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Original Article

Characterization of a starch-hydrolyzing α-amylase produced by *Aspergillus niger* WLB42 mutated by ethyl methanesulfonate treatment

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Abstract: *Aspergillus niger* is the most commonly used fungus for commercial amylase production, the increase of amylase activity will be beneficial to the amylase industry. Herein we report a high α-amylase producing (HAP) *A. niger* WLB42 mutated from *A. niger* A4 by ethyl methanesulfonate treatment. The fermentation conditions for the amylase production were optimized. The results showed that both the amylase activity and total protein content reached highest after 48-h incubation in liquid medium using starch as the sole carbon source. The enzyme production reached maximum at temperature of 30°C, pH 7, with 40 g/L starch in the medium inoculated with 1.4% v/v spore. When 0.3% w/v urea was added to the liquid medium as a nitrogen source, the amylase activity was elevated by 20%. Nine monosaccharides and derivatives were tested for α-amylase induction, glucose was the best inducer. Furthermore, the enzymology characterization of amylase was conducted. The molecular weight of amylase was determined to be 50 kD by SDS-PAGE. The amylase had maximum activity at 45°C and pH 7. The activity could be dramatically triggered by adding 1 mM Co^{2+}, increased to 250%. The activity was inhibited by detergents SDS and Triton X-100. Six different brands of starch were tested for amylase activity, the results demonstrated that the more soluble of the starch, the higher hydrolyzability of the substrate by amylase.

Keywords: *Aspergillus niger*, amylase, fermentation parameters, enzymology, EMS mutation

Introduction

The enzyme α-amylase E.C 3.2.1.1 catalyzes hydrolysis of α-D-(1, 4) glycosidic linkages in starch and related carbohydrates [1]. Amylase is extensively used in the food, paper, pharmaceutical, and detergent industries. The foremost application is hydrolysis of starch for the production of sweeteners, syrups, and chemicals (e.g., ethanol, acetone, and lactic acid) [2-4]. The global market for amylase is approximately US $156 million annually, and the cost of amylase in starch liquefaction represents 24% of the total process cost [2]. Thus, there has been growing interest in decreasing amylase costs by increasing enzyme production yield and/or activity.

Although amylase can be acquired from many plants and animals, microbial amylase generally meets industrial demand [5, 6]. *Aspergillus niger* is the most commonly used fungus for commercial amylase production [7] because it has a high acid tolerance and bacterial contamination can be easily avoided [8]. As a filamentous fungus, *A. niger* is suitable for solid state fermentation because its morphology facilitates colonization and penetration into solid substrates [8]. More importantly, *A. niger* is "Generally Recognized As Safe" by the United States Food and Drug Administration and therefore can be applied in the food industry [2, 7].

Among methods to improve amylase production by *A. niger*, treating conidiospores with mutagens to search for advanced mutants among the surviving progeny is regarded as the best means [9]. In this work, we mutated an amylolytic industry strain, *A. niger* A4, with ethyl methanesulphonate (EMS) and isolated stable

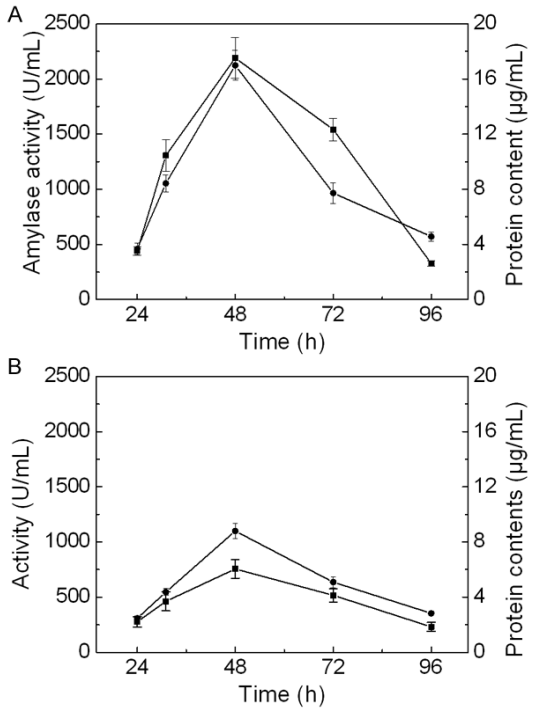

Figure 1. Amylase activity (■) and total protein content (●) in the supernatant of *A. niger* WLB42 (A) and the wild type *A. niger* A4 (B) cultures as a function of incubation time.

mutants for α-amylase hyperactivity using starch as the sole carbon source. We comprehensively evaluated the fermentation parameters of mutant strain *A. niger* WLB42 for α-amylase production and enzymology. Findings could facilitate the application of amylase in the food industry.

Materials and methods

Mutation and screening

Mutagenesis of *A. niger* A4 with EMS (Sigma Aldrich, St. Louis, MO) followed Khattab and Bazaraa [10]. Conidiospores grown in potato dextrose agar (potato, 200 g; dextrose, 20 g; agar, 15 g; deionized water to 1 L) were harvested using phosphate buffer solution (PBS: 0.1 mol/L, pH 7.0), counted under a light microscope (2×10^9 spores/mL), stored at 4°C, and used as stock inoculum. Spores were treated with 200 mmol/L EMS for 3 h, harvested by centrifuging at 3000 *g* and 4°C for 10 min, and washed twice with PBS. Viability dropped by 99% after EMS treatment.

To conduct primary screening, the mutated spore suspension was spread onto the surface of starch agar plates (starch, 20 g; KNO_3, 1 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 15 g; deionized water to 1 L) (the starch, purchased from Fleischmann's (Toronto, ON), was used throughout the study, unless stated otherwise) and incubated at 30°C for 7 days. In total, 284 mutants were selected. The diameters of the starch degradation halos (D_p) and of the colonies (D_c) were measured. The D_p value and potency index (D_p/D_c) were used as the standard to screen for high α-amylase-producing (HAP) mutants.

After primary screening, four mutants with higher potency indexes (>3) and D_p values (>10 mm) underwent secondary screening in submerged fermentation liquid starch medium (same recipe as starch agar without the agar) at 30°C and 200 rpm. At 24, 48, and 72 h, aliquots of fermentation broth were withdrawn and centrifuged at 12000 *g* and 4°C. The supernatant served as the crude amylase. Enzyme activity was determined by measuring the release of reducing sugar using the 3, 5-dinitrosalicylic acid (DNS) method [11]. Briefly, 10 μL crude enzyme solution was mixed with 0.1 mL 1% w/v starch and bathed at 40°C for 20 min, followed by addition of 0.3 mL DNS reagent. The mixture was heated in a boiling water bath for 5 min, cooled, and then 0.3 mL of the solution was injected into a 96-well plate to measure absorbance at 520 nm. One unit of amylase activity was defined as the amount of the enzyme in 1 mL fermentation broth that hydrolyzed 1 μmol/min reducing sugar (here maltose) under standard assay conditions. The crude amylase was also evaluated for total protein content with the Bradford Assay (Bradford reagent from Bio-Rad, Mississauga, ON) at a wavelength of 595 nm, using a bovine serum albumin standard calibration curve [12]. Among the four strains, WLB42 showed the highest amylase activity and protein content; this strain was chosen as the HAP strain.

Optimization of amylase production

For all treatments, *A. niger* WLB42 was cultured in liquid starch medium (0.5 mL spore solution (2×10^9 spores/mL) was inoculated in 50 mL medium). In the optimization tests, the response variable was amylase production, assayed using the DNS method. The incubation time was first optimized by incubating *A. niger*

Table 1. Comparison of the strain A. niger A4 and its four mutants

Strains	Primary screening			Secondary screening	
	D_p (mm)	D_c (mm)	D_p/D_c	Relative activity (U/mL) (48 h)	Protein content (μg/mL) (48 h)
A. niger A4 (WT)	6.5±0.6	2.7±0.2	2.4	755±84	8.8±0.5
A. niger WLB34	10.6±0.5	3.2±0.4	3.3	1536±139	11.3±1.2
A. niger WLB41	10.3±0.6	2.1±0.3	4.9	1348±134	10.4±0.9
A. niger WLB42	16.1±0.8	5.0±0.4	3.2	2189±185	17.0±1.1
A. niger WLB43	15.2±1.2	4.9±0.5	3.1	1789±68	15.1±1.3

at 30°C and 200 rpm. At 24, 48, 72, and 96 h, amylase activity was assayed, and the total protein content in the supernatant was measured using the Bradford Assay. Amylase showed maximum activity (2189 ± 185 U/ml) at 48 h, as did the total protein content in the supernatant (17.0 ± 1.1 μg/mL) (**Figure 1A**). The optimal incubation time of 48 h was used in subsequent tests, unless otherwise noted.

The effect of temperature was determined by culturing *A. niger* at 25, 30, 35, 40, and 45°C. The impact of pH was determined by culturing *A. niger* at 30°C and six pH levels (pH 3, 4 and 5 in citrate buffer; pH 6, 7 and 8 in PBS buffer). To determine the effect of starch amount, *A. niger* was incubated at starch concentrations of 10, 20, 30, 40, and 50 g/L at 30°C and pH 7. The impact of inoculum concentration was investigated at 0.2, 0.6, 1.0, 1.4, 2.0 and 4.0% v/v inoculum concentration. Amylase activity was measured at 24, 48, and 72 h. To evaluate the impact of nitrogen source, the *A. niger* growth medium was augmented with yeast extract, peptone, urea, and ammonium nitrate separately as sole nitrogen source (0.2% w/v) and incubated at 30°C and pH 7. The effects of nine monosaccharides and derivatives (arabitol, sorbitol, galactose, xylose, mannose, glucose, mannitol, xylitol, and adonitol) were measured by individually adding the nine compounds to the liquid medium at the beginning of the incubation at a concentration of 5 mmol/L. Finally, the effect of glucose concentration was determined by adding 0, 5, 10, 20 or 25 mmol/L glucose to the medium.

Characterization of purified amylase

To purify the amylase, mutant strain *A. niger* WLB42 was incubated in submerged medium for 48 h. The fermentation broth was centrifuged at 12000×*g* and 4°C for 10 min. The supernatant was filtered through a 0.22 μm polyethersulfone membrane (Sterlitech Corp., Kent, WA) followed by dialysis with a dialysis tube (molecular weight cutoff, 1 kD) at 4°C for 24 h. The solution was transferred to another dialysis tube with a molecular weight cutoff of 25 kD to be concentrated by 10% w/v polyethylene glycol at 4°C for 24 h. The enzyme protein was then purified by trichloroacetic acid/acetone protein precipitation [12]. Simply, 1:4 v/v mixture solutions of trichloroacetic acid/acetone were put into the concentrated enzyme solution to precipitate the enzyme at -20°C for 12 h. The enzyme solution to organic solvent volume ratio was 1:4. After precipitation, the enzyme solution was centrifuged at 12000×*g* for 10 min. The protein pellet was washed three times with 100% acetone. The dried pellet served as the crude enzyme for subsequent experiments. The enzyme protein content was monitored by the Bradford Assay method.

The molecular weight of purified amylase was measured by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [13] following the method described by Laemmli et al. [14] using a Bio-Rad electrophoresis apparatus. The protein marker and amylase were run simultaneously. The gel was stained in Coomassie Brilliant Blue R-250.

The gel containing 1% starch was used to detect amylase activity. The gel was washed with 2% Triton X-100 and rinsed 3 times by distilled water. The gel was transferred to 100 mmol/L PBS buffer (pH 7), incubated at 45°C for 20 min., and stained with 0.1% Congo Red. The gel was destained with 1 mol/L NaCl to visualize the clear bands that indicate amylase activity.

As in the amylase production experiments above, the enzymology characteristics of the purified amylase used amylase activity as the response variable, assayed using the DNS method. The incubation time for all tests was 20 min. The effect of temperature was investigated at 5°C temperature intervals from 40-80°C. The impact of pH was determined at

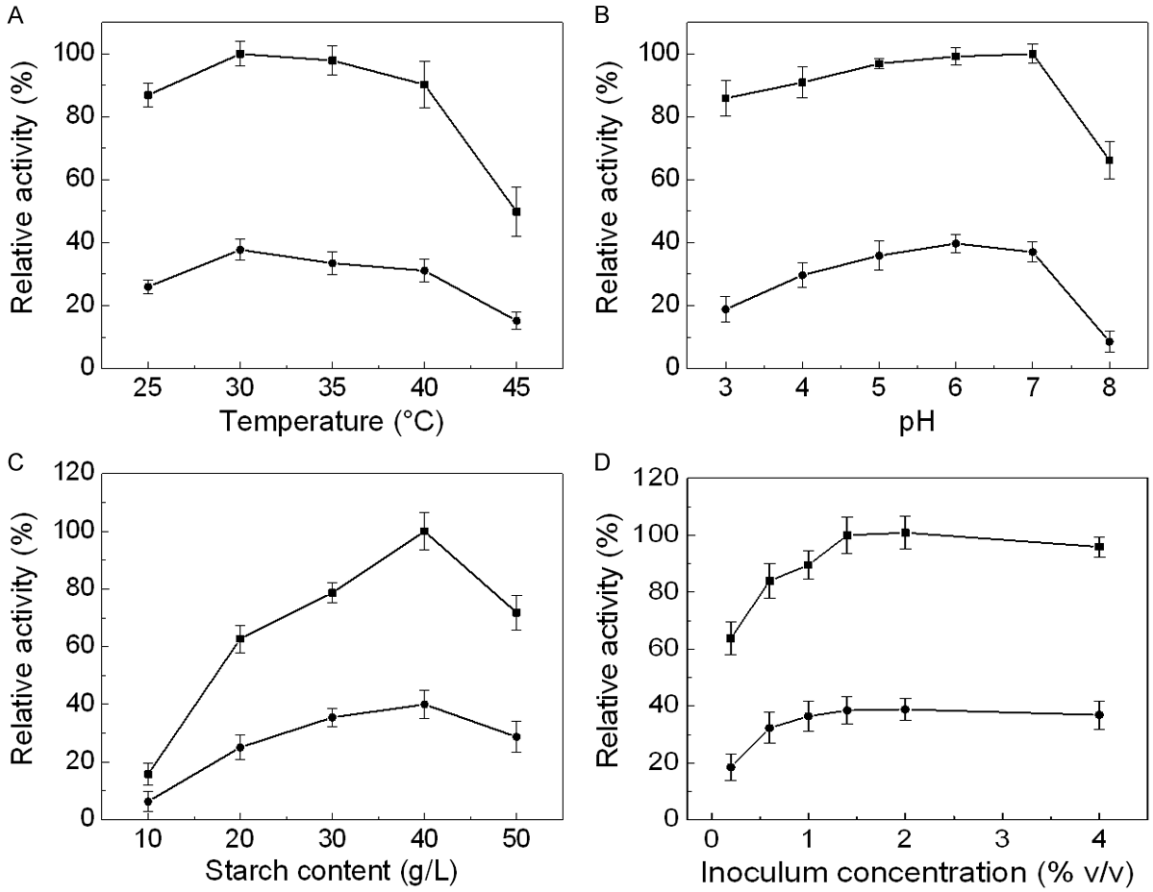

Figure 2. Effects of (A) temperature, (B) pH, (C) starch concentration, and (D) inoculum concentration on the production of amylase by *A. niger* WLB42 (■) and the wild type *A. niger* A4 (●).

pH 3, 4 and 5 in citrate buffer and pH 6, 7 and 8 in PBS buffer. The effects of the metal ions Cu^{2+}, Ca^{2+}, Co^{2+}, K^{+}, Na^{+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} in their chloride and sulphate salts (2 mmol/L) and of detergents (SDS at 2 mmol/L and Triton X-100 at 2%) were measured at 45°C and pH 7. The same assay was used to test the effect of Co^{2+} concentration from 1-5 mmol/L. The effect of starch substrate was determined after incubation at 45°C and pH 7 with six brands of starch (1% w/v): S1 (Item No. 64548, Real Canadian Superstore, Thunder Bay, ON); S2 (Great Value, Wal-Mart, Thunder Bay, ON); S3 (Item No. 64549, Real Canadian Superstore, Thunder Bay, ON); S4 (Fleischmann's, Toronto, ON); S5 (ACS879, BDH Chemicals, Toronto, ON); and S6 (A0343248, Acros Organics, Toronto, ON).

After testing the effect of starch substrate, the relative solubility of the 6 brands of starch was determined by individually measuring the OD600 values of 1% w/v starch solution prepared in water. Therefore, the higher the OD-600 value, the lower solubility of the starch. Furthermore, the reducing sugar contents of the six brands of starch were determined by DNS method except that the amylase was not added.

Statistical analysis method

For each experiment, the results for the wild type *A. niger* A4 was provided for comparison. Every test was repeated three times and the mean value with the standard error was provided.

Results

Mutation and isolation of A. niger

The original α-amylase-producing strain *A. niger* A4 was an industry strain. We mutated spores of strain A4 using 200 mmol/L EMS to obtain HAP mutants. After mutation, the spores

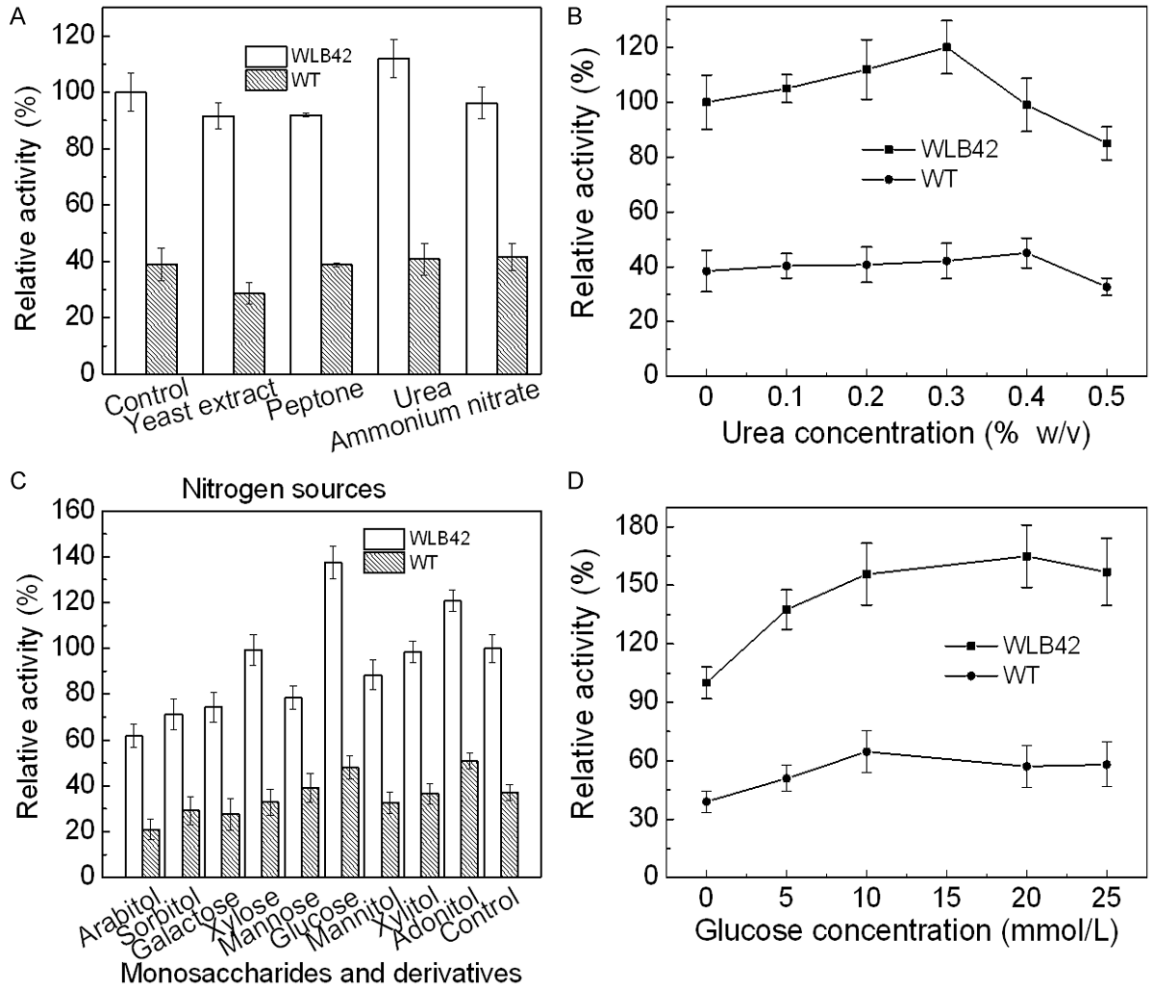

Figure 3. Effects of (A) four nitrogen sources, (B) different concentrations of urea, (C) nine monosaccharides and derivatives, and (D) different concentrations of glucose on amylase production by *A. niger* WLB42 and the wild type *A. niger* A4.

were cultured on starch agar medium using starch as the sole carbon source, and a total of 284 single colonies of mutants were obtained. For primary screening, the D_p and D_p/D_c values of the 284 colonies and the wild type *A. niger* A4 were measured and compared. As listed in **Table 1**, four mutant strains, i.e., *A. niger* WLB34, WLB41, WLB42 and WLB43, with higher D_p (>10 mm) and D_p/D_c (>3) values were chosen for secondary screening.

In the secondary screening, the four mutants (*A. niger* WLB34, WLB41, WLB42 and WLB43) as well as the wild type were inoculated into liquid starch medium and the amylase activities and the total protein contents were determined at different time intervals. It was found that both the maximum activities and protein contents of the four mutants and the wild type were achieved after 48-h incubation, and the related data were listed in **Table 1**. Among the four mutants, the *A. niger* WLB42 showed the highest activity of 2189±185 U/mL and the highest protein content of 17.0±1.1 μg/mL, which were 2.9 and 1.9 folds of those of the wild type *A. niger* A4, respectively. As a result, the *A. niger* WLB42 strain was chosen as the HAP mutant. Further, the fermentation parameters and enzyme characteristics were comprehensively investigated.

Optimized fermentation parameters

Amylase production by *A. niger* WLB42 was similar at 25, 30, 35, and 40°C with a maximum at 30°C (**Figure 2A**). At 45°C, the amylase production dramatically declined. Similarly, amylase production was relatively stable at pH

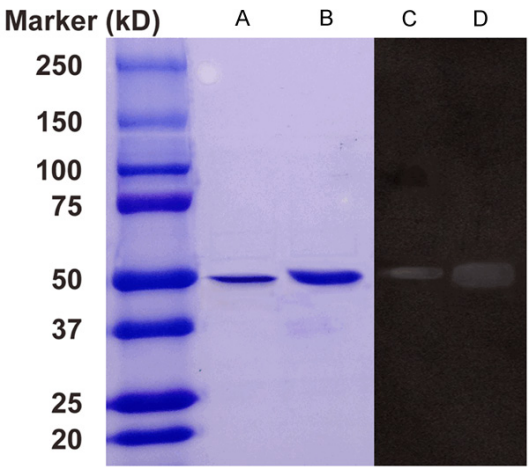

Figure 4. SDS-PAGE gel of the supernatant produced by A. niger WLB42 and the wild type A. niger A4. (A and B) are the regular SDS-PAGE for strains A4 and WLB42, respectively; (C and D) show the clear bands indicating amylase activity for strains A4 and WLB42, respectively.

3-7, but with an increasing trend such that relative activity was 14% higher at pH 7 than pH 3 (**Figure 2B**). However, the mean amylase production was dramatically lower at pH 8, indicating that amylase production was the highest at neutral and acidic conditions. The mean relative activity of amylase increased with increasing starch concentrations, was highest at 40 g/L starch, and then lower at 50 g/L starch (**Figure 2C**). For the six inoculum concentrations tested, amylase activity peaked at 1.4% inoculum concentration (**Figure 2D**). The corresponding trends of wild type *A. niger* A4 were similar with those of *A. niger* WLB42 except that the optimum pH for amylase production was pH 6.

Among four nitrogen sources, urea addition (0.2% w/v) was associated with the highest amylase production (**Figure 3A**). Subsequent testing of four additional urea concentrations showed that maximum enzyme production was achieved when 0.3% w/v urea was added to the liquid medium (**Figure 3B**). Among nine monosaccharides and derivatives, glucose stimulated amylase production the most (**Figure 3C**). Consequently, different concentrations of glucose were tested; addition of 20 mmol/L glucose yielded the maximum amylase activity (**Figure 3D**). The related results of the wild type *A. niger* A4 were exhibited in the same figure for comparison, and the optimum concentrations for the amylase production by urea and glucose were 0.4% w/v and 10 mmol/L, respectively.

Enzymology of purified amylase

Purified amylase secreted by *A. niger* WLB42 has a molecular weight of 50 kD (**Figure 4B**, **4D**), which was the same as that of the wild type (**Figure 4A**, **4C**). The optimum temperature for amylase activity was 45°C, though activity was only slightly lower up to 65°C (**Figure 5A**). Even at 80°C, this amylase showed 82% activity after 20 min-incubation. Amylase showed maximum enzyme activity at pH 7.0, but as was seen in the experiments above, this enzyme shows high activity under neutral and acidic conditions (**Figure 5B**). Correspondingly, the optimum temperature and pH for the wild strain *A. niger* amylase were 45°C and pH 6-7, respectively.

Among the metal ions tested, the addition of Co^{2+} (2 mmol/L) to the enzyme enhanced amylase activity 2-fold relative to the control, whereas the addition of Cu^{2+}, Ca^{2+}, K^{+}, Mg^{2+}, and Zn^{2+} suppressed activity (**Figure 6A**). Subsequent testing at different Co^{2+} concentrations demonstrated that a lower Co^{2+} concentration (0.5 and 1 mmol/L) could elevate amylase activity approximately 2.5-fold relative to the control (**Figure 6B**). The two detergents SDS and Triton X-100 inhibited amylase activity (**Figure 6C**). Among the six starch brands tested, amylase activity was lowest for S6 (**Figure 7A**), which also had a very low OD600 (indicating high solubility) (**Figure 7C**). For the other five starches, amylase activity was negatively related to the OD600 of the starch solution, suggesting that the hydrolyzability of the substrate by amylase increased with increase of starch solubility. Given that results for S6 did not obey the rule, we further tested the reducing sugar contents of the six brands of starch, which were similar for S1-S5 but 2.5 times higher in S6 (**Figure 7D**). The results implied that the high reducing sugar content of S6 significantly prevented amylase activity through production inhibition.

Discussion

The maximum amylase production of the HAP *A. niger* WLB42 mutant (2189±185 U/mL) was approximately 2.9 folds of the maximum activity produced by the wild type *A. niger* A4 (**Figure**

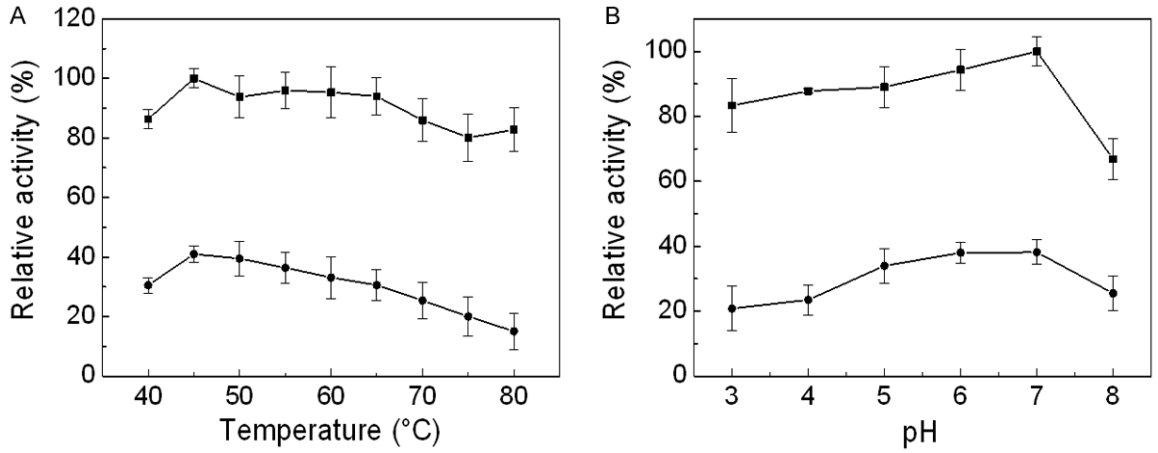

Figure 5. Effects of (A) temperature and (B) pH on the activity of purified amylase from *A. niger* WLB42 (■) and the wild type *A. niger* A4 (●).

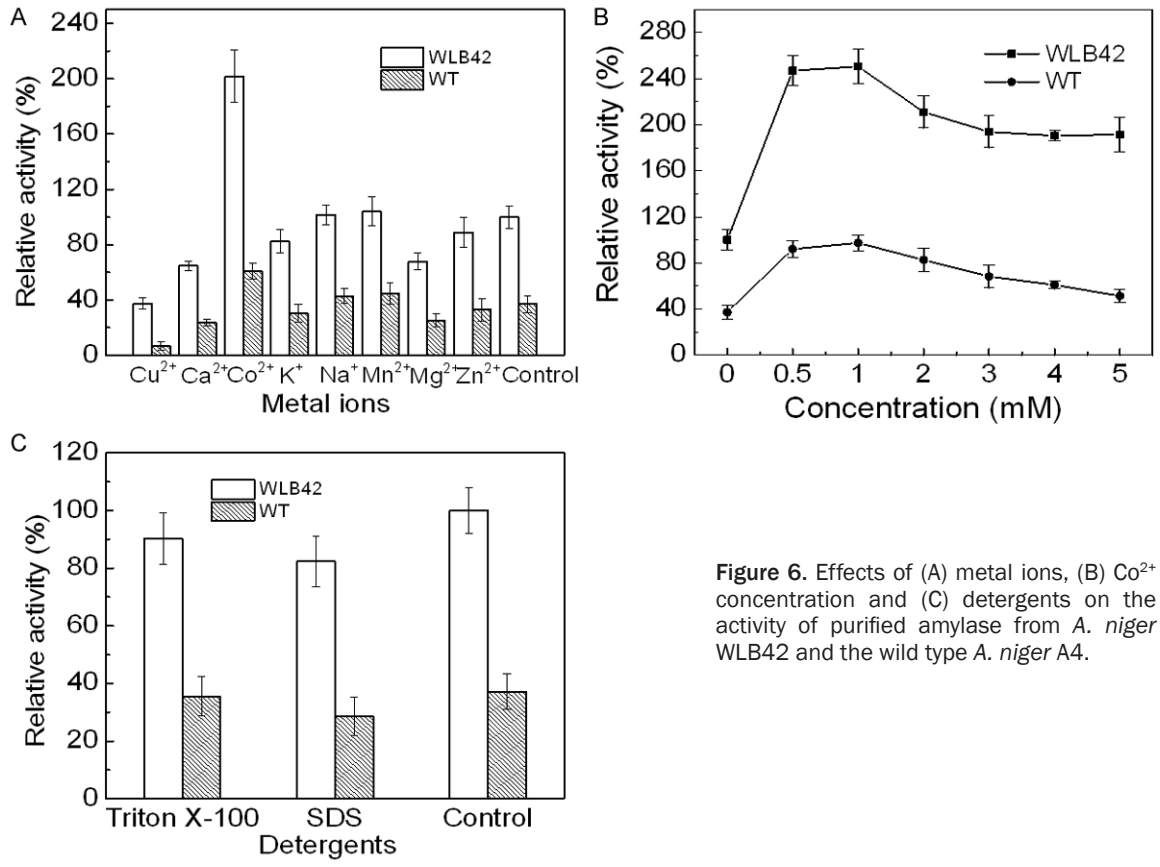

Figure 6. Effects of (A) metal ions, (B) Co^{2+} concentration and (C) detergents on the activity of purified amylase from *A. niger* WLB42 and the wild type *A. niger* A4.

1), 44 times the maximum activity produced by *A. niger* 34 [15], 24 times the maximum activity of *A. niger* FCBP-198 [2], and almost 3 times the amylase activity of *A. niger* AM07 [16]. Also, the fermentation time that achieved the maximum amylase activity in this work (48 h) was shorter than that for *A. niger* NRRL 3112 (120 h) [17], *Aspergillus* sp. JGI 12 (96 h) [6], and *A. niger* 34 (72 h) [15].

The molecular weight of amylase produced by *A. niger* WLB42 was 50 kD, the same as that of the wild type (**Figure 4**), indicating that the EMS treatment may have induced amylase gene

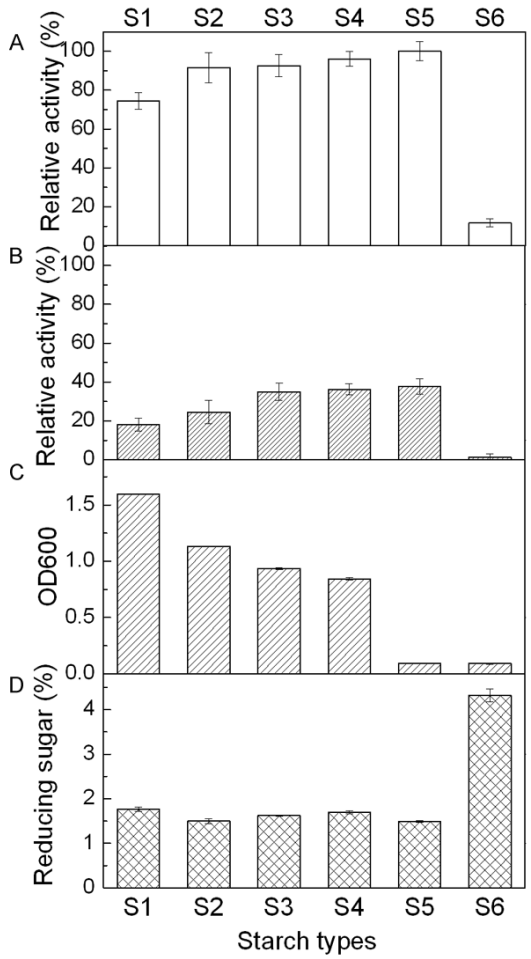

Figure 7. Effects of different starch brands on amylase activity. A and B: Relative amylase activity from *A. niger* WLB42 and A4 (the wild type) under six brands of starch substrates. C: OD600 of 1% w/v starch. D: Reducing sugar (maltose) content (w/w) in six brands of starch.

base mutation. The molecular weight was higher than values reported in the literature for other *A. niger* strains. For example, the molecular weights of amylases produced by *A. niger* BAN 3E [18] and *A. niger* JGI 24 [19] were 43 kD.

The optimum starch concentration for amylase production from the HAP *A. niger* WLB42 mutant was 40 g/L, agreeing with that for the wild type. This optimum starch concentration differed from the optimum of 10 g/L determined by Kammoun et al. [20] for *A. niger* ATCC 16404 and 30 g/L determined by Omemu et al. [16] for *A. niger* AM07. Based on the higher molecular weight of amylase from the *A. niger* WLB42 and A4, we speculate that the high utilization of starch by these two strains was associated with the relatively high molecular weight of the amylases.

The HAP *A. niger* WLB42 mutant had the highest amylase production at 30°C, but showed a relative activity higher than 85% within a broad range of temperatures from 25 to 40°C (**Figure 2A**). The corresponding trend for the wild type was similar with that of *A. niger* WLB42, except that the relative activity was ca. 20-40% as shown in **Figure 2A**. Alva et al. [6] also reported that the optimum temperature for the amylase production by *Aspergillus* sp. JGI 12 was 30°C. However, the amylase production sharply declined to zero at 40°C. Varalakshmi et al. [19] found that the optimum temperature for amylase production by *A. niger* JGI 24 was approximately 22°C, and the production rapidly decreased with the further increases in temperature. The enzyme obtained from the HAP *A. niger* WLB42 mutant shows excellent thermostability at relatively high temperature.

The HAP *A. niger* WLB42 mutant could produce amylase at pH 3-7 (relative activity higher than 85%) with the optimum of pH 7. Correspondingly, the optimum pH for amylase production of the wild type was pH 6, and the strain showed good amylase production within pH 3-7. Whereas in the literature, most *Aspergillus* spp. showed optimum amylase production at pH 3-5 and production was significantly declined at pH 7 [6, 15, 21, 22].

Nitrogen source is a crucial factor for the fungal growth and amylase production. Urea could elevate the amylase production from the HAP *A. niger* WLB42 mutant, but yeast extract, peptone, and ammonium nitrate inhibited amylase production. Similarly, Acourene and Ammouche [23] found that amylase production by *A. niger* ANSS-B5 could be increased by the addition of urea and inhibited by ammonium nitrate. However, in that study, peptone and yeast extract also enhanced amylase production. Urea also enhanced amylase production by *A. niger* AN-9, but peptone also had a stimulatory effect [24]. A third strain reported in the literature, *A. niger* JGI 24, showed decreased amylase production after addition of urea to the medium [19]. Clearly, different amylase-producing *A. niger* strains prefer different nitrogen sources.

The amylase secreted by *A. niger* WLB42 tolerated a very broad temperature range. Relative activity was higher than 90% at 45-65°C. Even at 80°C, this amylase showed 82% activity. Comparing with the wild type, the thermostability of the amylase at high temperature (>70°C) increased to some extent (**Figure 5A**). By comparison the maximum activity of amylase from *A. niger* L119 occurred over a range of only 50-55°C; outside this temperature range, the activity rapidly decreased such that at 30 and 80°C, activity was approximately 20% and 5%, respectively [25]. Similarly, amylase from *A. niger* AM07 showed the highest activity at 60°C; however, the activity declined to less than 40% at 80°C [16]. Amylase from the HAP *A. niger* WLB42 mutant appears well suited to applications involving high temperatures.

Moreover, the purified amylase had an activity exceeding 80% within pH 3-7, which is broader than the pH range (4-6) reported by other authors; at pH 7, the activity usually declined [6, 26, 27]. The amylase produced by *A. niger* WLB42 has strong potential uses in both neutral and acidic conditions.

Further, the addition of Co^{2+} could stimulate amylase activity to 250%, as was found for the wild type *A. niger* (**Figure 6B**) A4 and *A. niger* isolate JGI 24 [6]. Cu^{2+} and Zn^{2+} have often demonstrated an inhibitory effect on amylase activity [25, 28], a finding that was supported in the present study. However, we found an inhibitory effect of Ca^{2+}, whereas it is commonly reported that Ca^{2+} elevates amylase activity [1, 6, 28].

In summary, HAP *A. niger* WLB42 obtained by EMS mutation and screened using starch as the sole carbon source showed peak amylase production after 48 h, at which time the total protein content in the supernatant also reached a maximum. Optimal amylase production was achieved at 30°C and pH 7, with 40 g/L starch in the liquid medium and 1.4% v/v inoculum concentration. Amylase activity increased to 120% upon the addition of 0.3% w/v urea as a nitrogen source. Glucose increased amylase production to a maximum of 165% at a concentration of 20 mmol/L. Purified amylase had a molecular weight of 50 kD. The optimum temperature and pH for the enzyme were 45°C and pH 7, respectively. The enzyme showed good activity over a board range of temperatures (40-80°C) and pH values (3-7), indicating it has potential use in a broad range of food industry applications. Amylase activity was dramatically enhanced by Co^{2+} addition and slightly increased by Na^{+} and Mn^{2+} addition. The detergents SDS and Triton X-100 inhibited amylase activity to different extents. The more soluble the starch, the higher the hydrolyzability of the substrate by amylase, unless the reducing sugar concentration in the starch was high. Ongoing work in our laboratory focuses on potential safety issue of the mutant, gene cloning, protein structural biology, and application testing.

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Disclosure of conflict of interest

None.

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