Chapter 3: Purification and kinetic characterization of the acidophilus amylase isolated from *Aspergillus niger* RBP 7

3.1. Introduction

The composition of different enzymes obtained from plant and animal tissues are almost similar with the enzymes produced by microorganisms. The purification of enzymes must be required to study the characteristics of any particular enzyme. Until the enzyme in question has been purified, it is sometimes difficult to specify exactly what reaction it catalyses. If an enzyme catalyzes some reactions which are differing from its normal reaction there will be some probability of the presence of another enzyme which catalyzes the reaction (Dixon and Webb, 1979). Therefore, for the study of the properties and behavior of an enzyme as a chemical catalyst, it is necessary to have it as pure as possible. Enzymes vary from each other in size, shape, charge, solubility and biological activity and these inherent similarities and differences were considered to purify them away from other non- protein contaminants. Employing successive chemical and physical fractionation procedure, enzymes can be purified. Each step of the purification is given by yield or recovery and a purification factor. Once an enzyme is purified, it is possible to study its enzymology, to understand its affinity for particular substrate and detect its ability to catalyze enzymatic reactions. For the purification of enzymes different chromatographic techniques are required such as ion-exchange chromatography, gel filtration chromatography, affinity chromatography etc.

Alpha amylases have been purified from different acidophiles (Kumari et al., 2013), alkalophiles (Prakasham et al., 2006), thermophiles (Rao et al., 2002). The properties and specificity of an alpha amylase is generally depends on its source and each α- amylase produces a characteristic oligosaccharide on hydrolysis of glucan (Forgarty and Kelly, 1979).

The purification of α- amylases from different microbes was reported previously which are as follows *Aspergillus carbonarius* (Okolo et al., 2000), *Monascus sanguineus* (Tallapragada et al., 2017), *Aspergillus flavus* (Sidkey et al., 2011), *Bacillus thuringiensis*

(Smitha et al., 2015), *Bacillus subtilis* (Gbenga et al., 2017) *Bacillus cereus* (Annamalai et al., 2011), *Bacillus amyloliquefaciens* (Demirkan and Utsumi, 2005).

Among fungal amylases mostly amylase producing fungi is *Aspergillus* sp. and it has many industrial importances. The amylase producing *Aspergillus* sp. are *Aspergillus niger, Aspergillus flavus, Aspergillus awamori* etc. The pH of the medium played a major role on the activity of enzyme. Not only the pH but also the other factors like temperature, substrate concentration, and inhibitory agent also affect the activity of enzyme.

The present chapter deals with the purification of acidophilus amylase from *Aspergillus niger* RBP7. Some properties of the purified enzyme have also been characterized.

3.2. Materials and methods

3.2.1. Acidophillus amylase extraction from the end product of SSF

Enzyme production by *Aspergillus niger* RBP7 was carried out using potato peel as substrate as described in chapter 1. In this fermentation process 1.25 g of potato peel was used as substrate and moistened with 1 ml of liquid medium (%, w/v): [$NaNO_3$ (0.3), $MgSO_4$ (0.05), KCl (0.05), $FeSO_4$ (0.002), K_2HPO_4 (0.1)] and pH was adjusted to 3.0. These flasks were autoclaved and cooled, then inoculated with 1 ml (2×10^2 spore) of *Aspergillus niger* RBP7 spore, and subsequently incubated at 45 °C for 96 h. After fermentation 15 ml of sterile distilled water was added to each flask and vigorously agitated in rotary shaker for 30 min at 100 rpm. The mixture was filtered through cheese cloth and centrifuged at 8,000 rpm for 10 min. The supernatant was used as the crude enzyme preparation and used for subsequent study.

3.2.2. Assay of amylase activity and protein content

As described in chapter 2 (2.2.4.4 and 2.2.4.5)

3.2.3. Enzyme Purification

The crude enzyme that was obtained after 96 hours fermentation was saturated with 80 % ammonium sulfate [$(NH_4)_2SO_4$] and kept overnight at 4 ºC to precipitate the proteins. The

precipitate was recovered by centrifugation at 10,000 rpm for 10 minutes. The obtained precipitate was dissolved in 0.2 M acetate buffer (pH 3.0) followed by dialysis against distilled water for 24 h at room temperature. The dialysate (5 ml) was loaded onto Sephadex G- 100 column (2.5cm×70cm) equilibrated with acetate buffer (pH 3.0, 0.2M) and enzyme was eluted with the same equilibrating buffer at a flow rate of 1 ml/min. The fractions of 2 ml were collected using an auto-fraction collector (Eyela, Japan). All the steps of purification were carried out at 4 ºC. The amylase activity and protein content in each fraction was estimated accordingly.

3.2.4. 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 slab with Coomassie brilliant blue R250 (Laemmli, 1970). For zymography, non-denaturing 10 % (w/v) PAGE was performed (Halder et al., 2016) and the electrophoresis was carried out at 4 ºC. After that, the gel was rinsed with deionised water and washed at 40 ºC in 0.2 M acetate buffer (pH 3.0). The washed gel was incubated in fresh buffer containing 1% (w/v) soluble starch at 40 ºC for 30 min. After being washed with distilled water, the gel was subjected to staining with iodine solution for the appearance of clear zones (of starch hydrolyzation) against a dark blue (starchy) background.

3.2.5. Substrate specificity and enzyme kinetics

Alternative substrates 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 (HiMidia, India) at a concentration of 1.0 % (w/v) in 0.2 M acetate buffer (pH 3.0). All sets of assay were performing following the standard protocol DNS method by Miller (1959).

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 Enzyme kinetics software module 1.3, 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 turn over number and catalytic efficiency were estimated from the following formula-

Turn over number (K_{cat}) = V_{max}/E_t (total enzyme)

Catalytic efficiency = K_{cat}/K_m

3.2.6. Measurement of optical rotation

The optical rotation of starch hydrolyzed products by purified amylase was determined by following method of Hyun and Zeikus (1985). A reaction mixture (1 ml) consisting of 1g % starch solution in 0.2 M acetate buffer (pH 3.0) and 100 µl of purified enzyme in a 1 cm cuvet was incubated at room temperature. The mutarotation of the mixture was measured after 30 min by adding 30 mg of solid sodium carbonate to the reaction mixture in a Perkin- Elmer polarimeter (model 174) by using sodium light.

3.2.7. Measurement of dextrinizing activity

Hydrolysis of soluble starch was made with purified amylase under standard assay condition. The reduction of blue colour complex of starch in presence of iodine was determined colorimetrically (Smith and Roe 1949).

3.2.8. Effect of inhibitors on enzyme activity

Enzyme activity was measured in the presence of inhibitors like iodoacetamide and p-chloromercuro benzoate at the concentration of 10^{-2}M.

3.2.9. Determination of optimum pH and Temperature and stability

The effect of pH on the activity was estimated by assaying with starch (1%) dissolved in acetate buffer at a pH range of 2.0 - 6.0 every 0.5 unit interval. The pH stability was determined by pre-incubating the enzyme with a range of pH (2.0 - 6.0) for 1 h - 6 h. In this condition the residual activity was measured. Assays were performed according to the standard protocol as described earlier in chapter 1.

For determination of optimum temperature, the assay was performed with a range of temperature (20 - 60 ºC) every 5°C interval. The high temperature stability of purified α-

amylase was measured by incubating at different temperatures (40 -70 ºC) for different time intervals (20-120 min). The residual amylase activity was measured according to standard protocol.

3.2.10. Effects of additives on enzyme activity

To study the effects of various additives on purified enzyme, the enzyme was pre-incubated for 1 h at optimum assay condition and then incubated with the following salt solutions:

Metal ions: Ca^{2+} ($CaCl_2$), Mg^{2+} ($MgSO_4$), Zn^{2+} ($ZnSO_4$), Mn^{2+} ($MnSO_4$), Cu^{2+} ($CuSO_4$), K^+ (K_2SO_4), Ag^+ ($AgSO_4$), Fe^{3+} (Fe_3SO_4), Co^{2+} ($CoCl_2$) at final concentration of 1 mM and 5 mM.

For determining the stability with surfactants, SDS, Tween-60, Tween-80 and TritonX-100 were used with different concentrations (1%, 2%, 3%, w/v).

Chelating agents like ethylene diamine tetra acetic acid (EDTA) with the concentration of 1, 2 and 3 (%, w/v) were used.

β- Mercaptoethanol and phenylmethylsulfonyl fluoride (PMSF) (1%, 2%, 3%, w/v) were used as inhibitor for testing the enzyme stability.

In all cases, the residual activity was measured.

3.2.11. Detection of temperature quotient (Q_{10})

Temperature quotient (Q_{10}) denoted that the changes the rate of an enzymatic catalytic reaction at every 10 °C rise in temperature.

$$Q_{10} = (R_2/R_1)^{10/(T_1 - T_2)}$$

Where T_1 and T_2 are the initial and final temperatures at which experiment was conducted.

R_1 and R_2 are the amylase activity at T_1 and T_2 respectively.

3.2.12. Storage stability and light sensitivity

For determining the storage stability, enzyme was incubated at room temperature and as well as in refrigerator (4 ºC) for 15 days. The enzyme activity was determined by removing

the samples after 7th day and 15th day. The enzyme assay was performed by following standard method with starch as substrate (DNS method by Miller, 1959).

The light sensitivity test was also performed by incubating the enzyme under sunlight for 15 days. The enzyme activity was checked by removing the samples at 7th day and 15th day. The enzyme assay was performed by following standard protocol with starch as substrate (DNS method by Miller, 1959).

3.3. Result and discussions

3.3.1. Purification and characterization of α-amylase

Proteins were precipitated with saturation to 20%, 40%, 80 % by ammonium sulfate and the α- amylase from *Aspergillus niger* RBP7 was subsequently purified with size exclusion chromatography using a Sephadex G100 column. Table 3.1 showed that at the initial step (ammonium sulphate) fractionation, the recovery of enzyme activity was 41.86%. Finally the enzyme was purified about 9.31 fold with specific activity of 335.61. The activity recovery was 7.19%. The active fraction from gel filtration were concentrated and applied on SDS-PAGE. The ammonium sulphate precipitation process of amylase was studied by many workers (Nguyen et al., 2002; Azad et al., 2009). Bolton et al. (1997) purified α- amylase of *Bacillus flavothermus* by ammonium sulphate precipitation and the specific activity of protein of purified amylase was 335.61 U/mg. Priest et al. (1984) reported that α-amylase from *Lipomyces kononenkoae* purified by ammonium sulphate treatment, affinity binding on cross-linked starch and DEAE- Biogel chromatography.

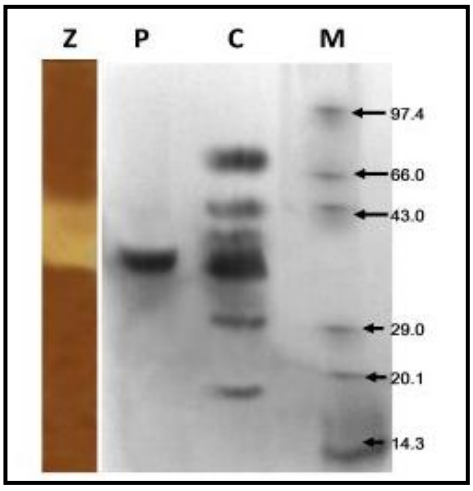

Fig 3 .1. PAGE analysis of each fraction during purification: Z, Zymogram; P, Purified enzyme; C, Crude enzyme; M, Molecular weight marker (kD) after column chromatography.

The isolated enzyme was homogeneous, as seen by a single protein band both in native and reducing condition advocating the nature of the enzyme. The protein band of purified α-

amylase was confirmed by the zymogram analysis. The purified enzyme (single band) hydrolyzed the starch present in the zymogram showing a clear zone around the blue starch iodine complex background (Fig. 3.1). The molecular weight of enzyme was found to be approximately 37.5 kDa (Fig. 3.1). Literature survey attested that the α-amylase has a wide range of molecular weight. The α- amylases of molecular weight ranging from 42 to 150 kDa from different *Bacillus sp* were studied by Pandey et al (2000). Similarly fungal amylase have molecular weight of 43 kDa from *Aspergillus niger* JGI 24 (Varalakshmi et al., 2009), 56 kDa from *Monascus sanguineus* (Tallapragada et al., 2017), 66 kDa from *Aspergillus oryzae* (Patel et al., 2005), 50 kDa from *Engyodontium album* (Ali et al., 2014) were also reported.

Table 3.1. Purification profile of acidophilus α- amylase from *Aspergillus niger* RBP7

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

3.3.2. Substrate specificity and enzyme kinetics

The enzyme was hydrolytically active on a number of substrates. It was found that the hydrolytic efficiency was differing substrate to substrate and the descending order of efficiency as follows: soluble starch > amylose > amylopectin. The enzyme was unable to cleave pullulan. The activity was estimated at various concentration of soluble starch (Fig. 3.2 a, b). 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

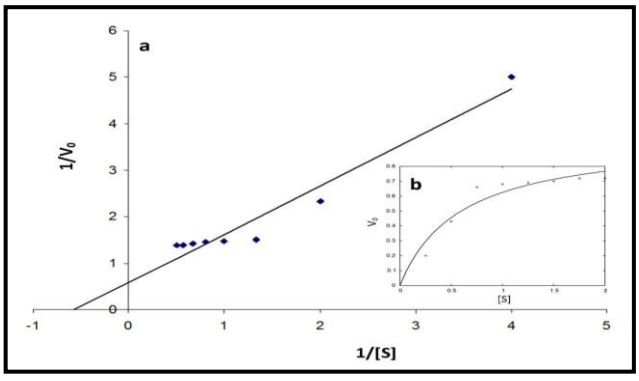

Fig 3 .2. Lineweaver–Burk (a) and Michaelis–Mentens (b) plots of enzyme kinetics of purified α- amylase at optimum pH and temperature.

increase when the concentration exceeded 4 mg/ml indicating that substrate saturation was taking place. Kinetic parameter (V_{max}, $K_{m,}$ K_{cat} and catalytic efficiency) values for the hydrolysis of soluble starch by α-amylase were illustrated here. According to the result revealed that α-amylase gave K_m (1.4 mg/ml), V_{max} (0.992 µmol/ min), K_{cat} 2.9796 min^{-1} and catalytic efficiency 5.0693 mg/ml/min for soluble starch . The production of amylase by *Monascus sanguineus* showed K_m value 0.055 mM and V_{max} value 22.075U/ml was reported by Tallapragada et al. (2017) and Negi and Banerjee, (2009) reported that the K_m and V_{max} value of amylase was 9.8 mg/ml and 56.2 mg/ml/min respectively where amylase was produced by *Aspergillus awamori*.

3.3.3. Determination of the type of amylase

To know the type of amylase (α/β) produced by *Aspergillus niger* RBP7 dextrinizing activity, optical rotation of hydrolytic product studies were carried out. The amylase showed a rapid loss of intensity of starch- iodine colour complex (blue loss) (Fig. 3.3). The mutarotation of the enzymatic starch hydrolytic products were estimated and represented in Fig. 3.4. It revealed that the value of optical rotation of hydrolyzed products decreased in respect to time of hydrolysis. Previous study showed that rapid loss of percentage of iodine colour and is due to the multiple attack of substrate by the enzyme (Samanta et al., 2017). Thus, this enzyme acted on substrate by endo- attacking fashion. Bailey and Whelan (1961) stated that minimum 18 glucose units are required in a chain length for starch iodine colour formation. The anomeric configuration of the oligosaccharide formed by the action of the purified enzyme was shown in Fig 3.4. This result indicated that anomeric configuration of the hydrolytic product was α-configuration.

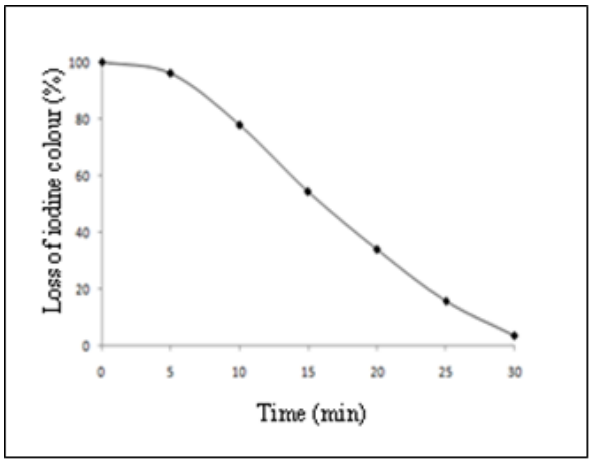

Fig.3.3. Percentage loss of starch iodine colour complex in presence of starch as substrate after enzymatic degradation by amylase from *Aspergillus niger* RBP7.sample were withdrawn at 0, 5, 10, 15, 20, 25, 30 min and processed for formation blue colour in presence of iodine.

The similar findings of anomeric products were obtained by Jana et al. (2013), Samanta et al. (2017).

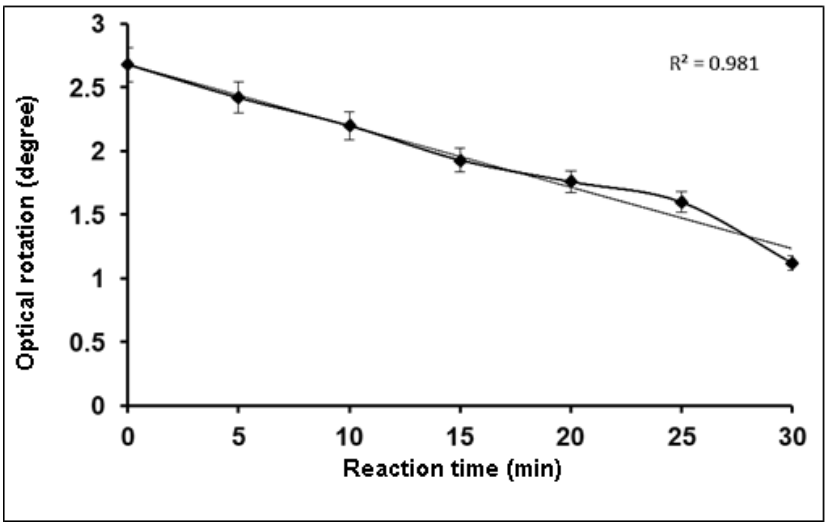

Fig.3.4. Optical rotation of the products formed by the action of the purified amylase from *Aspergillus niger* RBP7 in presence of solid sodium carbonate

The enzyme was not inhibited by p-chloromercuro benzoate (10^{-2}M) and iodoacetamide (10^{-2}M). Haifeng et al. (2007) reported that α-amylase was not inhibited by iodo-acetic acid. From the above study it can be established that the purified enzyme is endo-attacking α- amylase and not inhibited by β- specific inhibitor (Fig. 3.5). Basically these two inhibitors, p- chloromercuro benzoate and iodoacetamide inhibit the thiol group if present in the active site (these are common in case of β- amylase).

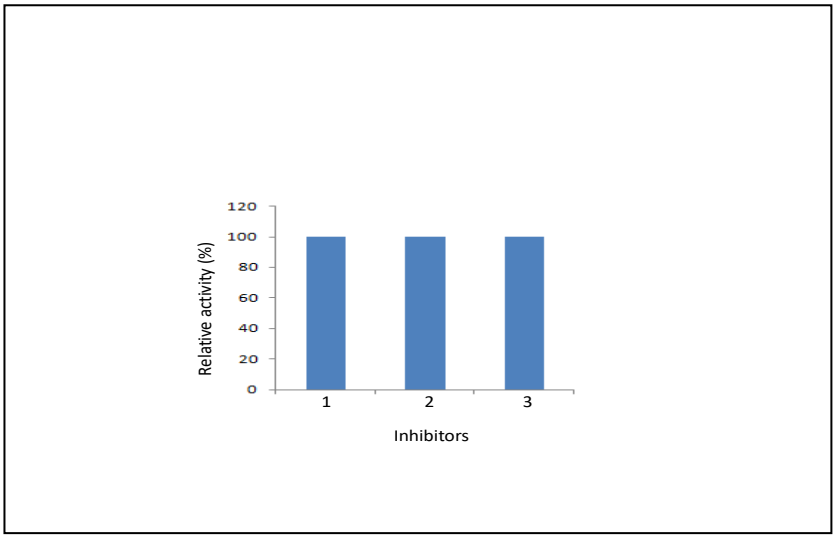

Fig.3.5. Effect of enzyme inhibitors on amylase activity of acidophilic amylase from *Aspergillus niger* RBP7. 1. Control, 2. P-chloromercuro benzoate (10^{-2}M), 3. Iodoacetamide (10^{-2}M). Amylase activity was measured at pH-3.0 and temperature 40 ^{o}C.

In case of α- amylase three amino acids like aspertate-206, glutamate-230, and aspertate-297 are present in the active site (Matsuura 1991). But in case of β- amylase two cysteine residues are present at the position of 95 and 343 at its active site (Mikami et al., 1980). Addition of thiol group

inhibitors blocks the cysteine residue and inhibits the β- amylase activity. The acidophilic amylase from *Aspergillus niger* RBP7 was not inhibited by thiol group inhibitor, increased rapid loss of iodine color and produced α- anomeric products, then, it was definitely an endo- attacking α- amylase.

3.3.4. Effects of pH and temperature on activity and stability of the enzyme

The maximum activity of α- amylase of the experimental organism was showed at pH 3.0 (Fig. 3.6). The enzyme showed the stability in a broad range of pH (2.0 - 6.0) at 60 to 300 min of incubation period (Fig. 3.7). Below and above the optimum pH not only the shape and structure of enzyme is changed but also the structure of substrate is also changed, so the substrate cannot bind with the enzyme active site and the catalysis is halted. The purified amylase showed maximum activity at 45 °C (Fig. 3.8) and the stability of the enzyme was observed between 40 - 70 °C at 20 to 120 min of incubation (Fig. 3.9). Enzyme catalyzed reaction rate initially increases as temperature rises owing to increase kinetic energy of the reacting molecule. However, if the kinetic energy of environment exceeds the energy barrier of enzyme, that can able to disrupt the weak hydrogen and hydrophobic bonds. As a result enzyme becomes denatured with an accompanying loss of catalytic activity (Samanta et al., 2009). Yandri et al. (2012) reported that α- amylase from *Bacillus subtilis* ITBCCB 148

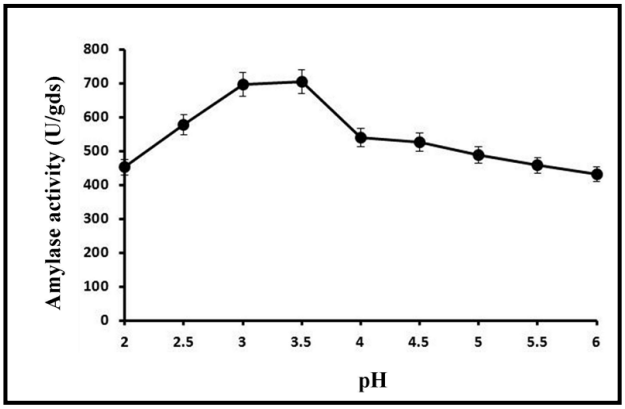

Fig. 3 .6. Maximum activity of acidophilus amylase RBP7 at pH- 3.0

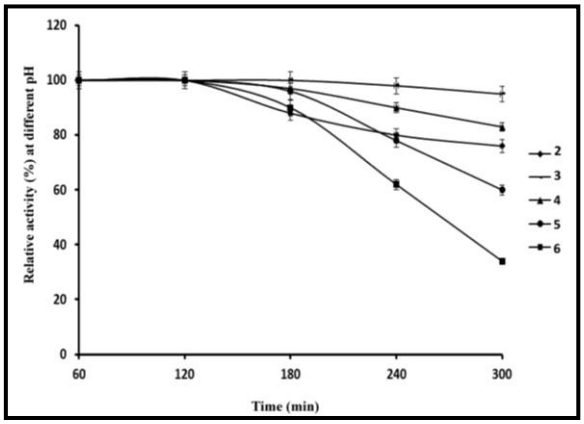

Fig. 3 .7. The stability of purified α- amylase of *Aspergillus niger* RBP7 at pHs 2-6 for 60-300 min of incubation

can work between pH 5.0-9.0 and maximum activity showed at pH 6. Oyewale et al. (2012) found that at pH 7.0, amylase showed its maximum activity and enzyme was produced by *Aspergillus flavus*. Fungi *Aspergillus terreus* have its optimum activity at pH 5.0 but it is stable in ranges between pH 3.0 - 10.0 (Sethi et al., 2016). Sidkey et al. (2011) reported that amylase from *Aspergillus flavus* have optimum pH 3.0 and optimum temperature 30 ^{o}C. Aygan et al. (2008) reported that enzyme obtained from *Bacillus sp* AB 68 was active in a broad range of temperature 20 to 90 °C, with an optimum of 50 °C. Odibo and Ulbrich-Hofmann, (2001) reported that α- amylases produced by *Thermomyces lanuginosus* showed its highest activity at 60^{o}C.

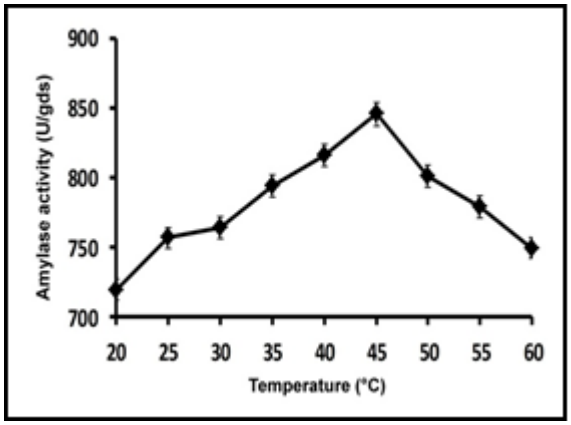

Fig. 3.8. Maximum activity of acidophilus amylase RBP7 at temperature 45 ^{o}C

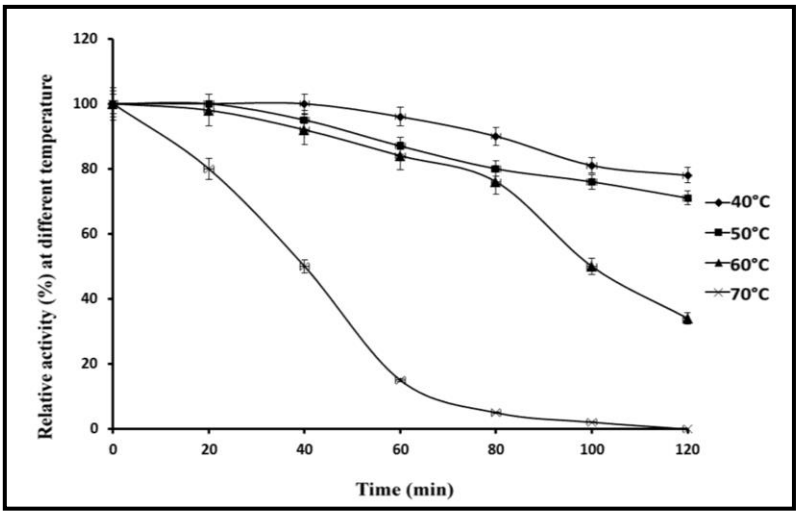

Fig. 3 .9. The stability of purified α- amylase of *Aspergillus niger* RBP7 at temperatures 20-80 ^{o}C for 60-120 min of incubation

3.3.5. Temperature quotient (Q_{10})

Temperature quotient (Q_{10}) is mainly used to study the dependence of enzyme catalytic reaction rate on temperature. Result showed that temperature quotient (Q_{10}) of the acidophilic amylase is 1.065 at 45 ^{o}C. Samanta et al. (2014) reported that amylase

production from *Bacillus licheniformis* SKB4 have temperature coefficient value (Q_{10}) 1.0. 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 the Q_{10} value is 1.065, which indicates that temperature is crucial factor for enzymatic action (Samanta et al., 2014).

3.3.6. Effect of additives on α-amylase

Metals have both influencing and inhibitory effect on enzyme activity and stability (Pandey et al., 2000; Gupta et al., 2003). 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 tested metal had mild inhibitory effect on enzyme activity (Table 3.2). So, this α-amylase is not a metallo enzyme. The activity of amylase retained 70% in presence of all the tested salt at 5mM concentration. The activity amylase RBP7 was not fully metal dependent. This result is comparable with the findings of Rao et al. (2002). The Ca^{2+} independent amylase from *Trichoderma pseudokoningii* was reported by Abdullal et al. (2018)

The relative activity of enzyme showed more than 80% in presence of 1% EDTA. Therefore, metal chelation has no major inhibitory effect on enzyme activity. The inhibitory agents like phenylmethylsulfonyl fluoride (PMSF) is a serine protease inhibitor. Phenylmethylsulfonyl fluoride (PMSF) at 3%, had no immense effect on the relative activity of amylase which was 87.82%. Kumari et al. (2013) also reported that PMSF had no effect on amylase activity produced from *Bacillus cereus* MTCC 10205. Beta mercapto ethanol is mainly used to reduce disulfide bonds. In presence 3% β- mercaptoethanol the relative activities were 92.84% therefore, it had no major effect on amylase activity. No major inhibition of enzyme activity in presence of β- mercapto ethanol indicating that there was any involvement of SH- group at the active site of enzyme. This result is also similar with the report of Kumari et al. (2013).The amylase activity more than 30% was retained in presence of different surfactant (SDS, tween 60, tween 80, triton X). Previous study

showed that the amylase activity was decreased by 85% in presence of different surfactant SDS, tween 60, tween 80, triton X (Jaiswal and Prakash, 2013).

Table 3.2. Effect of additives and metal ions on the activity of purified acidophilus α-amylase of *Aspergillus niger* RBP7

Metal ion	**Concentration**	**Relative activity (%)**
Control is 100%	1 mM	73.53
K+	5 mM	73.12
Cu^{2+}	1 mM	75.2
	5mM	72.1
Fe^{3+}	1 mM	76.86
	5 mM	75.61
Mg^{2+}	1 mM	79.35
	5mM	78.9
Zn^{2+}	1 mM	81.01
	5 mM	79.34
CO^{2+}	1 mM	80.6
	5 mM	76.54
Ca^{2+}	1 mM	93.6
	5 mM	90.6
Ag^{+}	1 mM	79.8
	5 mM	77.6
Mn^{2+}	1 mM	78.17
	5 mM	75.5
Surfactant	**Concentration**	**Relative activity (%)**
SDS	1%	34.03
	2%	32.8
	3%	23.24
Tween60	1%	43.6
	2%	37
	3%	35.7
Tween80	1%	51.05
	2%	40.7
	3%	40.26
TritonX	1%	40.26
	2%	33.6
	3%	30.7
Chelating agent	**Concentration**	**Relative activity (%)**
EDTA	1%	88.8
	2%	76.12
	3%	65.21
Inhibitory agent	**Concentration**	**Relative activity (%)**
PMSF	1%	90.4
	2%	88.48
	3%	87.82
β- mercapto ethanol	1%	95.75
	2%	93.33
	3%	92.84

3.3.7. Storage stability and light sensitivity

The purified amylase could retain about 90 % of its activity after 15 days of incubation at room temperature (Fig. 3.10) and retained 97% activity in freezing condition. Samanta et al. (2017) stated that amylase from *Bacillus* sp was stable for six months at 4°C. Enzyme retains its 95.4 % activity after 15 days of incubation under sunlight. Due to the presence of high storage stability and less light sensitivity the enzyme can be preserved for a long time in presence of light. These two properties of enzymes are very essential for the digestive syrup production. The comparative characteristics of different fungi were studied (Table 3.3).

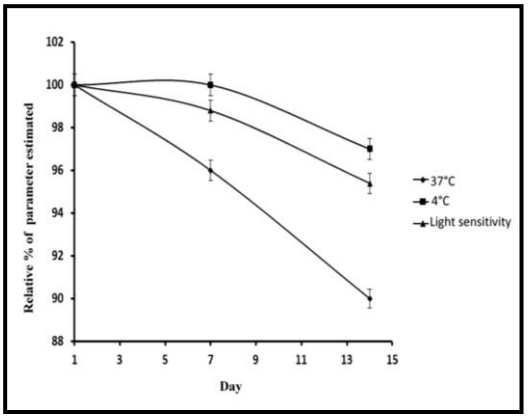

Fig.3.10. Effect of storage time and light sensitivity on stability of α- amylase RBP7

Table 3.3. Comparison of different characters of fungi

Organism	Molecular weight (KDa)	Optimm pH for activity	Optimum temperature for activity (°C)	Metal ion(ca^2)Relative activity	*Km*	*Vmax*	References
Aspergillus carbonarius	32	6.0-7.0	40	72%	0.194 mg/ml	0.024 µmol/ml/min	Okolo et al. (2000)
Monascus sanguineus	56			65%	0.055 mM	22.07 U/ml	Tallapragada et al.(2017)
Aspergillus flavus	56	6.5	30		0.5 mg/ml	17.78 mg/ml/min	Sidkey et al. (2011)
Trichoderma pseudokoningii	30	4.5-8.5	50	152%	4 mg/ml	0.74 µmol	Abdulaal (2018)
Penicillium camemberti	60.5	6.0	30		0.92 mg/ml	38.5 µmol/min	Nouadri et al. (2010)
Bacillus licheniformis	60	6.5	90	90%	6.2 mg/ml	1.04 µmol/mg	Samanta et al. (2017)

Bacillus thuringiensis	59	6.0	70		2.9 mg/ml	0.053 µmol/ml/min	Smitha et al. (2015)
Bacillus subtilis	51	4.0	60	100%			Gbenga et al.(2017)
Bacillus cereus	42	8.0	65	90%			Annamalai et al. (2011)
Engyodontium album	50	9.0	60	110.48%	6.28mg/ml	15.36U/mg	Ali et al. (2014)
Escherichia coli	52	7.0	30				Hassan et al. (2018)
Aspergillus niger RBP7	37.5	3.0	45	93%	1.4 mg/ml	0.992 µmol/min	

3.4. Conclusion

The acidophilus amylase obtained from *Aspergillus niger* RBP7 shows some unique features. The enzyme is highly acidophilic, remain stable during storage and also active in presence of light. The activity of enzyme is metal independent. These features make the enzyme a promising commercial exploitation especially in digestive syrup producing industries, food industries and textile industries. It is also useful for waste water management.