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Full Length Research Paper

Purification and characterization of a thermostable glucoamylase produced by *Aspergillus flavus* HBF34

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Glucoamylase (GA) from *Aspergillus flavus* HBF34 strain was partially purified 120 folds using starch affinity chromatography. Two isoenzymes (GA1 and GA2) were identified by polyacrylamide gel electrophoresis (PAGE) zymography. Sodium dodecyl sulfate (SDS)-PAGE analysis revealed that one of the enzymes consist of one subunit and the other, two subunits. The optimum pH of the purified GA was 6.0 and the optimum temperature was 60°C. GA was found to be stable at temperatures up to 50°C and at a pH range between 3.0 and 9.0. Km and Vmax values of the enzymes were determined using soluble potato starch, glycogen, amylopectin and amylose as substrates and calculated to be 0.046, 0.075, 0.1 and 0.125 mg/ml and 769, 1250, 3333 and 2500 U/mg protein, respectively. While GA was activated by Mn^{2+}, Ca^{2+}, Co^{2+} and Ba^{2+}, it was inhibited by Hg^{2+}, Fe^{3+}, Al^{3+}, Zn^{2+} and Cu^{2+}. The activity of GA was found to be tolerant up to 5 M NaCl concentration. *N*-bromosuccinimide (NBS) and phenylmethanesulfonylfluoride (PMSF) inhibited the enzyme, suggesting the involvement of tryptophan and serine residues in the catalytic process. Raw corn starch adsorption of GA was found to be 93%. Thin-layer chromatography (TLC) results showed that amylase was in fact a glucoamylase.

Key words: *Aspergillus flavus*, glucoamylase, characterization.

INTRODUCTION

One of the most abundantly distributed polysaccharides in nature is starch, which is produced by plants. It is composed of two high molecular weight polymers, amylose and amylopectin. Amylose is a linear chain of glucose residues linked with α-1,4 bonds. Amylopectin is a branched polymer where the α-1,4-linked glucose residues are branched at 17-26 residue intervals with α-1, 6 bonds (James and Lee, 1997; Norouzian et al., 2006). A wide variety of microorganisms are able to degrade and utilize this natural high molecular weight biopolymer by secreting starch-degrading enzymes such as the α-amylases (EC 3.2.1.1), β-amylases (EC 3.2.1.2), glucoamylases (GA) (EC 3.2.1.3), isoamylases (EC 3.2.1.68), pullulanases (EC 3.2.1.41) and cyclodextrin glucanotransferases (EC 2.4.1.19). GA (1,4-α-D-glucan glucohydrolase) is an exo-acting enzyme that catalyses the production of β-D-glucose from the non-reducing ends of glucose containing amylose, amylopectin and glycogen by consecutively hydrolyzing α-1,4; α-1,6 and rare α-1,3 linkages. GA is present almost exclusively in filamentous fungi and far less in bacteria and yeasts (Fogarty and Kelly, 1990).

GA is important group of enzymes in starch processing. They are second to the proteases in worldwide distribution and sales among industrial enzymes. GA find many applications in industry. This enzyme is used in dextrose production, in the baking industry, in the brewing of low-calorie beer and in whole grain hydrolysis for the alcohol industry (James and Lee, 1997). The primary

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Abbreviation: TLC, Thin-layer chromatography; **NBS,** *N*-bromosuccinimide; **PMSF,** phenylmethanesulfonylfluoride; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis, **APA,** aflatoxin-producing-ability; **DNS,** 3,5-dinitrosalicylic acid; **GA,** glucoamylase; **EDTA,** ethylenediaminetetraacetic acid; **DTT,** 1,4- dithiothreitol; **DTNB,** 5,5'-dithiobis-(2-nitrobenzoic acid); **CMC,** 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodimide methyl *p*-toluenesulphonate; ***AR*,** adsorption rate.

commercial application of glucoamylases is the production of glucose syrups from starch. These applications require enzymes with high activity and thermostability. There are many advantages of using thermostable enzymes, such as, an increased reaction rate and decreased contamination risk through the use of high temperatures. The aim of the present study is to describe the methods for production, purification and characterization of an extracellular GA from *Aspergillus flavus* HBF34 strain for use in industrial applications.

MATERIALS AND METHODS

Chemicals

Analytical reagent grade chemicals were obtained from commercial sources. Unless mentioned otherwise, all chemicals were purchased from Merck (Darmstadt, Germany). Amylopectin, amylose, glycogen, rice starch, raffinose, wheat starch, dextrin, orcinol, maltotriose, maltotetraose, maltopentaose, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), *N*-bromosuccinimide (NBS), 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodimide methyl *p*-toluenesulphonate (CMC) and phenylmethanesulfonylfluoride (PMSF) were purchased from Fluka (Buchs, Switzerland). Bile salt, corn starch, *N,N'*-methylenebisacrylamide, 2-(*N*-morpholino)-ethanesulphonic acid (MES), $AlCl_3$, 1,4-dithiothreitol (DTT), yeast nitrogen base, 3,5-dinitrosalicylic acid (DNS), molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and bovine serum albumin (Fraction V) were purchased from Sigma (Taufkirchen, Germany). Na_2O_3Se and LiCl were purchased from AVOCADO (Karlsruhe, Germany). NaCl, KH_2PO_4, sucrose, sodium potassium tartrate and sodium citrate were purchased from Carlo Erba (Italy). KI, glucose and n-propanol were purchased from Riedel-de Haën (Seelze, Germany). Malt extract agar was purchased from Lab M (Bury, England).

Selection of fungi

All fungi used in this study were provided from the microbial culture stock in the Department of Biology at Adnan Menderes University. Screening of the 17 fungi samples was carried out using the starch plate medium containing 0.67% yeast nitrogen base, 1% soluble starch, 0.5% bile salt, and 2% agar-agar (Møller et al., 2004). Fungi on starch plates were inoculated to test for amylase secretion, incubated for 3 days and stained with an iodine solution (0.3% I_2, 0.6% KI). Amylase positive strains were detected by the presence of a clear zone of starch hydrolysis around the colony on the starch plates. The diameter of these clear zones, which was an indication of amylase activity, was measured. The *A. flavus* HBF34 was found to be the strain with highest amylase activity and was therefore selected for amylase production, purification and characterization. In addition, *A. flavus* HBF34 strain was tested for the ability to produce aflatoxin in aflatoxin-producing-ability (APA) test medium (Pitt et al., 1983) and results showed that, this strain did not produced aflatoxin.

Enzyme production

The culture medium used for amylase production contained (g/l): $NaNO_3$ 3.0, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.\ 7H_2O$ 0.01, KH_2PO_4 1.0, peptone 10; soluble potato starch 20. The pH was adjusted to 5.4 and aforementioned basal medium was sterilized by autoclaving at 121°C for 15 min. Approximately 10^8 spores/ml from 5-day-old cultures were inoculated into 500 ml Erlenmeyer flasks containing 100 ml of the culture medium. The incubation was carried out at 27°C for 7 days in a rotary shaker rotating (Memmert, Germany) at 150 rpm. The culture was sampled with 24-h intervals and the sample was filtered through a Whatman filter paper No. 1. The filtrate was dried at 80°C for 24 h and the dry weight was measured. The final pH, reducing sugar content and enzyme activity of the filtrate were measured. The culture filtrate with highest enzyme activity was lyophilized (Labconco, USA) and kept at -20°C prior to further purification.

Enzyme assay

Glucoamylase activity was determined according to Bernfeld's (1955) method using soluble potato starch (final concentration: 0.5%) as a substrate. Two hundred µl of 1.0 % soluble potato starch in 50 mM McIlvaine buffer (pH 6.0) with 200 µl of partially purified enzyme was mixed and incubated at 60°C for 30 min. The enzymatic reaction was stopped by the addition of 100 µl of (1%) DNS. After 5 min of heating at 100°C, the reaction mixture was chilled on ice for 2 min and then diluted to 1.0 ml by adding distilled water. The absorbance measurements were performed at 530 nm using spectrophotometer (Shimadzu UV–1700, Japan). A blank was prepared by adding DNS before the enzyme was added. The assay in each case was done in triplicate. The enzyme activities were calculated using a calibration curve prepared with D-glucose as a standard by following the same procedure described above. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of glucose per min from soluble starch at 60°C and pH 6.0. Specific activity was expressed as units of enzyme activity per mg of protein.

Protein determination

The protein concentrations of the enzyme samples were estimated using the method of Lowry et al. (1951) and bovine serum albumin was used as a standard. All measurements were performed three times and the average value was taken.

Purification of glucoamylase

The enzyme purification process was performed by the starch affinity method described in Najafi and Kembhavi (2005). Two gram insoluble corn-starch was washed with 50 mM McIlvaine buffer (pH 6.0) for 10 min at room temperature in order to remove all soluble parts and then centrifuged at 3000 × *g* (Sigma 3K-30, Germany) for 10 min at room temperature. One gram of lyophilized enzyme was dissolved in 10 ml of 50 mM McIlvaine buffer (pH 6.0). The denatured proteins were removed by centrifugation at 3000 x *g* for 1 min at 4°C. The supernatant was incubated on ice for 20 min with continuous shaking to achieve a homogenous temperature. The starch pellet was kept on ice for 20 min then mixed with the chilled supernatant. The suspension was incubated on ice and swirled slowly. After 120 min, the suspension was centrifuged at 3000 × *g* for 10 min at 4°C. The starch pellet was filled to a glass column (3 x 10 cm). A peristaltic pump (ATTO AC-2110, Japan) was connected to the column and then the starch column was washed with chilled 0.1 M NaCl at 4°C. After the washing process, starch was removed from the column and 50 mM McIlvaine buffer (pH 6.0, 10 ml) was

added to the starch and the suspension was incubated at 40°C for 60 min. Following incubation, the suspension was centrifuged at 3000 × *g* for 10 min at 4°C and washed three times with 50 mM McIlvaine buffer (pH 6.0). The supernatant was analyzed for enzyme activity and protein concentration. All experiments were conducted in triplicate.

Electrophoresis

Non-denaturing PAGE was used for the determination of the number of glucoamylase released to the culture medium by the *A. flavus* HBF34 strain. In addition, the purity of the enzyme and its sub-unit molecular mass were determined by SDS-PAGE and SDS-PAGE zymogram. PAGE and SDS-PAGE were carried out with 10% polyacrylamide gel as described by Laemmli (1970) using the Mini-Protean III electrophoresis system (Bio-Rad, Richmond, CA, USA). Molecular weight standards were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). The samples were not heated prior to loading of the SDS-PAGE to allow activity staining after migration. Electrophoresis was performed at a constant current of 20 mA per gel. Samples were run on SDS-PAGE gels in duplicate; one to be stained for protein detection and the other to be used for enzyme activity detection. The proteins were stained with Coomassie brilliant blue G-250.

To detect amylase activity on gels, zymograms were performed according to the method used by Wanderley et al. (2004). After non-denaturing PAGE and SDS-PAGE, the gels were washed with distilled water for 20 min, these were incubated with 50 mM McIlvaine buffer (pH 6.0) for 1 h at room temperature and then further incubated at 4°C for 1 h in a solution containing 1% of soluble potato starch (in 50 mM McIlvaine buffer, pH 6.0). Then, the gels were incubated at 50°C for 1 h. Starch-containing gels were washed with distilled water and stained with iodine solution (1 mM I_2 in 0.5 M KI). Amylase activity, which appears as clear bands on dark blue background, was visualized. Stained proteins and zymograms were compared to determine the molecular weight of the amylases.

Effect of temperature and pH on activity and stability of the GA

The effect of temperature on GA activity was measured at different temperatures (10 - 80°C) in pH 6.0 McIlvaine buffer solution under standard assay conditions. To determine the thermal stability, the enzyme was incubated for various periods of time at various temperatures (27, 40, 50, 60, 70 and 80°C) in 50 mM McIlvaine buffer (pH 6.0). Residual activity was then measured under standard assay conditions.

To determine the optimum pH, GA activity was measured under standard assay conditions by performing the assays at different pH values in the following buffers (50 mM): McIlvaine buffer (pH 3.0 - 6.50), Sørensen phosphate buffer (pH 7.0 - 7.5) and Tris-HCl buffer (pH 8.0 - 9.0). To estimate pH stability, the purified enzyme was preincubated at various pH values (pH 3.0 - 9.0) at 40°C. Samples were removed at different time intervals and residual activity of the enzyme was detected under standard assay conditions.

Effect of metal ions and ethylenediaminetetraacetic acid (EDTA) on GA activity

For determining the effect of metal ions (NaCl, KCl, LiCl, NH_4Cl, $CoCl_2$, $MnCl_2$, $MgCl_2$, $NiCl_2$, $HgCl_2$, $CuCl_2$, $BaCl_2$, $ZnCl_2$, $CaCl_2$, $AlCl_3$, $FeCl_3$ and Na_2O_3Se) and the chelating agent EDTA on GA activity, enzyme assays were performed in the presence of the metal ions and EDTA at final concentrations of 1, 5 and 10 mM in 50 mM MES buffer (pH 6.0) using soluble potato starch as substrate. The relative enzyme activity was measured under standard assay conditions.

Effect of inhibitors and denaturants on GA activity

The following compounds were tested for inhibition effect on GA activity: PMSF, a serine specific inhibitor; β-mercaptoethanol and DTT, sulfhydryl reducing agents; DTNB, a cysteine specific inhibitor and sulfhydryl oxidizing agent; NBS, a tryptophan specific inhibitor; CMC, a carboxyl specific inhibitor. Furthermore, urea and SDS were used as protein denaturant agents. The purified enzyme was incubated at 40°C for 30 min in 50 mM McIlvaine buffer (pH 6.0) containing inhibitor or denaturant agent at final concentrations of 1, 5 and 10 mM. The relative enzyme activity was measured under standard assay conditions.

Kinetic parameters

The effect of different concentrations (0.025 - 2 mg/ml) of the test starches (soluble potato starch, glycogen, amylopectin and amylose) on GA activity was determined under standard assay conditions. The kinetic rate constants, Km and Vmax values were calculated using Lineweaver-Burk plot.

Substrate specificity

The substrate specificity of the GA was determined using sucrose, maltose, maltotriose, raffinose, dextrin, amylose, amylopectin, glycogen, rice starch, wheat starch, corn starch and soluble potato starch. Substrate solutions were prepared in 50 mM McIlvaine buffer (pH 6.0) at a concentration of 1%. The relative activity was measured by the standard enzyme assay using the respective substrate.

Salt tolerance

In order to determine salt tolerance of the GA, the purified enzyme was incubated at final concentrations of 0.05, 1, 2, 3, 4 and 5 M NaCl for 24 h at 4°C. After incubation, the relative enzyme activity was measured under standard assay conditions.

Raw starch adsorption of GA

Two ml of purified GA (3630.28 U/ml) and 1 g of raw starch (wheat, rice or corn) was mixed and gently stirred for 20 min at 4°C. Following centrifugation at 2500 x *g* for 10 min, the activity in the supernatant was determined under standard assay conditions. The adsorption rate (*AR*) was calculated according to the following equation (Morita and Fujio, 2000).

AR (%) = [(O - R)/O] x100

Where, R indicates the residual amylase activity in the supernatant and O indicates the original enzyme solution.

Thin-layer chromatography (TLC) of hydrolysis products

A chromatographic analysis of the reaction end products of soluble

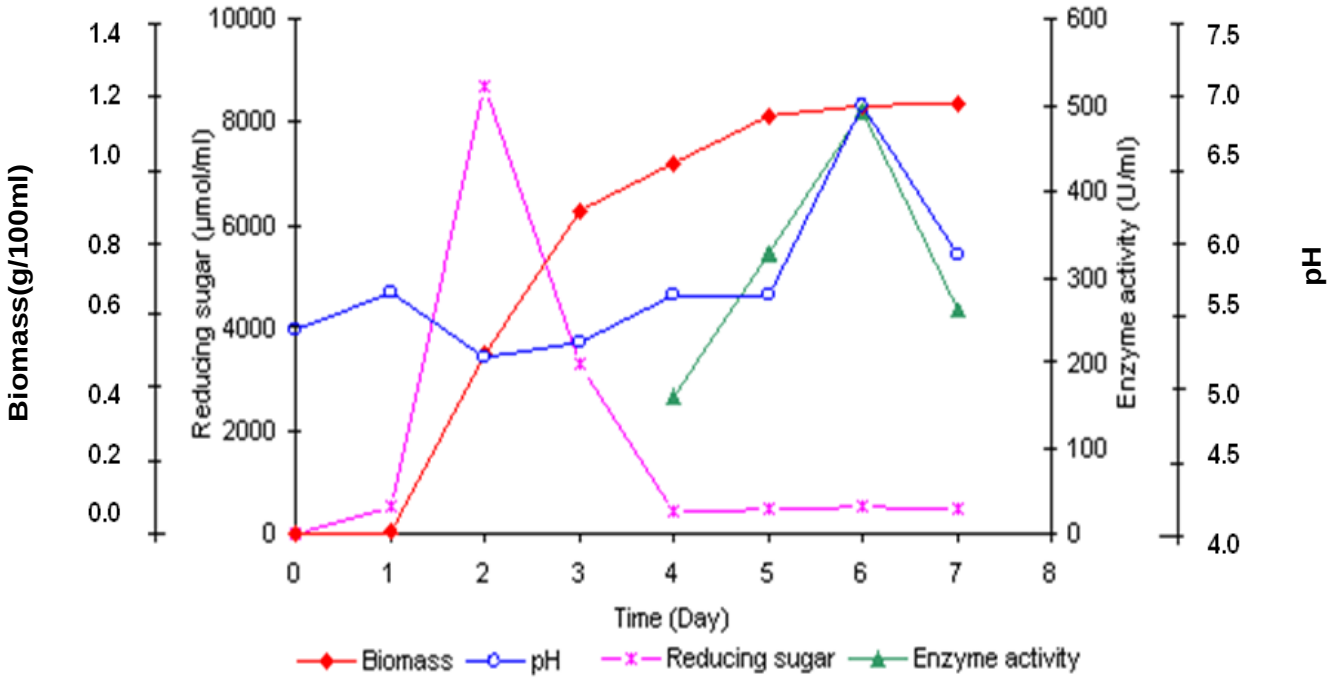

Figure 1. Glucoamylase production during growth of *A. flavus* HBF34.

potato starch was carried out using TLC following the methods of Fontana et al. (1988). The reaction mixture containing 1 ml of 1% (w/v) soluble starch and 1 ml of enzyme was incubated for 48 h at 40°C. Samples were withdrawn periodically and a volume of 10 µl of the reaction mixture was applied on silica gel plate (Alugram® Sil G/UV_{254} 0.20 mm, 5 x 10 cm, Sigma-Aldrich, Germany). The chromatogram was developed with solvent system of butanol/ethanol/water (5:3:2). After the plate was air-dried, the spots were visualized by spraying H_2SO_4 and methanol (1:9) containing 0.2% orcinol, and heating at 100°C. Glucose, maltose, maltotriose, maltotetraose and maltopentaose were used as standards.

RESULTS AND DISCUSSION

Growth and glucoamylase production of *A. flavus* HBF34

Amylolytic activity was detected in 8 out of 17 fungi isolates screened. Among these 8 fungal isolates, *A. flavus* HBF34 exhibited higher amylolytic activity in starch agar medium and was selected for further studies. The progress of reducing sugar concentration, amylase production, pH and biomass values of *A. flavus* HBF34 growth medium against time are shown in Figure 1. Amylolytic activity measurement was not accomplished by DNS method until 3rd day of fermentation due to the excess amount of reducing sugar in the medium. The highest amylase activity (491.36 U/ml) was detected during the stationary phase on the 6th day of cultivation and decreased on the 7th day which corresponds to 53% of the activity detected on the 6th day. The reducing sugar amount increased until the 2nd day of cultivation. However, after this period it was significantly reduced. The mycelia production increased until the 7th day of cultivation and the pH values increased up to 6.93 on the 6th day of cultivation and then decreased the following day.

Purification of glucoamylase

Purification of glucoamylase from *A. flavus* HBF34 was carried out by using the substitute affinity method in one step purification. The one step purification method gave a 25% yield with a specific activity of 6619 U/mg and 120 fold purification. Purification results are summarized in Table 1. Najafi and Kembhavi (2005) who applied this method, purified *Vibrio* sp. amylase with 163.5 fold and 78% yield. In a study carried out on *A. flavus*, a different starch affinity and acetone precipitation method for purification of α-amylase was employed and purification fold and yield were determined as 262.32 and 0.57%, respectively (Abou-Zeid, 1997). Starch adsorption method, affinity chromatography and ion exchange chromatography were used for the purification of *Streptomyces* sp. No.4 amylase with 126 fold purification and a 2% yield (Primarini and Ohta, 2000). Purification steps of ammonium sulphate precipitation, fast protein liquid chromatography (FPLC)-hydrophobic interaction chromatography and corn starch affinity adsorption used in a study on *Thermus* sp.

Table 1. Purification of GA from *A. flavus* HBF34.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Culture filtrate	16455	298.56	55.14	100	1
Starch affinity	4104	0.62	6619	25	120

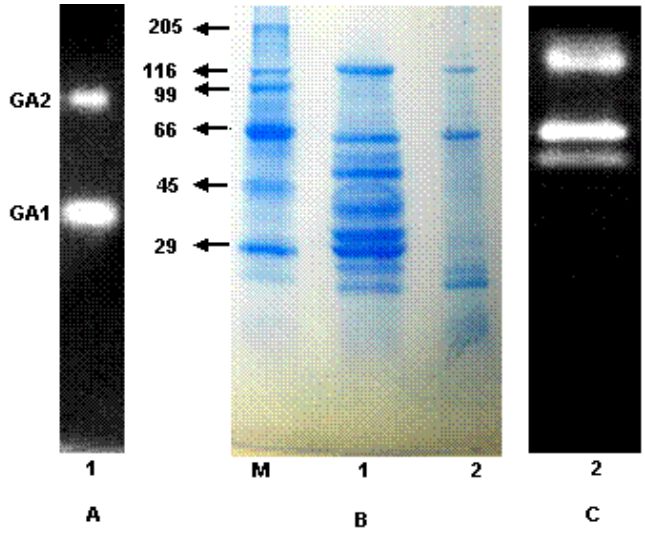

Figure 2. Electrophoresis profile. A, PAGE zymogram; B, SDS-PAGE of purified extracellular glucoamylase; C, SDS-PAGE zymogram; 1, Crude filtrate; 2, purified glucoamylase; M, molecular weight markers.

α-amylase produced 399 fold purification and a 2.6% yield (Shaw et al., 1995).

Electrophoresis

Non-denaturing PAGE showed two bands after activity staining (Figure 2A) indicating that *A. flavus* HBF34 releases two glucoamylases (GA1 and GA2) to the culture medium. These were determined to be coded for by two different *genes*. As reported by many researchers, most fungi produce more than one glucoamylase, while some of them produce both α-amylase and glucoamylase (Selvakumar et al., 1996; Morita and Fujio, 2000; Odibo and Ulbrich-Hofmann, 2001; Nguyen et al., 2002; Cereia et al., 2006; Negi and Banerjee, 2009). It is known that in some *Bacillus* strains a precursor amylase with high molecular weight (Mamo and Gessesse, 1999b) divides into two or more amylases as a result of proteolytic degradation.

As can be shown in Figure 2B, appearance of a number of protein bands, apart from those of glucoamylases, in electrophoresis indicates a partial purification of glucoamylases. Results of SDS-PAGE zymography revealed three bands (64, 70 and125 kDa) indicating that one of the GA is composed of two subunits (Figure 2C).

In order to determine which enzyme has two subunits, further purification steps are needed to be carried out. On the other hand, the molecular weight of GA purified from the same species was reported to be 51.3 kDa in a study done by El-Abyad et al. (1994). In previous studies, molecular weights of GA in fungi were reported to be in a range of 25 - 112 kDa (Vandersall et al., 1995; Silva and Peralta, 1998; Odibo and Ulbrich-Hofmann, 2001; Nguyen et al., 2002; Kusuda et al., 2004; Cereia et al., 2006).

Effect of temperature and pH on activity and stability of the GA

GA activity was tested at temperatures ranging from 10 - 80°C (Figure 3). Activity increased linearly from 10 - 60°C and reached a maximum at 60°C. Almost 95% of maximum activity was retained at 65°C, suggesting a high thermal stability. The GA activity decreased rapidly above 65°C and 24% of maximum activity was observed at the highest temperature (80°C) tested. These results indicated that the GA of *A. flavus* HBF 34 could be used at any temperature between 40 and 65°C in biotechnological applications. Most raw starch-digesting glucoamylases are known to exhibit optimum temperatures between 50 and 70°C and are remarkably stable at high temperatures (Norouzian et al., 2006). Optimum temperature of purified GA (60°C) was determined to be same as that of *A. flavus* (El-Abyad et al., 1994) and *Aspergillus niger* NRRL 3135 (Vandersall et al., 1995). In a few cases, higher optimum temperatures were reported. For example, the optimum temperature for glucoamylase activity of *A. flavus* A1.1 (Gomes et al., 2005), *Aspergillus fumigatus* (Silva and Peralta, 1998) and *Aspergillus* sp GP-21 was reported to be 65°C (Mamo and Gessesse, 1999a). Additionally, a study with *Thermomyces lanuginosus* A13.37 reported 70°C for optimum temperature (Gomes et al., 2005).

Thermostability is considered as an important and a useful criterion for industrial applications of glucoamylases from microorganisms. The enzyme from *A. flavus* HBF34 was stable for 2 h when incubated at 27, 40 and 50°C (Figure 4). It was determined that the enzyme still main-

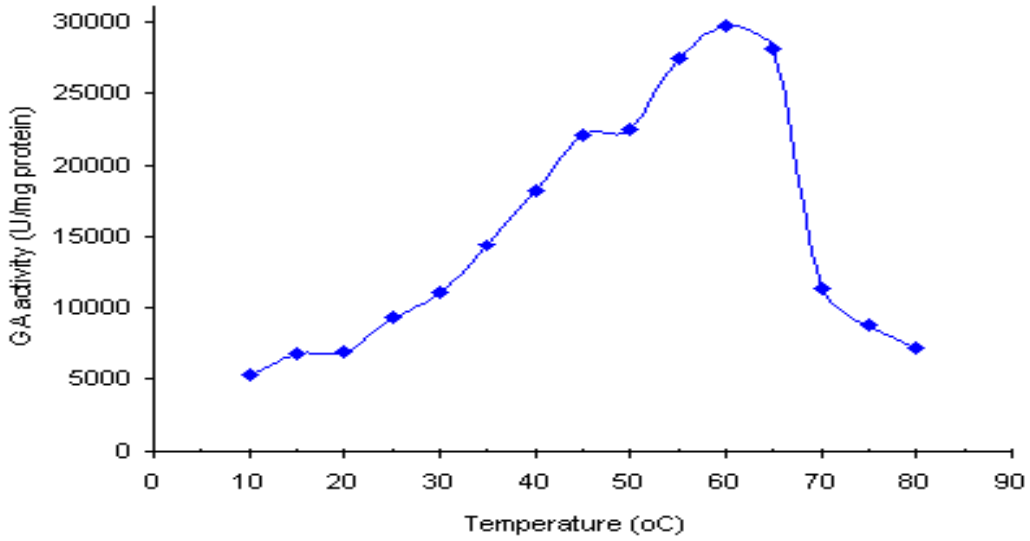

Figure 3. Effect of temperature on activity of glucoamylase from *A. flavus* HBF34.

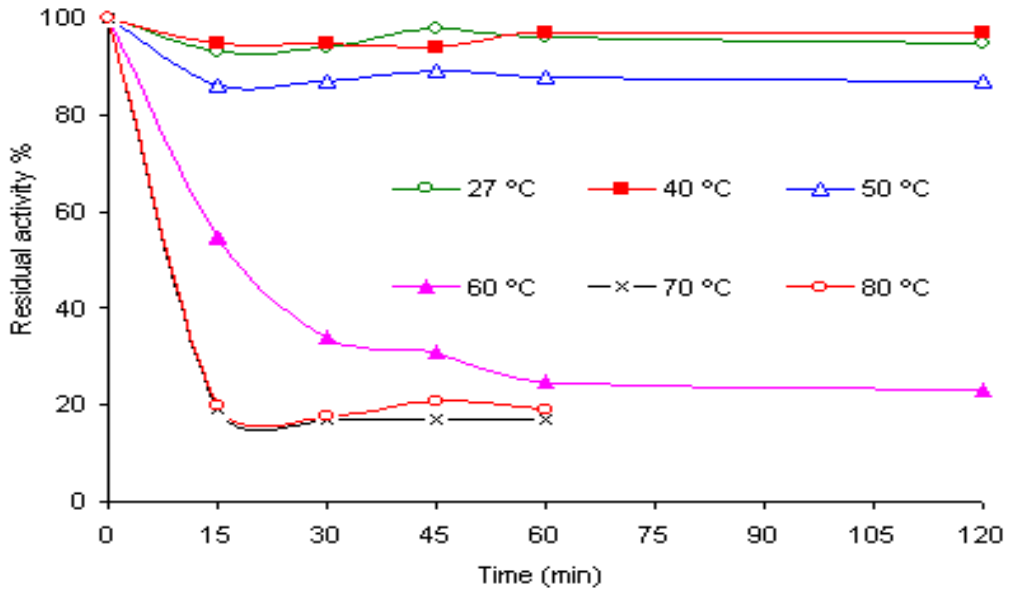

Figure 4. Effect of temperature on stability of glucoamylase from *A. flavus* HBF34.

tained its activity at 27 and 40°C after 72 h incubation while 56% of activity was retained at 50°C for the same time period (data not shown). However, 75, 83 and 81% of the original GA activity disappeared in the first hour of incubation at 60, 70 and 80°C, respectively, and no detectable activity was left after 2 h at 70 and 80°C. The high thermostability of *A. flavus* HBF34 GA makes it more suitable for its industrial applications. Many fungal glucoamylases have been reported to be stable at considerably high temperatures only for short time period (Norouzian et al., 2006).

The maximum enzyme activity of GA was measured at pH 6.0 (Figure 5). More than 64% of maximum activity was determined between pH 3.5 - 5.5 and 79% at pH values ranging between 6.5 and 7.5. GA activity started to decrease at pH values over 8.0. This data shows that the enzyme is active in a wide range of pH values from 3.5 - 7.5, which indicates that high activity is associated with acidic pH values. Obtaining glucose from starch is an important process of the starch industry. α-Amylase and glucoamylase enzymes are used in starch liquefaction and saccharification and the process is carried out at pH

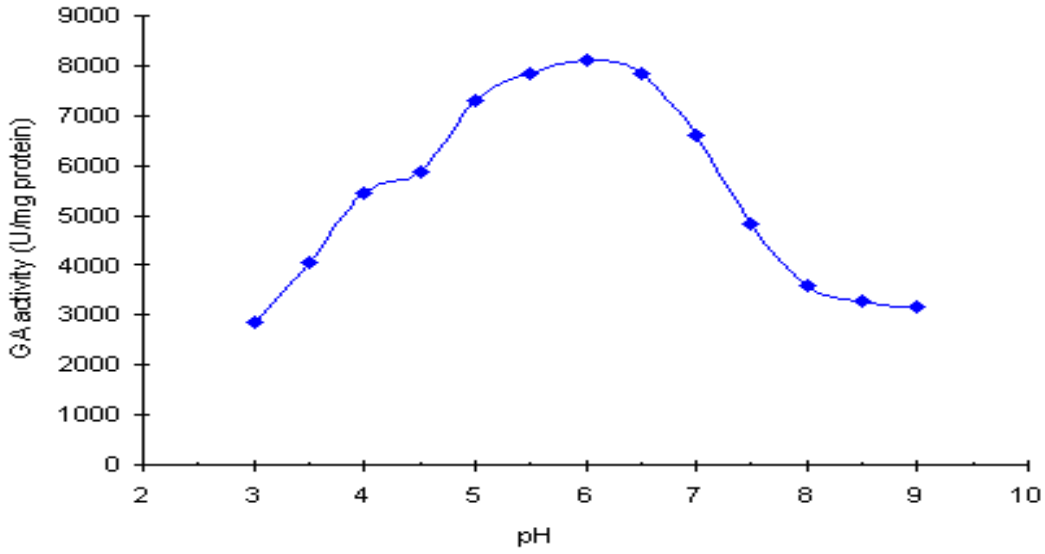

Figure 5. Effect of pH on activity of glucoamylase from *A. flavus* HBF34.

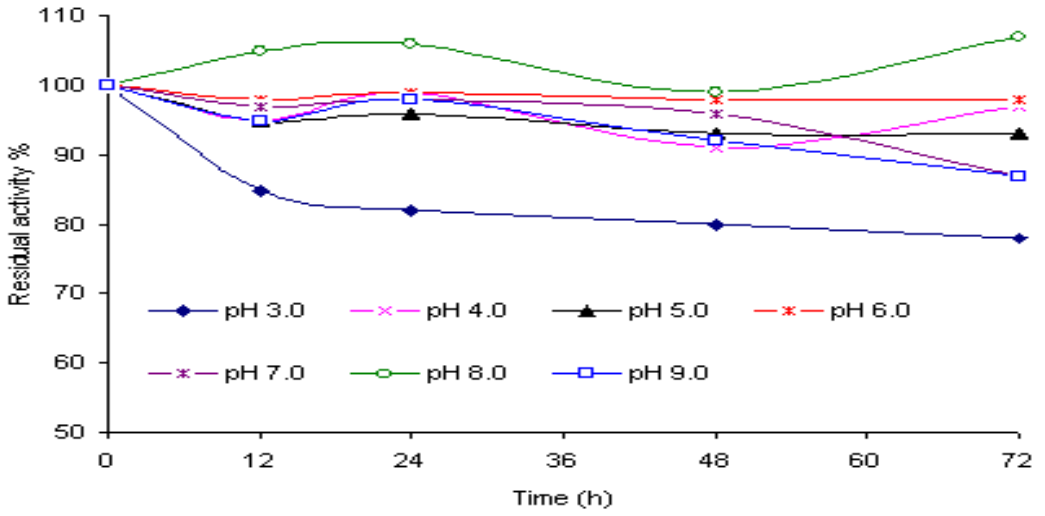

Figure 6. Effect of pH on stability of glucoamylase from *A. flavus* HBF34.

4.2 - 6.2 (Moreira et al., 2004). It has been reported that fungi glucoamylases are active at acidic pH (Norouzian et al., 2006). pH 4.0 was found to be optimum for the GA of *A. flavus* (El-Abyad et al., 1994; Gomes et al., 2005) whereas, the optimal pH values for other *Aspergillus* GAs were found to be between 4.5 and 7.0 (Mamo and Gessesse, 1999a; Moreira et al., 2004).

Enzyme stability is an important criterion for long lasting industrial processes with extreme pH levels making it highly desirable in the enzyme industry. The stability of glucoamylases in a wide range of pH values was reported by a number of researchers (Fogarty and Kelly, 1990; Moreira et al., 2004; Norouzian et al., 2006). Also, it was reported in a few studies that *A. flavus* GAs are stable at pH values between 4.0 and 9.0 (El-Abyad et al., 1994; Gomes et al., 2005). In the present study, GA from *A. flavus* HBF34 showed high stability in a wide range of pH (pH 3.0 - 8.0) for a relatively long time (78% of its activity was retained after 72 h) (Figure 6). This indicates that the enzyme is suitable for industrial purposes.

Effect of metal ions and EDTA on GA activity

The GA activity was significantly stimulated by metal ions, especially by Mn^{2+}, Ca^{2+}, Ba^{2+} and Co^{2+} which increased

Table 2. Effect of metal ions and chemical agents on glucoamylase activity.

Chemical agent	Relative activity (%)		
	1 mM	5 mM	10 mM
Control	100	100	100
$FeCl_3$	109	13	0
$AlCl_3$	90	41	12
$MnCl_2$	239	269	214
$CaCl_2$	134	164	167
$CoCl_2$	120	111	102
$NiCl_2$	99	81	71
$BaCl_2$	98	113	124
$MgCl_2$	88	81	63
$HgCl_2$	50	0	0
$CuCl_2$	79	59	32
$ZnCl_2$	60	56	12
LiCl	101	104	95
KCl	99	103	98
NH_4Cl	99	94	90
Na_2O_3Se	97	81	24
NaCl	97	99	98
EDTA	83	81	61

Table 3. Effect of inhibitors and denaturants on the activity of GA from *A. flavus* HBF34.

Inhibitors and denaturants	Relative activity (%)			
	None	1 mM	5 mM	10 mM
NBS	100	0	0	0
PMSF	100	84	78	45
β-mercaptoethanol	100	90	84	84
DTNB	100	111	152	174
CMC	100	109	117	141
DTT	100	90	119	120
Urea	100	84	86	86
SDS	100	84	45	0

the activity between 20 - 169% (Table 2). Similar results were reported for glucoamylases from *T. lanuginosus* (Odibo and Ulbrich-Hofmann, 2001; Nguyen et al., 2002). GA is used together with *α*-amylase, which requires calcium in the liquefaction process. The fact that GA is not inhibited by Ca^{2+} ions makes it suitable for use in the starch industry. The enzyme activity was not affected by Ni^{2+}, Mg^{2+}, Li^{+}, K^{+}, NH_4^{+}, Se and Na^{+}. However, among the metal ions tested, Fe^{3+}, Al^{3+}, Hg^{2+}, Cu^{2+} and Zn^{2+} had a profound inhibitory effect on the enzyme activity. This inhibitory effect has also been reported for different glucoamylases from other species (El-Abyad et al., 1994; Odibo and Ulbrich-Hofmann, 2001; Najafi and Kembhavi, 2005; Cereia et al., 2006). The inhibition by mercuric ions may indicate the involvement of indole amino acid residues on enzyme function (Inokuchi, 1999). EDTA, an ion chelate, was not effective on GA activity suggesting that this enzyme does not require metallic ions in its active site for activity.

Effect of inhibitors and denaturants on GA activity

As indicated in Table 3, the GA activity was totally diminished in the presence of either 10 mM PMSF (55%) or 1 mM NBS (100%), suggesting that accessible serine and tryptophan residues play an important role in the catalytic action. Similar results have also been reported for glucoamylases from *Rhizopus niveus* and other fungal glucoamylases (Shenoy et al., 1985; Inokuchi, 1999). DTT, DTNB and CMC also stimulate enzyme activity. It can be concluded from this result that sulfhydryl and carboxyl groups do not take part in catalysis. β-Mercaptoethanol and urea had little effect on enzyme activity while SDS resulted in 100% inhibition at 10 mM concentration.

Kinetic parameters

The dependence of the rate of enzymatic hydrolysis on substrate concentration followed Michaelis-Menten kinetics and the linear relationships of I/V versus I/[S] were obtained. Km and Vmax values for soluble potato starch, glycogen, amylopectin and amylose substrates of GA from *A. flavus* HBF34 were determined to be 0.046, 0.075, 0.1 and 0.125 mg/ml and 769, 1250, 3333 and 2500 U/mg protein, respectively. According to these results, the highest activity of the GA was measured for soluble potato starch followed by glycogen, amylopectin and amylose. The rate of substrate hydrolysis by GA is affected both by molecular size and structure of the substrates as well as by the next bond in sequence. GA has a greater affinity for longer chains, and while Michaelis constants decrease, the maximum rate increases with the chain length of the molecule (Fogarty and Kelly, 1990). The decreased value of Km for starch may be due to an increased number of interactions between the active site of the enzyme and the substrate molecule, resulting in an increased affinity of the enzyme (Selvakumar et al., 1996).

The Km value found for soluble potato starch in the present study was approximately 62 fold lower than that reported for the GA from *A. flavus* (2.85 mg/ml) (El-Abyad et al., 1994) as well as those given for the GA from *Aspergillus* (Vandersall et al., 1995; Silva and Peralta., 1998). This result shows that, the enzyme under investigation had a higher catalytic activity for starch compared

Table 4. Substrate specificity of glucoamylase from *A. flavus* HBF34

Substrate	Relative activity (%)
Soluble potato starch	100
Glycogen	102
Amylopectin	84
Corn starch	72
Rice starch	70
Wheat starch	69
Maltose	68
Amylose	59
Dextrin	57
Maltotriose	50
Raffinose	42
Sucrose	36

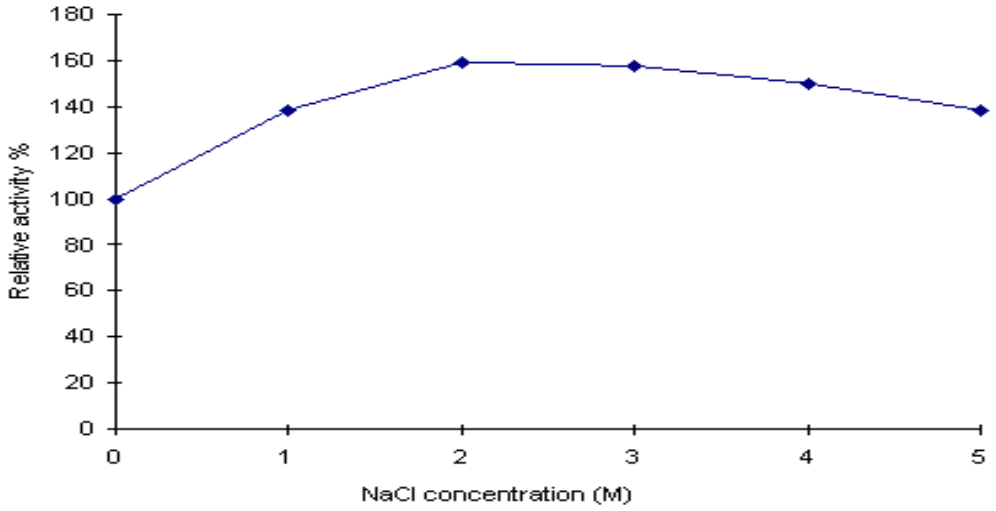

Figure 7. Effect of NaCl concentration on stability of glucoamylase from *A. flavus* HBF34.

to other glucoamylases. Since lower Km values allow for faster and easier industrial processes, it can be concluded that its industrial potential is high.

Substrate specificity

We can suggest from the data shown in Table 4 that GA of *A. flavus* HBF34 has a wide range of substrate specificity. Although, the GA has low activity against sucrose, it has a higher activity against long chain substrates such as starch and glycogen, which is congruent with literature on *A. flavus*, *A. fumigatus* and *Lyophyllum shimeji* (El-Abyad et al., 1994; Silva and Peralta, 1998; Kusuda et al., 2004). Despite the high affinity of GAs to α-1,4 bonds, they also display a little affinity for hydrolysis of α-1,6 and α-1,3 bonds. Hydrolysis rate of the substrates are affected not only by its molecular size and structure but also by the types of the bonds in its chain (Fogarty and Kelly, 1990).

Salt tolerance test

This test is important in saccharification of starch and in treatment of effluent with high salinity containing starch or cellulose residues in pollution control mechanism (Cordeiro et al., 2002; Al-Qodah et al., 2007). The previous studies done on the α-amylase reported a 60% loss of activity at different salt concentrations (Cordeiro et al., 2002; Al-Qodah et al., 2007). Data from the present study showed no loss in all of the salt concentrations examined, but rather, showed a 59% increase in activity (Figure 7). From this result, it can be suggested that GA could be

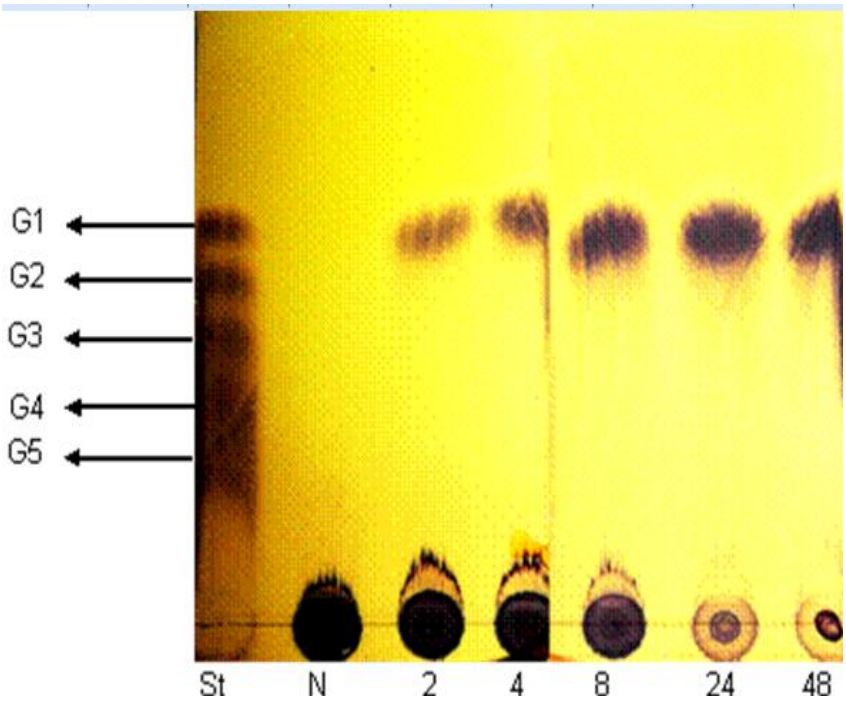

Figure 8. Thin-layer chromatography of the reaction product of soluble potato starch hydrolyzed by the glucoamylase from *A. flavus* HBF34. Hydrolysis time were 0, 2, 4, 8, 24 and 48 h. A mixture of glucose and maltooligosaccharides was used as standards (St): glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4) and maltopentaose (G5).

employed in industrial processes that require higher salt concentrations.

Raw starch adsorption

The GAs ability to digest raw starch resources and its immobilization is very important from industrial point of view. GA produced by the *A. flavus* HBF34 was strongly adsorbed on corn starch (93%) but weakly adsorbed on wheat starch (47%) and rice starch (19%). It was reported that amyloglucosidase produced by the *Aspergillus* sp. GP-21 had the adsorption rate of over 70% on raw corn and potato starch (Mamo and Gessesse, 1999a). It was also reported that glucoamylase from *Rhizopus* sp. MKU 40 and *Rhizopus* sp. A-11 were strongly adsorbed onto raw starches (Morita and Fujio, 1997; Morita and Fujio, 2000).

TLC of hydrolysis products

Glucoamylase is an exo-acting enzyme that catalyses the production of β-glucose from the non-reducing ends of amylose, amylopectin and glycogen. It consecutively hydrolyzes α-1,4; α-1,6 and rare α-1,3 linkages (Cereia et al., 2006). TLC (Figure 8) revealed only glucose as the hydrolysis product of starch which indicates that the purified enzyme was a glucoamylase (EC 3.2.1.3).

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