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Full Length Research Paper

Purification and characterization of α-amylase from *Ganoderma tsuage* growing in waste bread medium

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The objective of this study was to purify and characterize the α-amylase for industrial perspective. The production of α-amylase through solid-state fermentation by *Ganoderma tsuage* was investigated by using waste bread as substrates. Production parameters were optimized as 2 mL of inoculum size, moisture 50%, additional carbon source (glucose) and nitrogen source (ammonium nitrate) 10:1, 1 mM/mL $MgSO_4$, 0.75 mM/mL $CaCl_2$ and 0.50 mM/mL KH_2PO_4. The purification value of α-amylase was observed as 1.2 fold with specific activity of 112 U/mg having a yield of 22%. Specific activity of α-amylase increased up to the level of 143 U/mg and had 1.5-fold purification factor having a yield of 6% after Sephadex gel filtration. Optimum value of α-amylase was obtained at 35°C and at pH 6 for the time duration of 72 h. The K_m and Vmax values for α-amylase were 1.3 mg and 39 mg/min, respectively. Calcium chloride ($CaCl_2$) was found to increase the activity of α-amylase while all other compounds seemed to have inhibitory action against α-amylase. Silver nitrate ($AgNO_3$) was the strongest inhibitor and therefore would not be advised for use in future research against α-amylase production.

Key words: α-Amylase, purification, characterization, waste bread, *Ganoderma tsuage*

INTRODUCTION

The sources of α-amylases are quite diverse such as plants, animals and microbes. The major advantages of using microorganisms for production of amylases are the bulk production capability and to obtain enzymes of desired characteristics (Aiyer, 2005). Amylases are enzymes that break down starch or glycogen into simple monomers of glucose. To meet the growing demands in the area of industry, it is essential to improve the performance of enzyme extraction techniques and thus increase the yield without increasing the expenses of production. α-Amylase purification has mainly been restricted to a few species of fungi (AbouZeid et, 1997). It is produced by a variety of living organisms ranging from bacteria, fungi to plants and humans (Pandey et al., 2000). α-Amylase (endo-1, 4-Dglucose- D glucohydrolase 3.2.1.1.) fits into the family of endo amylases that randomly slice the 1,4 – D glycoside linkage between adjoining glucose units in the product chain retaining the a anomeric carbon configuration in the product (Sasi et al., 2008). A great level of interest has paid attention on the potential of converting protein from agricultural waste like *Cocos nucifera* meal to microbial protein or single cell protein (Ravinder and Chaqndey, 2005).

Microbial amylases especially produced by *Ganoderma tsuage* are utilized for food industry now days. Using current technologies, microbial amylases are commercially produced which have almost completely replaced chemical hydrolysis processes of starch in starch processing industries (Alva et al., 2007). Microorganisms can grow under controlled conditions in large number to yield amylases that are relatively easy to isolate and purify (M9). α-Amylase utilizes starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide, high fructose syrup and maltotetraose syrup. α-Amylase preparations are mainly used in food industry,

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brewing processes and continuous process for de-sizing of textile fabrics. Other applications include modification of starches suitable for preparation of adhesives, sizes and coatings for the paper industry, as well as manufacture of glucose and glucose syrup (Hanes and Stedt, 1988). Spectrum of applications of αamylase has extended in many sectors such as clinical medicinal and analytical chemistry (Ramachandran et al., 2004).

The expenditure of enzyme production in submerged fermentation (SmF) is high, which lessens the cost by substitute methods. Baysal et al. (2003) have reported α-amylase production in solid-state fermentation with wheat bran and rice husk as substrates. Ikram-ul-Haq et al. (2003) have illustrated the selection of an appropriate low cost fermentation medium for the production of α-amylase by using agricultural by-products. The optimizations of fermentation parameters for α-amylase production establish a relationship between *G. tsuage* and waste bread medium. The purpose of this research was to study the purification and characterization of amylase by *G. tsuage* through the process of solid-state fermentation (Imran et al., 2011).

MATERIALS AND METHODS

Screening for amylase producing fungi

The medium was prepared and the pH of that medium was adjusted to 5.5 with 1 M HCl/1 M NaOH. It was autoclaved at 121°C for about 15 min. The media were then poured into the germ-free test tubes and some Petri plates; the test tubes were placed in slanting position. When medium became solidified, the slants were inoculated with the spores of *G. tsuage* aseptically in laminar airflow. These slants were incubated aerobically at 30°C for sporulation until 72 h, and by making little adjustments in the method, the sporulated slants was freezed at 4°C in refrigerator. Organism was sub cultured bimonthly (Imran et al., 2011) and its pH was maintained at 5.5 with M HCl and M NaOH. It was autoclaved for 15 min at 121°C and at a pressure of 1.1 kg/cm. A loopful culture of *G. tsuage* from potatoes dextrose agar (PDA) slants was transferred aseptically in laminar air floor into the conical flask (500 mL) containing 100 mL sterilized inoculum medium. It was incubated on orbital incubator shaker with 120 rpm at 37°C.

After 72 h, the number of spores was counted in the medium with the help of haemocytometer by the method of Kolmer (1959). The spore concentration was adjusted at 1.52×10^8 spores per mL in the homogenous spore suspension. Five millliter (5 mL) of this inoculum was added to the fermentation flasks (500 mL) containing 70% moist substrate to optimize different parameters for amylase production. Fresh inoculum was prepared for each parameter under investigation (Imran et al., 2011).

Microorganism and fermentation

G. tsuage obtained from the Department of Biochemistry and Molecular Biology, University of Gujrat, Pakistan was maintained on potato starch-agar slants at pH 4 and 32°C (Asghar et al., 2000). Conical flasks with 100 mL of waste bread medium containing different concentrations of micronutrients inoculated with 5 mL of homogenous spore suspension (3 x 106 spores/mL) were incubated at pH 4 and 32°C on a shaker (120 rpm) for optimum fermentation period. The fermented biomass in each case was filtered and then centrifuged. The supernatant was ultra-filtered through millipore filter and then filtrate examined for α-amylase production.

α-Amylase production in solid-state protocol

Triplicate flasks (500 mL) containing 10 g waste bread medium were adjusted to 70% moisture (w/w) with pre-optimized medium of pH 5.5, containing glucose as carbon source and NH_4NO_3 as nitrogen source in 10:1 C:N ratio and 1.5 mM $MgSO_4$. The flasks were autoclaved, inoculated with 2 mL of inoculum, and incubated at 30°C for four days under stationary solid-state conditions.

Enzyme extraction

The fermented biomass was harvested after four days by adding 100 mL of 100 mM sodium citrate buffer containing 1 mL Tween 80 of pH 5 and shaking at 150 rpm for 15 min. The contents of flask were filtered by Whatman No.1 filter paper (125 mm). The filtrate was centrifuged at 14,000 rpm at 4°C for 15 min and the supernatant used for enzyme assay and purification process (Mehboob et al., 2011).

Analysis of enzyme

α-Amylase activity was determined as described by Okolo et al. (1995). The reaction mixture consisted of 1.25 mL of 1% soluble starch, 0.5 mL of 0.1 M acetate buffer (acetic acid and sodium acetate) of pH=5.0, and 0.25 mL of crude enzyme extract. After 10 min of incubation at 50°C, the dinitrosalicylic acid (DNS) method of Miller (1959) was used to estimate the liberated reducing sugars (glucose equivalents).

Determination of protein content

Protein contents of the crude and purified enzyme extracts was estimated by the method of Bradford (1976) by using Bovine serum albumin as standard.

Precipitation ammonium sulphate and dialysis

In order to achieve maximum precipitation of enzyme, several $(NH_4)_2SO_4$ concentrations (30, 40, 50, 60 and 70%) were used. Ammonium sulphate was used for salting out preferentially because it is soluble in water and high ionic strength can be attainable. At high ionic strength, salt may remove water of hydration from proteins and reduce solubility, hence proteins are coagulated. Crude enzyme extract was saturated to different concentrations of $(NH_4)_2SO_4$ by adding calculated amount of $(NH_4)_2SO_4$ in 10 mL crude extract under constant day. Then it was kept overnight at 4°C and centrifuged as 15000 rpm at 4°C by the centrifuge. The pellets were collected, dissolved in minimum quantity of buffer and dialyzed against distilled water while the supernatant was discarded (Irshad et al., 2011; Imran et al., 2011).

Gel filtration chromatography

A pooled fraction from dialysis loaded on Sephadex G-50 gel filtration column (16×2 cm) was equilibrated with 50 mM malonate buffer of pH 4.5. The 200 µL/run of sample applied and 100 mM phosphate buffer (pH 6.0) having 0.15 M NaCl was used as elution buffer and 12 major positive fractions collected with the flow rate of

Table 1. Purification summary for α-amylase produced by *G. tsuage*.

S/N	Purification step	Total volume (mL)	Total enzyme activity (IU)	Total protein content (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
1	Crude α-amylase	500	44000	458	96	100	1
2	$(NH_4)_2SO_4$ Ppt	30	9881	88	112	22	1.2
3	Dialysis	30	6659	51	130	15	1.4
4	Sephadex-100	10	2715	19	143	6	1.5

0.5 $mLmin^{-1}$. After each purification step, the total protein content and enzyme activity were determined to calculate specific activity and purification factor (Irshad et al., 2011).

Characterization of α-amylase

The purified α-amylase was subjected to characterization through kinetic studies by studying the following processes

Effect of pH on α-amylase activity

α-Amylase was checked at different pH levels ranging from 3 to 9. α-Amylase was analyzed at pH 3 to 3.5 in 100 mM succinate buffer, pH 4 to 5 in 100 mM citrate buffer, pH 6 to 7 in 100 mM phosphate (Na_2HSO_4 and NAH_2SO_4) buffer, and pH 8 to 9 in 100 mM sodium phosphate buffer.

Effect of temperature on α-amylase activity

Purified α-amylase was evaluated at different temperature ranging from 25 to 70°C at optimum pH 5.5; the enzyme was incubated at varying temperatures for 15 min.

Effect of substrate concentration: Determination of Km and Vmax

The Michalis-Menten kinetic constants (*K*m, *V*max) were determined by using varying concentrations of starch ranging from 0.5 to 3 mg/mL following the method described by Metin et al. (2010).

Effect of activators / inhibitors

Effects of different metal ions including calcium chloride ($CaCl_2$), silver nitrate ($AgNO_3$) and organic compounds including tetramethylethylenediamine (TEMED), ethyl-enediamine tetra acetic acid (EDTA) in 1 to 50 mM concentration range on α-amylase activity were investigated. The enzyme was incubated at 35°C (assay temperature) in the presence of varying concentration solutions (pH 5.5) of the respective compounds for 10 to 15 min (Metin et al., 2010).

RESULTS

Purification of α-amylase

The purification summary for α-amylase production is shown in Table 1. α-Amylase was purified using 60% saturation with $(NH_4)_2SO_4$ having yield up to 1.2 fold purification. The specific activity for α-amylase production was reasonable (112 U/mg) with yield of 22%. After Sephadex G-100 filtration (Figure 1), the specific activity of α-amylase increased up to 143 U/mg with 1.5 fold purification having activity yield of 6%. The activity of α-amylase can be increased using various activators.

Effect of pH on the activity of the amylase

The effect of various level of pH on α-amylase production was investigated in buffer of pH ranges from 3.0 to 9.0 using starch as substrate. The pH-activity profile (Figure 2) showed that the activity of α-amylase revealed its highest activity (86 U/mL) at pH 6, which was its optimum pH. However, a further increase in pH caused denaturation of enzyme as a gradual decline of the enzyme activity was revealed.

Effect of temperature on activity of the α-amylase

The incubation temperature of α-amylase production was checked for 15 min before the routine activity assessed. The optimum value for α-amylase production was observed at 80 U/mL at 35°C, which was reasonable for this particular study (Figure 3).

Km and Vmax

Effect of different concentrations of starch on the activity of α-amylase studied and reciprocal plot of 1/S Vs 1/V was constructed for the determination of maximum velocity and Michaelis–Menten constant Km (Figure 4).

The results reveal that the K_M value for α-amylase was observed as 1.3 mg and Vmax value was observed to be 39 mg/min.

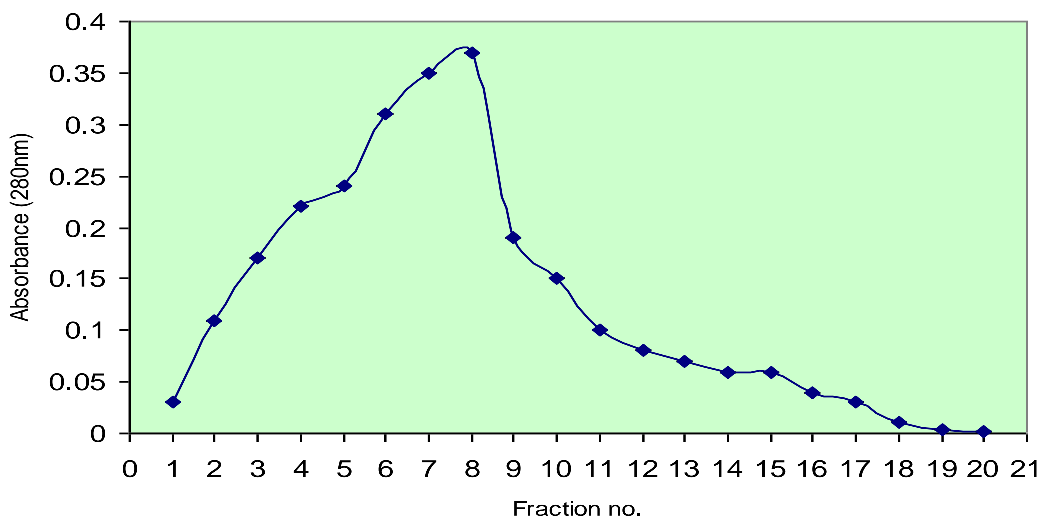

Figure 1. Gel filtration chromatography of α-amylase produced by *G. tsuage*.

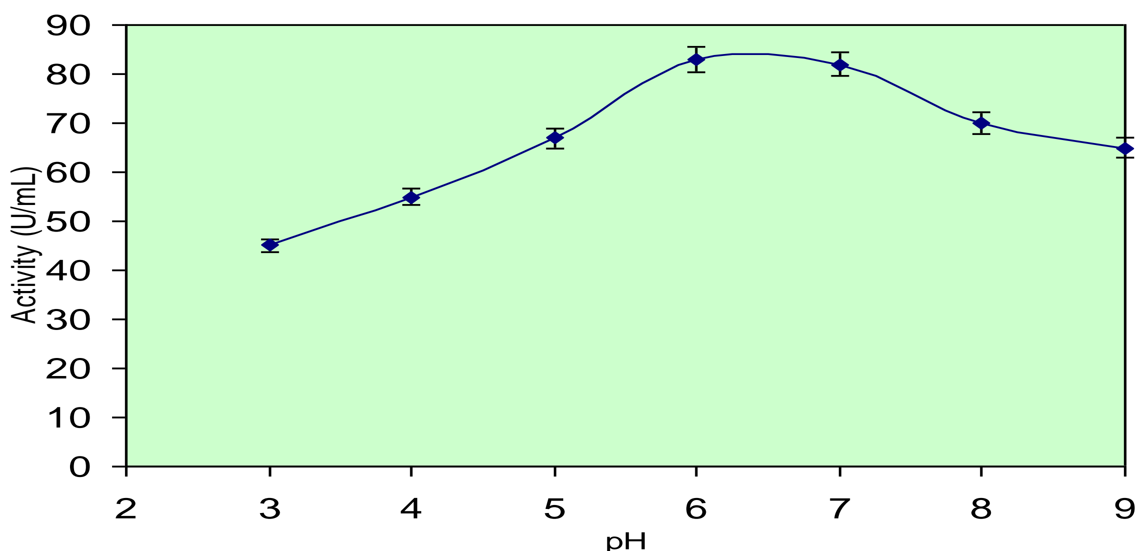

Figure 2. Effect of pH on the activity of α-amylase from *G. tsuage*.

Effect of inhibitors and denaturants on α-amylase activity

The effects of varying concentrations of different activators and inhibitors such as $CaCl_2$, EDTA, TEMED and $AgNO_3$ on α-amylase activity were investigated using starch as a substrate (AbouZeid, 1997). $CaCl_2$ was found to increase the activity of α-amylase. All other compounds had inhibitory action to α-amylase activity and $AgNO_3$ was the strongest inhibitor (Figure 5).

DISCUSSION

In this study, the production of α-amylase was investigated using waste breads as substrates and the results are quite reasonable and more or less productive for economic point of view. Production parameters having carbon, nitrogen and various mineral were optimized and checked for the production of α-amylase at best suitable conditions. The purification value of α-amylase was observed as 1.2 fold, which showed that it possessed some useful aspects in food industry. Reasonable specific activity of α-amylase (143 U/mg) after gel filtration revealed that this substrate had good utilization for α-amylase production. Optimum value of α-amylase was 80 U/mL which was obtained at 35°C and maximum activity of 83 U/mL achieved at pH of six for the duration of 72 h. The K_M value for α-amylase was 1.3 mg, which revealed an affordable bonding between substrate and

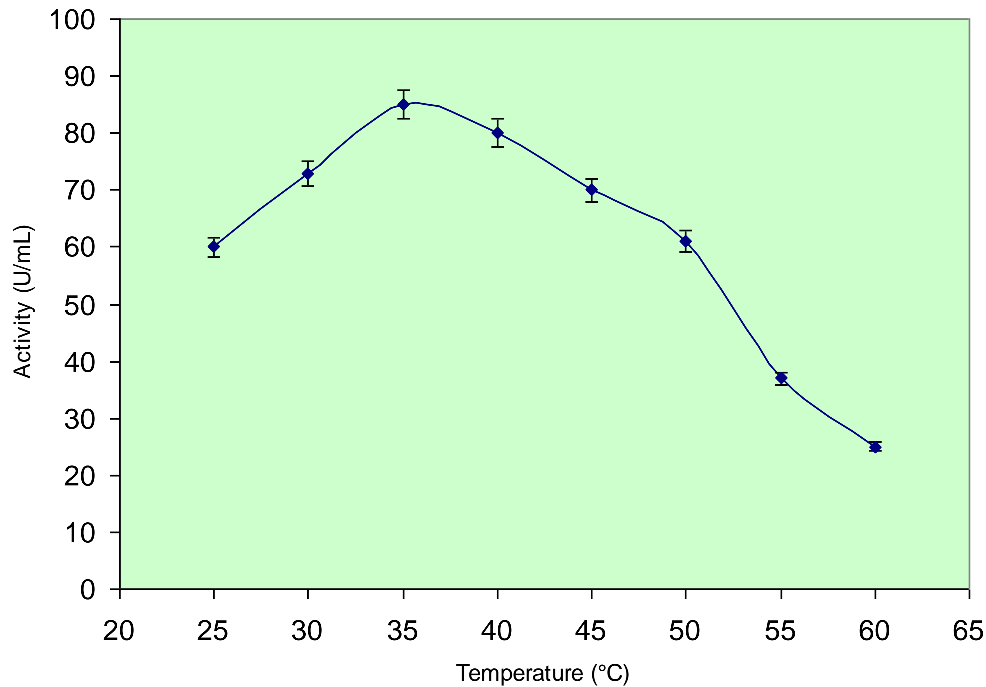

Figure 3. Effect of temperature on activity of α-amylase produced by *G. tsuage*.

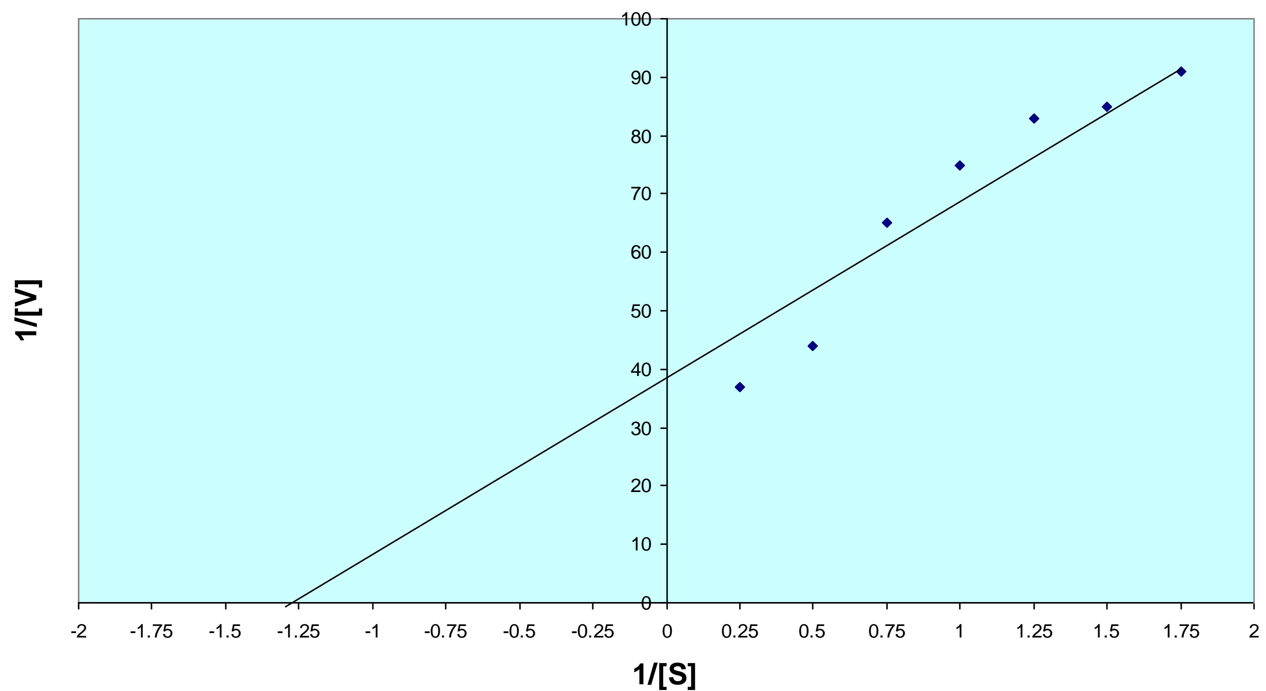

Figure 4. Reciprocal plot of 1/[S] vs 1/[V] for the determination of K_M and V_{max} of α-amylase.

enzyme. Maximum velocity value was observed as 39 mg/min. $CaCl_2$ was found to enhance the activity of α-amylase up to 92 U/mL while all other compounds especially $AgNO_3$ seemed to have inhibitory action against α-amylase production.

The highest activity of α-amylase from *Penicillium citrinum* HBF62 was determined at pH 5.5 by Metin et al. (2010). Nouadri et al. (2010) obtained α-amylase from

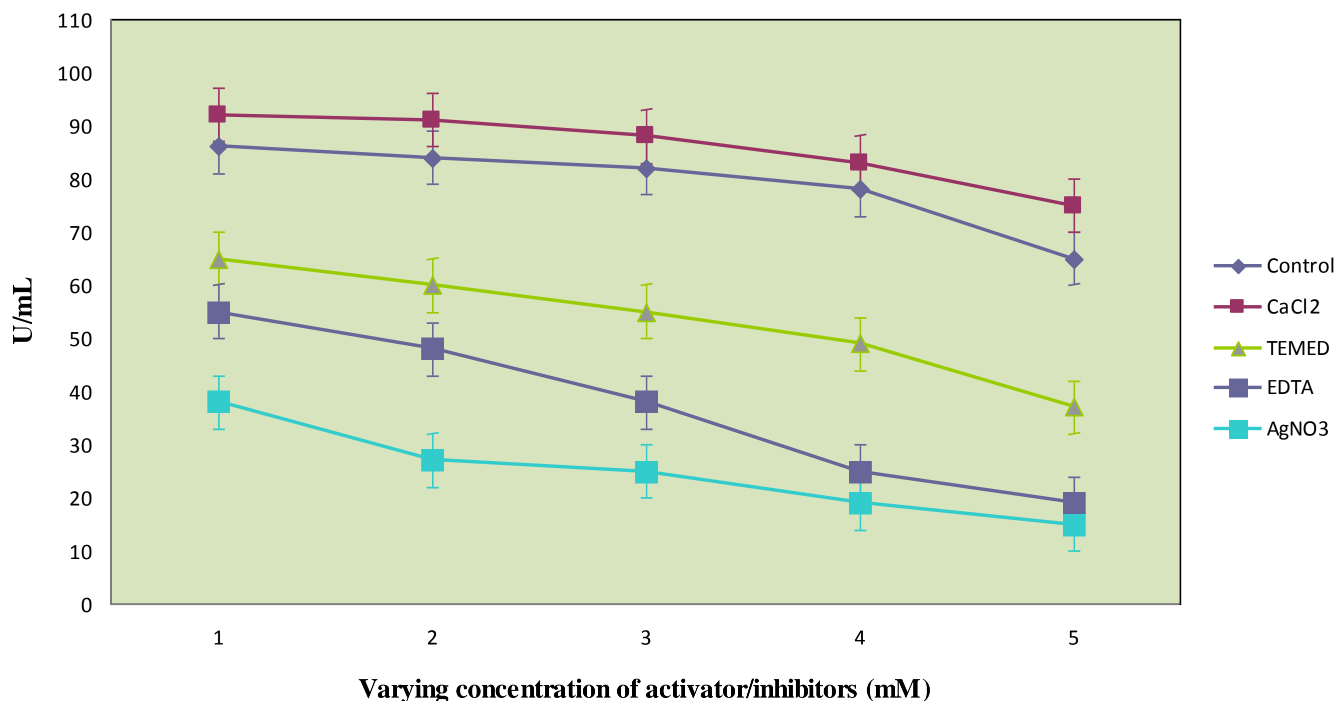

Figure 5. Effect of varying concentration of activator and inhibitors

Penicillium camemberti PL21 at pH 5.5. Purified α-amylase was isolated from novel *Bacillus cereus MS6*, used for 1% starch as a media in liquid culture and the optimum activity of amylase observed at 45°C and pH 7.0 as reported by Al-ZaZaee et al. (2011).

The enzyme activity produced 1% starch or urea by the *Bacillus* sp. isolated from paddy seeds, which was optimal at 30°C and pH 6.8, and increased by the presence of Ca^{2+} and Co^{2+} ions under submerged fermentation (Varalakshmi et al., 2008). The optimum temperature for α-amylases from fungal and yeast sources has generally been found to be between 30 and 70°C (Gupta et al., 2003; Sun et al., 2010). Optimum temperatures for α-amylase in earlier studies on the *Penicillium* species were reported between 30 and 60°C (Doyle et al., 1988; Ertan et al., 2006).

The Km and Vmax values were 0.92 mg/ml and 38.5 µmole/min, respectively at 30°C and pH 6.0 with 0.1 M phosphate buffer from *P. camemberti PL21* by Nouadri et al. (2010). The Km of α-amylase of *P. citrinum HBF62* was lower than those of *Penicillium amagasakiense* (1.12 mg/ml) (Doyle et al., 1988) and *Penicillium griseofulvum* (9.1 mg/ml) (Ertan et al., 2006). The Km of fungal and yeast α-amylases have been reported to be between 0.13 to 5 mg/ml (Pandey et al., 2000; Gupta et al., 2003).

The effect of 2 mM Ca^{2+} increased the α-amylase activity obtained from *P. camemberti PL21* by Nouadri et al. (2010) but other metals ions such as (Mg^{2+}, Hg^{+2}, Ag^{+} and Cu^{+2}) inhibited the enzyme activity. Similar results have also been reported for α-amylases from *Thermomyces lanuginosus* (Petrova et al., 2000), *Vibrio* sp. (Najafi and Kembhavi, 2005), *Thermococcus profundus* DT5432 (Chung et al., 1995) and other microbial amylases (Gupta et al., 2003). The enzyme activity was slightly stimulated in the presence of dithiothreitol (DTT), β-mercaptoethanol and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), indicating that cysteine residue(s) do not take part in catalysis. Also, the enzyme activation by DTT, β-mercaptoethanol and DTNB could be attributed to the reduction in aggregate size by destroying the intermolecular disulfide linkages and/or by the protection of thiol groups that stabilize the three dimensional structure of enzyme (Khedher et al., 2008).

REFRENCES

AbouZeid AM (1997). Production, purification and characterization of an extracellular α-amylase enzyme isolated from *Aspergillus flavus*. Microbios, 89(358): 55-66.

Aiyer PV (2005). Amylases and their applications. Afr. J. Biotechnol. 4(13): 1525-1529.

Alva S, Anumpama J, Savla J, Chiu YY, Vyshali P, Shruti M, Yogeetha BS, Bhavya D, Purvi J, Ruchi K, Kumudini BS, Varalakshmi KN (2007). Production and characterization of fungal amylase enzyme isolated from *Aspergillus sp.* JGI 12 in solid state culture. Afr. J. Biotechnol. 6(5): 576-581.

Al-ZaZaee, Abdu MM, Neelgund S, Gurumurthy DM, Rajeshwara AN (2011). Identification, Characterization of Novel Halophilic *Bacillus Cereus Ms6: a Source* for Extra Cellular A-Amylase. Adv. Environ. Biol. 5(5): 992-999.

Asghar M, Yuqub M, Sheikh MA, Baraque AR (2000). Optimization of cellulase production by *Arachniotus sp.* using corn stover as substrate. JAPS, 10: 37-40.

Baysal Z, Uyar F, Aytekin C (2003). Solid-state fermentation for production of a -amylase by a thermotolerant *Bacillus subtilis* from

hot-spring water, Process Biochem. 38: 1665-1668.

Bradford MM (1976). A rapid and sensitive method for quantification of microgram quantities of proteins utilizing the principles of protein dye binding. Anal. Biochem. 72: 248-254.

Chung YC, Kobayashi T, Kanai H, Akiba T, Kudo T (1995). Purification and properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus* DT5432. Appl. Environ. Microbiol. 61: 1502-1506.

Doyle EM, Kelly CT, Fogarty WM (1988). The amylolytic enzymes of *Penicillium amagasakiense*. Biochem. Soc. Trans. 16: 181-182.

Ertan F, Yagar H, Balkan B (2006). Some properties of free and immobilized α-amylase from *Penicillium griseofulvum* by solid state fermentation. Prep. Biochem. Biotechnol. 36: 81-91.

Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003). Microbial α-amylases: a biotechnological perspective. Process Biochem. 38: 1599-616.

Hanes B, Stedt S (1988). Milling and Baking Technology. John Willey and Sons USA. pp. 415-70.

Ikram-ul-Haq, Ashraf H, Iqbal J, Qadeer MA (2003). Production of alpha amylase by *Bacillus licheniformis* using an eco-nomical medium. Bioresour. Technol. 38: 8757-8761.

Imran M, Asad MJ, Gulfraz M, Mehboob N, Jabeen N, Hadri SH, Irfan M, Anwar Z, Ahmed D (2011). Hyper Production of glucoamylase by *Aspergillus niger* through chemical mutagenesis. Int. J. Physical Sci. 6(26): 6179-6190.

Irshad M, Asgher M, Sheikh MA, Nawaz H (2011). Purification and characterization of laccase produced by *Schyzophylum commune IBL-06* in solid state culture of banana stalks. Bioresour. 6(3): 2861-2873.

Khedher IBA, Bressollier P, Urdaci MC, Limam F, Marzouki MN (2008). Production and biochemical characterization of *Sclerotinia sclerotiorum* α-amylase ScAmy1: assay in starch liquefaction treatments. J. Food Biochem. 32: 597-614.

Khoo SL, Nazalan MN, Razip MS, Azizan MN (1994). Purification and properties of two forms of glucoamylase from *Aspergillus niger*. Folia Microbiol (Praha). 41(2): 65-174.

Mehboob N, Asad MJ, Imran M, Gulfraz M, Wattoo FH, Hadri SH, Asghar M (2011). Production of lignin peroxidase by *Ganoderma leucidum* using solid state fermentation. Afr. J. Biotechnol. 10(48): 9880-9887.

Metin K, Koç O, Ateslier ZBB, Bıyık HH (2010). Purification and characterization of α-amylase produced by *Penicillium citrinum HBF62*. Afr. J. Biotechnol. 9(45): 7692-7701.

Miller GL (1959). Use of Dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31: 426-428.

Mohammed MAA, Neelgund S, Gurumurthy DM, Rajeshwara AN (2011). Identification, Characterization of Novel Halophilic *Bacillus Cereus Ms6: a Source* for Extra Cellular A-Amylase. Adv. Environ. Biol. 5(5): 992-999

Najafi MF, Kembhavi A (2005). One step purification and characterization of an extracellular α-amylase from marine Vibrio sp. Enzyme Microb. Tech. 36: 535-539.

Nouadri T, Meraihi Z, Shahrazed DD, Leila B (2010). Purification and characterization of the α-amylase isolated from *Penicillium camemberti PL21.* Afr. J. Biochem. Res. 4(6): 155-162.

Okolo BN, Ezeogu LI, Mba CN (1995). Production of raw starch digesting amylase by *Aspergillus niger* grown on native starch sources. J. Sci. Food Agric. 69: 109-115.

Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000). Advances in microbial amylases. Biotechnol. Appl. Biochem. 31: 135-152.

Petrova SD, Ilieva SZ, Bakalova NG, Atev AP, Bhat MK, Kolev DN (2000). Production and characterization of extracellular α-amylases from the thermophilic fungus *Thermomyces lanuginosus* (wild and mutant strains). Biotechnol. Lett. 22: 1619-1624.

Ramachandran S, Patel AK, Nampoothiri KM, Chandran S, Szakacs G, Soccol CR, Pandey A (2004). Alpha amylase from a fungal culture grown on oil cakes and its properties. Braz. Arch. Biol. Technol. 47: 309-317.

Ravinder R, Chaqndey A (2005). Optimization of protein environment of deoiled rice bran by solid state fermentation using *A. flavus* MTCC 1846. 25: 1-30.

Sasi A, Baghyaraj R, Yogananth N, Chanthuru A, Ravikumar M (2008). Production of α-amylase in submerged fermentation by using *Bacillus sp*. Resear. J. Biol. Sci. 1: 50-57.

Varalakshmi KN, Kumudini BS, Nandini BN, Solomon JD, Mahesh B, Suhas R, Kavitha AP (2008). Characterization of Alpha Amylase from *Bacillus sp.1* isolated from paddy seeds. J. Appl. Biosci. 1(2): 46-53.