 h and 4 h, respectively (Figure 4A). No enzyme activity was detected after incubation for 2 h at 80 °C. The optimal temperature for β -mannanase of *Scopulariopsis candida* was ranged from 40 °C to 45 °C, and showed total loss of activity at temperature higher than 60 °C (Mudau and Setati, 2007). High stability of β -mannanase at high temperature is an important requirement for industrial application, particularly in animal feed industry. This is critical when there was a need to include enzymes in pelleted feed diets because heat is usually generated during the pelleting process which may cause enzymes deactivation (Inborr and Bedford, 1994).

The endoglucanase was stable at 40 °C and 50 °C, which retained more than 90% of its activity after incubation for 6 h and 5 h, respectively (Figure 4B). The estimated half life thermal stability value for endoglucanase from *A. niger* was at 60 °C and 70 °C for 6 h and 45 min, respectively. This enzyme was fully deactivated at 80 °C. The half life of β -mannosidase activity was about 1.5 h at its optimal temperature (70 °C) (Figure 4C). It was stable at 40 °C, 50 °C and 60 °C, and retained more than 80% of its activity for 6 h. The enzyme was fully deactivated after 0.5 h incubation at 80 °C.

The α -galactosidase was stable at 40 °C and 50 °C and the enzyme retained more than 80% of its original activity at both temperatures (Figure 4D). The half life of α -galactosidase was about 2.5 h at 60 °C. The α -galactosidase produced by *A. niger* was heat labile and completely destroyed by heating at 70 °C and 80 °C. The α -galactosidase from *A. niger* display a relatively low

thermostability (below 60 °C) in comparison to many α -galactosidase from other microorganisms that were stable at higher temperatures for long incubation period. The α -galactosidase from *Candida guilliermondii* was most active at 75 °C but has a half-life of only 20 min at this optimal temperature (Hashimoto *et al.*, 1993). The α -galactosidase from *Pycnoporus cinnabarinus* was thermostable without loss of activity at temperature below 75 °C (Ohtakara *et al.*, 1984). It was reported to have half life of 8.5 h at 85 °C and 3 min at 100 °C.

Characterization of enzyme essentially requires a purified preparation, not in a crude or mixed preparation. Although the activity and characterization of the enzymes conducted in this study were not in the purified form, the information obtained is sufficient to get better understanding on *A. niger* as the mannanase producing strain. In addition, the activities of the enzymes were assayed using specific substrate related to the activity of each enzyme. The information gathered is useful to identify the suitability of this strain in the modification of agrowaste, containing mannan, to animal feeds.

CONCLUSION

Results from this study have demonstrated that the easily grown fungus, *A. niger*, was capable to produce a cocktail of enzymes (β -mannanase, β -mannosidase, endoglucanase and α -galactosidase) required for the degradation of mannan-based polysaccharides. The enzymes activities were evaluated and defined as acidic proteins. The optimum pH for β -mannanase, endoglucanase and α -galactosidase were 3.5 while pH optimum for β -mannosidase was 3.0. The β -mannanase, endoglucanase, β -mannosidase and α -galactosidase was stable at pH 3.5 – 7, pH 3.5 – 6.5, pH 4 – 7 and pH 3.5 – 5.0, respectively. The β -mannosidase, endoglucanase and α -galactosidase displayed optimal activity at 70 °C, 60 °C and 50 °C – 60 °C, respectively. The β -mannosidase had half-life of 1.5 h at 70 °C, while α -galactosidase had a half-life of 2.5 h at 60 °C and endoglucanase had a half-life of 6 h at 60 °C and 45 min at 70 °C. This fungus may be used in the conversion of agriculture materials containing mannan-based polysaccharides, such as palm kernel cake, into animal feed. Palm kernel cake is an abundant waste produced by the palm oil industry and the potential use of this waste as animal feed have been reported (Sae-Lee, 2007).

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