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Characterization of an acidophilic α -amylase from *Aspergillus niger* RBP7 and study of catalytic potential in response to nutritionally important heterogeneous compound

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ABSTRACT An acidophilic α -amylase from *Aspergillus niger* RBP7 was purified after solid state fermentation on potato peel substrate. Molecular mass of the purified α -amylase was 37.5 kDa and it exhibited 1.4 mg/ml and 0.992 μ /mol/min K_m and V_{max} values, respectively. The enzyme was stable in the pH range from 2.0 to 6.0, at high NaCl concentration (3 M) and at temperatures between 40 °C and 70 °C. The enzyme showed an optimal activity at pH 3.0 and at 45 °C. The enzyme was inhibited by Hg²⁺ and was stable in the presence of different surfactants (Tween 60, Tween 80, and SDS at 1% level) and different inhibitory reagents (β -mercaptoethanol, phenylmethylsulfonyl fluoride, and sodium azide). This acidophilic amylase enzyme can digest heterogeneous food materials, i.e. the mixture of rice, fish, bread and curry with comparable activity to the commercial diastase enzymes available.

Acta Biol Szeged 62(1):75-82 (2018)

KEY WORDS

characterization
digestion of food material
molecular weight determination,
purification

ARTICLE INFORMATION

Submitted

7 December 2017.

Accepted

21 May 2018.

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Introduction

α -Amylase is a starch hydrolyzing enzyme generating glucose and maltose as end products. It has high market value in different sectors and the market size of amylase in baking industry was 67476.6 tons in 2015. The use of α -amylase in food and beverage industries is expected to be increased by 1.9% from 2016 to 2024 in North America. Acidophilic amylases are applied in different industries like the glucose and fructose syrup production, bakery industry and fruit juice and digestive syrup producing industries (Souza and Magalhães 2010; Parker et al. 2010).

Various bacterial and fungal isolates can produce acidophilic amylases; most of them are belonging to the genus *Bacillus* and *Aspergillus*. In general, the acidophilic amylases can digest different raw starchy food ingredients (Maity et al. 2010; Mukherjee et al. 2017), therefore, they are frequently used to remove such food materials from various surfaces (e.g., textile). In the starch processing industries, acidophilic amylases are used to produce glucose in high amount from the starch polymer (Konsula and Liakopoulou-Kyriakides 2004; Haq et al. 2010; Khan and Priya 2011; Raghu and Rajeswara 2015). In bakery

industry, acidophilic amylases are used to enhance the porosity, flavor and taste of the product (Gupta et al. 2003); amylases are also used for the clarification of beer and fruit juices (Gavrilescu and Chisti 2005; Ghorai et al. 2009).

In the present study, physicochemical characterization of an α -amylase produced by the *Aspergillus niger* RBP7 isolate was evaluated. In addition, the digestion of starchy materials present in nutritionally important heterogeneous foods was also studied comparing the activity of the purified amylase with some commercially available diastases.

Materials and Methods

Microorganism and culture condition

Aspergillus niger RBP7 (GenBank KX100578.1) was isolated from municipal garbage area of Midnapore, West Bengal, India (Mukherjee et al. 2017). The fungal culture was maintained on Czapek Dox agar slants (pH 3) and stored at 4 °C.

Amylase production and enzyme extraction

Enzyme production was carried out using potato peel

as substrate following the method of Mukherjee and co-workers (2017). In the fermentation process, 1.2 g of potato peel was moistened with 1 ml liquid medium (NaNO₃ 0.3 g, MgSO₄ 0.05 g, KCl 0.05 g, FeSO₄ 0.002 g, and K₂HPO₄ 0.1 g); pH and temperature were adjusted to 2.7 and 44 °C, respectively (Mukherjee et al. 2017). The flasks were autoclaved at 121 °C for 20 min at 15 psi pressure. The flasks were cooled, inoculated with 1 ml (2 × 10² spores) of *A. niger* RBP7, and incubated at 27 °C for 96 h. After fermentation, 5 ml of sterile distilled water was added to each flask and vigorously agitated in rotary shaker at 100 rpm for 30 min. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm for 10 min. The supernatant was taken as crude enzyme preparation and used for subsequent experiments.

Assay of amylase activity and protein content

α -Amylase activity was determined by incubating 1 ml reaction mixture containing 0.5 ml of enzyme source and 1% soluble starch dissolved in 0.2 M acetate buffer (pH 3.0) at 37 °C for 1 h. The reaction was stopped by 1 ml of 3,5-dinitrosalicylic acid (Merck, India) mixed with the above mixture. Then the mixture was boiled in water bath for 10 min. The produced reducing sugar was measured at 540 nm. One unit (U) of α -amylase activity was determined as the amount of enzyme that releases 1 μ mol of reducing sugar as glucose equivalent. The protein content of enzyme solution was estimated according to Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme purification

All purification steps were carried out at 4 °C. After the 96-h fermentation, the crude enzyme was salted out with 80% ammonium sulfate and kept overnight at 4 °C. Then, the solution was centrifuged at 10 000 rpm for 10 min and the precipitate was dissolved in 5 ml acetate buffer (0.2 M, pH 3.0). After dialysis against distilled water (24 h), the dialysate was loaded onto 2.5 cm × 70 cm Sephadex G-100 column (Sigma Aldrich, USA), equilibrated with acetate buffer (0.2 M, pH 3.0) and eluted with the same buffer at a flow rate of 1 ml/min. A volume of 2 ml fractions was collected and the amylase activity and the protein content in each fraction were determined.

Molecular weight determination and zymogram analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using a 10% (w/v) polyacrylamide gel. Protein bands were detected by staining the gel with Coomassie Brilliant Blue R250 (Laemmli 1970). For zymography, non-denaturing PAGE (10%, w/v) was performed (Halder et al. 2016) at 4 °C and the gel was subsequently washed with deionized water

and 0.2 M acetate buffer (pH 3.0) at 40 °C. Then, the gel was incubated in fresh acetate buffer containing 1% (w/v) soluble starch at 40 °C for 30 min. After washing with distilled water, the gel was stained with iodine solution until the clear zone of starch hydrolysis was appeared against the dark blue background.

Determination of the pH and temperature optimum and stability

The effect of pH on the activity was estimated in hydrochloric acid and sodium acetate buffer for pH 2.0 (Uchino 1982) and acetate buffer for pH range of 3.0 - 6.0 using 1% starch as substrate. The pH stability was determined by pre-incubating the enzyme under conditions between pH 2.0 - 6.0 for 1 to 6 hours. The residual activity was measured according to the standard protocol as described above. For determination of optimum temperature, the assay was performed at the temperature range of 20 °C - 60 °C. Temperature stability of the purified α -amylase was measured by incubating the enzyme at different temperatures between 40 °C and 70 °C for different time intervals (20 to 120 min). The residual amylase activity was measured under standard assay conditions described above.

Detection of temperature quotient (Q₁₀)

Temperature quotient (Q₁₀) denoted the changes in the rate of the enzymatic catalytic reaction at every 10 °C rise in temperature: $Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$

Where T₁ and T₂ are the initial and final temperatures at which experiment was conducted. R₁ and R₂ are the amylase activity at T₁ and T₂, respectively.

Effects of additives on enzyme activity

To study the effects of various additives on purified enzyme activity, the enzyme was pre-incubated for 1 h at optimum assay conditions with solutions of the following additives.

Metal ions: Ca²⁺ (CaCl₂), Mg²⁺ (MgSO₄), Zn²⁺ (ZnSO₄), Mn²⁺ (MnSO₄), Cu²⁺ (CuSO₄), K⁺ (K₂SO₄), Ag⁺ (AgSO₄), Fe³⁺ (Fe₃SO₄), and Co²⁺ (CoCl₂) at final concentration of 1 mM and 5 mM. After 1 h, we added substrate in each reaction mixture containing additives and incubated the reaction under standard assay condition to get the enzyme activity.

For determining the stability with surfactants, SDS, Tween 60, Tween 80 and Triton X-100 with different concentrations (1%, 2% and 3% w/v) were used. Chelating agents like ethylenediaminetetraacetic acid (EDTA) with the concentration of 1, 2 and 3 (% w/v) were used. Phenylmethylsulfonyl fluoride (PMSF), β -mercaptoethanol, and sodium azide (1%, 2% and 3% w/v) were used as inhibitor for testing the enzyme stability.

Table 1. Purification profile of amylases

Steps for purification	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Recovery (%)
Crude enzyme	292500	8117.5	36.03	1	100
Ammonium sulfate precipitation	122465	846.9	144.6	4.01	41.86
Sephadex G-100	8812.7	14.34	335.61	9.31	7.19

Solvents (1%, v/v) like acetone, methanol, butanol, propanol, toluene, hexane, ethanol, xylene, acetonitrile were used to estimate their effect on enzyme activity. In all cases, the residual activity was measured.

Halo-stability of purified acidophilic α -amylase

The purified enzyme was incubated with various concentrations of NaCl (0 - 5.0 M) for 1 h at room temperature, and the residual α -amylase activity was measured following the standard protocol.

Storage stability and light sensitivity

For determining the storage stability, the two sets of enzymes were incubated (in acetate buffer, pH 3.0) for 14 days at 4 °C and in room temperature (37 °C) as control. The light sensitivity test was investigated by incubating the enzyme in sun light (40 °C) for 14 days. The enzyme activity was checked following the standard assay protocol after sampling at the 7th and 14th days.

Substrate specificity and enzyme kinetics

Different polysaccharides were used for determination of the spectrum of substrate specificity of the enzyme. The tested substrates were soluble starch (Merck, India), amylose (Merck, India), amylopectin (Merck, India), dextran (HiMedia, India) and pullulan (HiMedia, India) at a concentration of 1.0% (w/v) in 0.2 M acetate buffer (pH 3.0). All assays were performed following the standard protocol for enzyme activity determination.

The kinetic constants (K_m and V_{max}) were estimated by double reciprocal plots of the data according to standard method of Lineweaver and Burk (1934) with Sigma Plot Enzyme kinetics software module 1.3 (Systat Software, USA). Michaelis-Menten plot was also drawn using the same software. Equation of Lineweaver Burk plot: $1/V_{max} = K_m/V_{max} \times 1/[s] + 1/V_{max}$. The turnover number and catalytic efficiency were estimated from the following formula: Turnover number (K_{cat}) = V_{max}/E_t (total enzyme), Catalytic efficiency = K_{cat}/K_m .

Study of enzymatic efficiency

Here we used the mixed food materials as substrate to know the activity of purified acidophilic α -amylase and different commercial amylase in heterogeneous condition

of food. In this study different commercial alpha amylases were used, e.g., CarmozymeTM (Mendine Pharmaceuticals, India), UnienzymeTM (Unichem Laboratories), AristozymeTM (Aristo Pharmaceuticals), VitazymeTM (East India Pharmaceutical). Briefly, a mixed preparation of food materials, *i.e.* rice, fish, bread and vegetable curry, was crushed into small pieces. Then the raw material was smashed with a homogenizer with the addition of acetate buffer (pH 3.0). The whole matter was considered as substrate (1 ml) and was incubated with 1 ml (150 U) of purified enzyme and different commercial enzyme preparations (150 U/ml). After incubation the reducing sugars of each experimental set were determined according to dinitrosalicylic acid method.

Results and Discussion

Enzyme production

The *A. niger* RBP7 produced 1112.25 U/gds of α -amylase in 72 h fermentation period at 40 °C and pH 3.0 (Mukherjee et al. 2017). This crude amylolytic solution was used for the enzyme purification.

Purification of the α -amylase

The α -amylase from *A. niger* RBP7 was purified by ammonium sulfate precipitation followed by a size exclusion chromatography step using Sephadex G100 resin. The purified enzyme had a specific activity of 335.61 U/mg of protein. The molecular weight of enzyme was found to be approximately 37.5 kDa (Fig. 1). The purification procedures are summarized in the Table 1. The isolated enzyme was homogeneous, as seen by a single protein both in native and with reduced and denaturing condition. The protein band of purified α -amylase was confirmed by the zymogram. The purified enzyme (single band) showed clear zone around the single band on starch containing zymogram when the zymogram was flooded with iodine solution (Fig. 1). The microbial α -amylases generally have their molecular weight between 23 and 150 kDa (Krishnan and Chandra 1983; Pandey et al. 2000). Fungal amylases from the *A. niger* JGI 24 and *Monascus sanguineus* have molecular weight of 43 kDa and 56 kDa, respectively (Varalakshmi et al. 2009; Tallapragada et al. 2017).

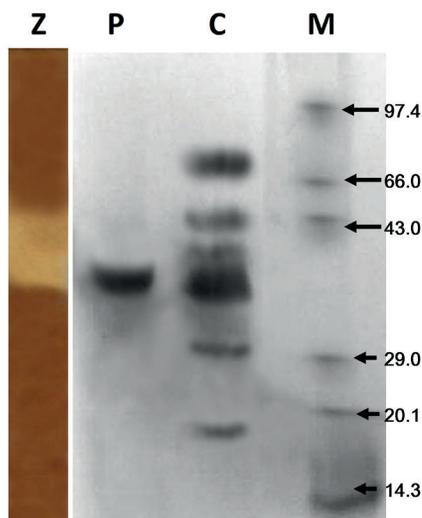


Figure 1. PAGE analysis of each fraction during purification. Z: zymogram; P: purified enzyme; C: crude enzyme; M: molecular weight marker (kD) after column chromatography.

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

The purified α -amylase exhibited the maximal starch hydrolysis at pH 3.0 (Fig. 2). The enzyme was stable in pHs between 2.0 and 6.0 for 60 to 300 min incubation period (Fig. 3). The purified α -amylase showed maximum amyolytic activity at 45 °C (Fig. 4) and it was stable during incubation at 40 - 70 °C for 20 to 120 min (Fig. 5). Yandri et al. (2012) reported that α -amylase from *Bacillus subtilis* ITBCCB 148 can work between pH 5.0 and 9.0 and maximum activity showed at pH 6.0. The *Streptomyces* sp. MSC702 α -amylase have optimum activity at 60 °C and the enzyme was stable between 20 and 80 °C (Singh et al. 2014). Aygan et al. (2008) reported that the α -amylase obtained from *Bacillus* sp. AB 68 was active in a broad range of temperature (20 to 90 °C), with an optimum of 50 °C. The *Aspergillus terreus* amylase have its optimum activity at pH 5.0, but it is stable at different pHs (pH 3.0 - 10.0) (Sethi et al. 2016).

Temperature quotient (Q_{10})

Temperature quotient (Q_{10}) is mainly used to study the dependence of enzyme catalytic reaction rate on temperature. Results showed that temperature quotient (Q_{10}) of the acidophilic amylase is 1.065 at 45 °C. The temperature coefficient value (Q_{10}) for *Bacillus licheniformis* SKB4 α -amylase was found to be 1.0 (Samanta et al. 2014). This Q_{10} value is calculated in order to know whether temperature is crucial factor for enzymatic reaction or not. For enzymatic catalysis range of Q_{10} value is generally 1-2, and any deviation from that there are other influencing factors which controlling the enzyme activity. In our case

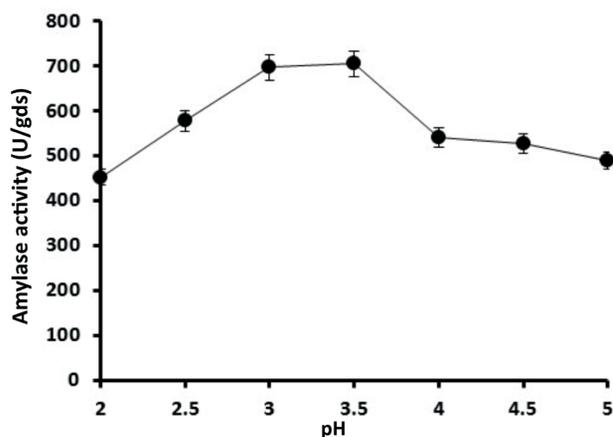


Figure 2. Activity of α -amylase at different pH

the Q_{10} value is 1.065, which indicates that temperature is crucial factor for enzymatic action.

Effect of additives on α -amylase

Metal salts have both enhancing and inhibitory effects on the enzyme activity and stability. Therefore, metal compatibility profiling of an enzyme is very important to find out its optimum catalytic conditions. In this study, it was observed that each metal had inhibitory effect on enzyme activity (Table 2). More than 40% of the activity was lost in presence of all the salt tested in 5 mM concentration. In the presence of 3% EDTA and β -mercaptoethanol, the relative activity of the enzyme decreased by 72% and 82%, respectively. The enzyme activity decreased drastically to about 75% in presence of the solvents tested. This may be due to the amino acid composition as well as three dimensional structures, which endorsed relative resistance of the enzyme against hydrophobic/non-polar micro environment (Halder et al. 2016).

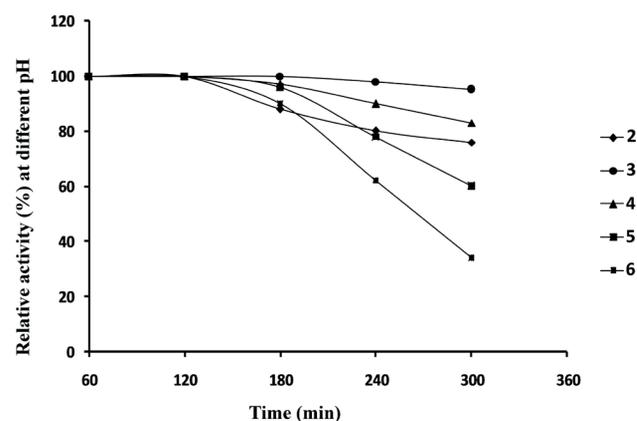
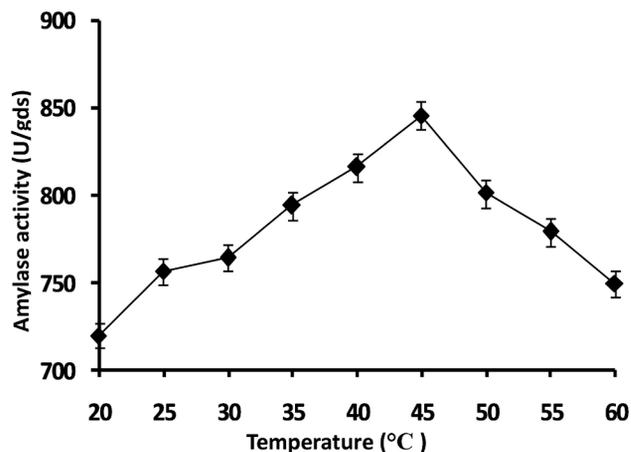


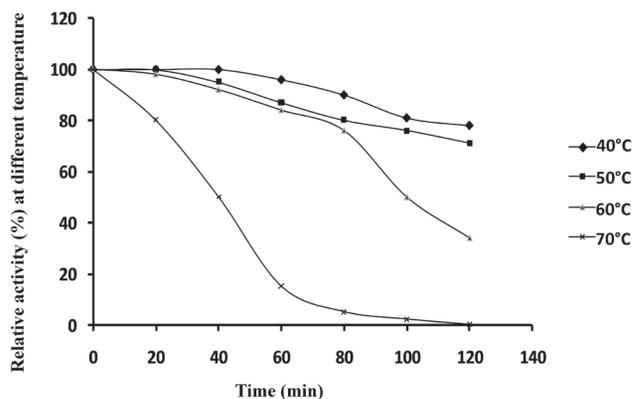
Figure 3. Effect of pH on the stability of purified α -amylase of *A. niger* RBP7

Table 2. Effect of additives on purified α -amylase of *A. niger* RBP7

Metal ion	Concentration	Relative activity (%)
K ⁺	1 mM	53.53
	5 mM	53.12
Cu ²⁺	1 mM	60.2
	5 mM	58.1
Fe ³⁺	1 mM	56.86
	5 mM	55.61
Mg ²⁺	1 mM	59.35
	5 mM	58.9
Zn ²⁺	1 mM	61.01
	5 mM	59.34
CO ²⁺	1 mM	60.6
	5 mM	53.54
Ca ²⁺	1 mM	60.6
	5 mM	57.6
Ag ⁺	1 mM	61.8
	5 mM	57.6
Mn ²⁺	1 mM	60.17
	5 mM	58.5
Surfactant		
SDS	1%	34.03
	2%	32.8
	3%	23.24
Tween 60	1%	43.6
	2%	37
	3%	35.7
Tween 80	1%	51.05
	2%	40.7
	3%	40.26
Triton X	1%	40.26
	2%	33.6
	3%	30.7
Solvent		
Acetone	1%	22.8
Methanol	1%	25.31
Butanol	1%	22
Propanol	1%	22.83
Toluene	1%	22
Hexane	1%	23.5
Ethanol	1%	23.65
Xylene	1%	22.4
Acetonitrile	1%	22.83
Chelating agent		
EDTA	1%	32.8
	2%	31.12
	3%	28.21
Inhibitory agent		
PMSF	1%	46.48
	2%	46.48
	3%	44.82
β -mercaptoethanol	1%	20.75
	2%	20.33
	3%	17.84
Sodium azide	1%	45.7
	2%	45.23
	3%	37.8

**Figure 4.** Activity of α -amylase at different temperature**Effect of NaCl on the purified α -amylase**

The high salt tolerance capability of enzyme is very important for its commercialization in bioprocess industry. Enzymatic activity of α -amylase was tested in presence of NaCl (0 - 5.0 M). Salt tolerant capacity of acidophilic amylase from *A. niger* RBP7 at various NaCl concentrations shown in Fig. 6. It was found that NaCl was not required for activity; however, maximum starch hydrolyzing activity (808 U/gds) was observed in the presence of 3 M of NaCl. The enzyme was found to be salt-tolerant since the hydrolysis also proceeded well in the presence of 5 M NaCl. Previous study showed that α -amylases produced by *Bacillus* sp MD124 was stable in 1 M NaCl concentration (Jana and Pati 1997). Amylase activity was 0.105 U/ml and inhibited by 44.7% NaCl produced by *Monascus sanguineus* reported by Tallapragada et al. (2017). There was a salt tolerant amylase produced by *Bacillus* sp. MD124 which was found to be retained 75% of its activity in 5.0

**Figure 5.** Effect of temperature on the stability of purified α -amylase of *A. niger* RBP7

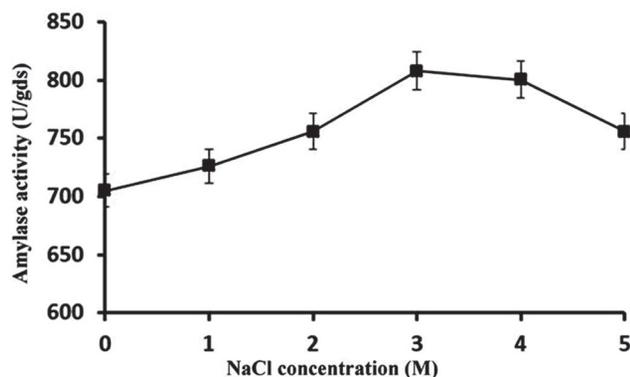


Figure 6. Activity of α -amylase at different concentration of NaCl

M of NaCl concentration (Jana et al. 1997).

Storage stability and light sensitivity

The purified amylase retained about 90% of its activity after 14 days incubation at room temperature (Fig. 7) and retain 97% activity in freezing condition. Enzyme retains its 95.4% activity after 14 days of incubation.

Substrate specificity and enzyme kinetics

The enzyme was active on soluble starch, amylose, amylopectin. It was found that the hydrolytic efficiency was different from substrate to substrate and the descending order of efficiency as follows: soluble starch > amylose > amylopectin. Pullulan was not cleaved by the enzyme. The enzyme showed Michaelis-Menten kinetic on soluble starch applied in various concentrations (Fig. 8). Kinetic parameter (V_{max} , K_m , K_{cat} and catalytic efficiency) values for the hydrolysis of soluble starch by α -amylase were K_m (1.4 mg/ml), V_{max} (0.992 μ mol/ min), K_{cat} 2.9796 min^{-1} and catalytic efficiency 5.0693 mg/ml/min (Fig. 8a, b; $V_0 = V_{max} [S] / K_m + [S]$). The result of the relationship between substrate concentration and enzyme activity of substrates reveal that the activity of the enzyme increased concomitantly with an increase in substrate concentration. But the enzyme activity stopped to increase when the concentration exceeded 4 mg/ml indicating that substrate saturation was taking place. Haq et al. (2010) reported that α -amylase with K_m value of 4.11 mg/ml and

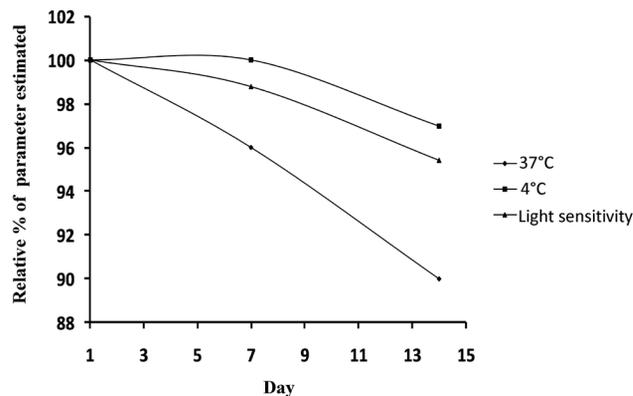


Figure 7. Effect of storage time and light sensitivity on stability of α -amylase of *A. niger* RBP7

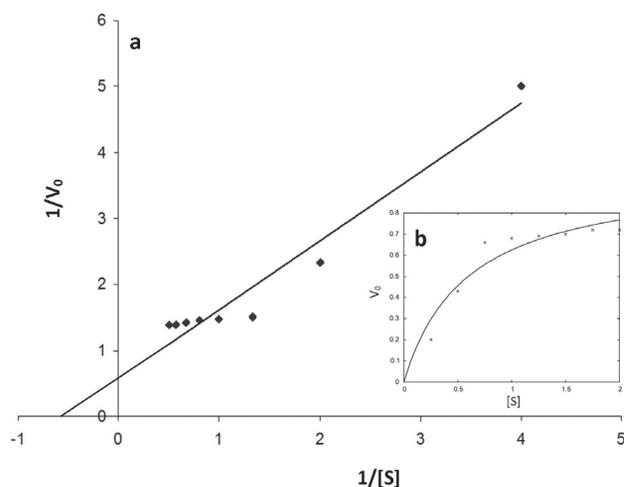


Figure 8. Lineweaver-Burk (a) and Michaelis-Menten (b) plots of enzyme kinetics of purified α -amylase at optimum pH and temperature

V_{max} value 0.45 mg with soluble starch as substrate from *B. licheniformis* EMS-6.

Enzymatic efficiency and bioconversion of heterogeneous nutritionally important compound

In these studies, mixture of food ingredients, *i.e.* rice, bread, fish and vegetable curry were digested with the

Table 3. Comparison of α -amylase from RBP7 with the commercially available Diastase

Enzyme	Enzyme activity (U/ml)	Specific activity (U/mg)	Reducing sugar (μ g/ml)
Carmozyme™	157.36	15.73	78.65
Vitazyme™	118.3	29.57	29
Aristrozyme™	121.73	12.17	60.84
Unienzyme™	115.79	17.39	57.88
α -Amylase from <i>A. niger</i> RBP7	155.9	5.5	77.91

purified amylase of *A. niger* RBP7 and the performance was compared with some commercially available amylolytic cocktails. Results showed that the reducing sugar concentration after treatment with *A. niger* RBP7 α -amylase was higher than those detected for most of the commercial enzymes tested. Only the Carmozyme cocktail resulted similar digestive capacity (Table 3). Anyway, the α -amylase from *A. niger* RBP7 have the potential to degrade starchy materials present in the mixture of different food ingredients.

Conclusion

Acidophilic α -amylase produced in SSF from *A. niger* RBP7 was purified by ammonium sulfate precipitation followed by gel filtration chromatography. The purified enzyme had molecular weight of 37.5 kDa detected on SDS-PAGE and exhibited active band after zymography. It can hydrolyze starchy materials present in the mixture of rice, bread, fish and vegetable curry food products. The broad pH and temperature stability and the activity remained after long-time storage are also validate the possible application of the *A. niger* RBP7 α -amylase as additive for digestive syrup cocktails.

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