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Full Length Research Paper

Purification and characterization of the α-amylase isolated from *PENICILLIUM CAMEMBERTI* PL21

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The amylase family of enzymes is a great significance due to its wide area of potential application. α-amylase from *PENICILLIUM CAMEMBERTI PL21* obtained from I. N. A. (The Technological Laboratory of Agriculture National Institute Paris- France) and using orange waste as substrates was produced under optimum conditions, after 168 h of incubation and subjected for purification and characterization. The enzyme was purified by ammonium sulfate precipitation, dialysis, sephadex G-100 and DEAE-Sepharose CL-6B column chromatography. A trial for the purification of α-amylase resulted in an enzyme with specific activity of (154.2 units/ml/mg protein) with (38.5 folds) purification .The α-amylase activity increased by enzyme concentration rise. The optimum substrate concentration for soluble starch was 1 % (w/v) while the optimum incubation temperature was 30ºC. The purified α-amylase enzyme had a maximum activity at pH 6 and the *K*m value for soluble starch was 0.92 mg/ml. Analyses of this enzyme for molecular mass was carried out by SDS-PAGE electrophoresis, which revealed one band 60.5 Kda.

Key words: *Penicillium camemberti PL21*, α-amylase, purification, SDS-PAGE electrophoresis.

INTRODUCTION

Alpha amylase (E.C 3.2.1.1) catalyses the hydrolysis of α -D-(1, 4) glycosidic linkage in starch components and related carbohydrates it is a key enzyme in the production of starch derivatives and also used in desiring fabrics, in the baking industry, pharmaceuticals and detergents. Submerged fermentation has been used for the production of industrially important enzymes because of the ease of handling and greater control of environmental factors such as temperature and pH. The utilization of agro-industrial residues, including orange waste powder as the substrate for the fermentation (Diomi et al., 2008; Oleivera et al., 2006) has growing interests as they are inexpensive energy rich resources and also eliminates large-scale accumulation of the biomass (Pandey et al., 2000; Henning et al., 2006; Ramachandran et al., 2007; Djekrif-Dakhmouche et al., 2006). To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production. α-amylase purification has largely been limited to a few species of fungi (AbouZeid et al., 1997; Khoo et al., 1994). Nevertheless, (Amirul et al., 1996) produced alpha-glucosidase, α-amylase and two forms of glucoamylase from *Aspergillus Niger* grown on a liquid medium containing raw tapioca starch as the carbon source.

On the other hand, as bacterial α-amylase has generally been produced from the strains belonging to genus *Bacillus* (Asgher et al., 2007,Hema et al., 2006) several attempts have been made at their purification and characterization, from both *mesophilic* and *thermophilic* strains (Pandey et al., 2000 and Kathiresan et al.,2006). An extra-cellular α-amylase produced by: *Penicillium chrysogenum* in solid state fermentation (Bel en orgaz et al., 2006, Bilal and Figen, 2007, Neuro et al., 1993). *P. olsonii* (Afifi et al., 2008) and *P. camemberti* (Isabelle et al., 2006; Nathalie et al., 1993) was partially purified using acetone and ammonium sulfate with of 60% of saturation, and 43.7% recovery of enzyme with 6.2-fold purification was recorded (Malhotra et al., 2002, Amirul et al., 1996) purified α-amylase, alpha-glucosidase, and

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glucoamylase to homogeneity by ammonium sulfate precipitation, ion-exchange and two cycles of gel filtration chromatography. In addition to that, an extra cellular α-amylase produced by *Lactobacillus fermentum* was purified by glycogen precipitation and ion exchange chromatography. The purification was approximately 28-fold with 27% yield (Talamond et al., 2002). The aim of this present work was to investigate the purification and partial characterization of the α-amylase enzyme which produced by *Penicillium camemberti PL 21*.

MATERIALS AND METHODS

Microorganism

Penicillium camemberti strain PL21 was provided by I.N.A (The technological laboratory of Agriculture National Institute Paris-France). Stock culture of *P. camemberti PL21* was stored on potato dextrose agar (PDA) at 4°C.

α-Amylase production

α-amylase from *P. camemberti PL 21* was produced in a 2-l fermenter (New Brunswick Scientific-USA) at 22°C, agitation 200 rpm, pH 5 and an aeration of 0.50 l per min .The culture medium (1.5 L) was prepared to include powder orange waste peel (20% w/v), and enriched by: yeast extracts 5 g/l, the corn-steep-liquor 20%, calcium chloride ($CaCl_2$) 0.125 mg/l, starch 1% and glycerol 10%, KH_2PO_4, 0.2 mg/l $MgCl_2 \cdot 6H_2O$. After 168 h of incubation, the cultures were kept in initial pH and temperature was controlled, (Fogarty et al., 1990). The extract was clarified by centrifugation at 10,000 rpm at 4°C for 15 min. The clear supernatant was used for enzyme activity measurements (Diomi et al., 2008).

α-Amylase assay

Amylase activity was determined by measuring the release of reducing sugar from soluble starch. The reaction mixture contained 0.5 ml of enzyme and 4.5 ml of 0.1 M phosphate buffer (pH 6.0) added to 2 ml of soluble starch (1%). After incubation at 30°C for 30 min in a shaking water bath, the reaction was stopped by the addition 2 ml of 3-5-Dinitrosalicylic acid method (Bernfeeld, 1955). The tubes were kept in boiling water for 5 min to develop the color and cooled. The absorbance was read at 540 nm after making up the volume to 10 ml. Maltose was used to construct a standard curve. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol maltose per min under the assay conditions Specific activity is expressed as amylase activity per mg of protein.

Protein assay

Protein was measured by the method of (Lowry et al., 1951) with bovine serum albumin (BSA) as standard. The concentration of protein during purification studies was calculated from the standard curve. The eluted fractions from the chromatographic separations were monitored at 280 nm.

Confirmation of amylase production

Amylase production by *P. camemberti PL 21* was confirmed on starch agar plates containing peptone 5 g, yeast extract 1.5 g, soluble starch 2.0 g, agar 15 g, a media composed with Na_2HPO_4 4 g, KH_2PO_4 2 g, NaCl 0.5 g, $MgSO_4$ 0.24 g, $CaCl_2$ 0.01 g, and (Burhan et al., 2003). The initial pH was adjusted with NaOH to pH 6.0 after autoclaving. Culture was maintained for 72 h at 30°C and pH 6.0. Amylase production was detected after flooding the plates with iodine solution (Hols et al., 1994), (Burhan et al., 2008, Saxena et al., 2007).

Enzyme purification

The α-amylase from *P. camemberti PL 21 was* purified by a combination of $(NH_4)_2SO_4$ precipitation, dialysis, gel filtration sephadex G-100 and DEAE Sepharose-CL-6B, and the clear supernatant was subjected to the following purification steps:

(1) After 168 h cultivation of *P. camemberti PL 21* in a 2-l fermenter containing 1.5 l of the amylase-producing medium, the fermentation broth was centrifuged at 10,000 rpm for 30 min to remove cells. The supernatant was then brought to 30-50% saturation with ammonium sulfate. The precipitate was removed by centrifugation at 12,000 rpm for 30 min, and then ammonium sulfate was added to the supernatant to 50 - 60% saturation.

(2) The precipitate protein was dissolved in 0.1M phosphate buffer, pH 6.0 and dialyzed against the same buffer. Precipitation, dialysis and centrifugation were carried out at 0 - 4°C, to remove the remaining salt. Specific activity of enzyme was estimated in the dialyzed fraction.

(3) The desalted enzyme solution was collected dissolved in phosphate buffer 0.1M; pH 6.0 and fractionated through Sephadex G-100 column (1.6 x 36 cm) (Frac-100.Pharmacia –Fine Chemicals) previously equilibrated with the same buffer. Elution was carried out with the respective buffer at 1ml/min. The protein content of each fraction was determined at 280 nm. The eluted enzymatically active fractions were pooled and used as the purified enzyme.

The purified enzyme was applied to a DEAE Sepharose CL-6B column chromatography (2.6 × 20 cm) which had been previously equilibrated with 0.1 M phosphate buffer (pH 6.0) containing 0.1 mM $CaCl_2$ and was then eluted at a flow rate of 1 ml/min.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and molecular weight determination

The molecular mass of the purified enzyme was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE (10 % polyacrylamide) (Laemmli, 1970) using BIO-RAD MINI PROTEAN apparatus. Phosphorylase b (97.0 Kda,), BSA (66.0 Kda, Carbonic anhydrase (30.0 Kda), Soybean trypsin inhibitor (20.1 Kda), α-Lactabumin (14.4 Kda) were used as molecular mass standards (molecular weight marker kit S7 were from Sigma Chemical Co., USA.).The electrophoresis was carried at 80 volt for 2 h. The gel was stained with 0.25% Coomassie Brilliant Blue R-250 and distained by washing overnight with a mixture of acetic acid-methyl alcohol-water (5:5:1 v/v).

Amylolytic activity in polyacrylamide gel electrophoresis (PAGE)

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed in 10% gels according to Davis (1964). After running, the gel was incubated with 0.1M phosphate buffer (pH 6.0) for 30 min and immediately immersed in 1% starch solution. The gel was

Table 1. Summary of purification procedures for α-amylase produced from *P. camemberti PL21.*

Purification step	Enzyme volume (ml)	Total activity (unit/ml)	Total protein (mg/ml)	Specific activity (unit/ml/mg protein)	Purification (x- fold)	Recovery (%)
Culture supernatant	500	3998.5	997.80	4.0	1.0	100
Ammonium $(NH_4)_2SO_4$ Fractionation	10	2024.7	36.50	55.4	13.8	50.6
Dialysis	10	1980.5	30.30	65.3	16.3	49.5
Sephadex G-100 chromatography	5	1386.2	13.30	104.2	26.0	34.6
DEAE-Sepharose CL-6B column chromatography	5	925.20	6.30	154.2	38.5	23.1

incubated for 20 min and amylolytic activity determined with a mixture 0.3% of KI and 0.1% I_2, and fixed with (3 %) acetic acid solution until the appearance of the bands with amylolytic activity (Silva et al.,2009). The gel was stained with 0.15% Coomassie Brilliant Blue R-250 and distained by washing overnight with a mixture of acetic acid-methyl alcohol-water (5:5:1 v/v). (Damodara et al., 2005)

pH optimum

The relative amylase activity was measured under the standard assay conditions, using (1% w/v) soluble starch; enzyme was incubated at room temperature 30°C for 30 minutes at various pH (3-10) in 0.1M acetate and phosphate buffer (McTigue et al., 1995).Relative activities at the test pH were calculated assuming the maximum activity observed as 100% (during the experiment).

Temperature optimum

Enzyme samples were incubated for 30 minutes at various temperatures between 10 and 90°C in 0.1M phosphate buffer pH 6.0 (Lo et al., 2001).The temperature at which maximum activity was observed was taken as 100% and relative activities at different temperatures calculated.

Thermal stability of enzyme

Thermostability of the amylase was performed by enzyme samples at optimum pH for 30 min pre-incubating at temperatures 30,40,50,60 and 70°C. The remaining activity was determined by incubating enzymes at optimum temperature for 30 min (Egas et al., 1998).

Kinetic determinations

Initial rates of starch hydrolysis were determined at various substrate concentrations (0.25 to 2 mg/ml). The kinetic constants Km and Vmax were estimated by the method of Lineweaver-Burk.

Effect of metal ions on specific activity

The purified enzyme was incubated for 10 min with different concentrations of each of the following substances (Table 2), all the metal ions added as chloride salts, then the relative activity was assayed.

RESULTS AND DISCUSSION

Purification of α-amylase

The results of the purification of α-amylase produced by the strain *Penicillium camemberti PL 21* is summarized in (Table 1). The first step in purification was conventional $(NH_4)_2SO_4$ fractionation. The fraction (50 - 60%) saturation of ammonium sulfate revealed maximum enzyme specific activity (55.4 unit/ml/mg protein). The α-amylase subjected to dialysis against resulted in specific activity of (65.3 units/ml/mg protein) , then it was further purified by Sephadex G-100 in order to desalt the solution (Xu and yan, 2007), remove the low molecular weight proteins This has induced the specific activity up to (104.2 units/ml/mg protein).

The purified enzyme obtained with the DEAE-Sepharose CL-6B column chromatography (Figure 1) exhibited 23.1% of the total initial activity and there was a 38.5 fold increase in specific activity (154.2 units/ml/mg protein) when compared with the crude culture filtrate.

Properties of the purified α-amylase

The α-amylase isolated from *P. camemberti PL 21* was approximately (38 folds) pure with a yield of 23%. As shown in Figure 2, the purified enzyme has migrated as a single protein band in both native PAGE and SDS-PAGE (10% gel), which indicates its homogeneity. The molecular mass of the α-amylase isolated from *P. camemberti PL 21* was estimated to be 60.5 kDa from its mobility relative to those of standard proteins on SDS-PAGE, indicating that the purified enzyme is a monomer. The enzyme containing amylolytic activity was applied in non denaturing polyacrylamide gel electrophoresis (PAGE) (Figure 2C); a clear-zone forming band indicated the presence of the active α-amylase in the native PAGE (Figure 2D). The purified enzyme could be stored at 0.5 M phosphate buffer, pH 6, at 4°C, for more than 3 months without any apparent loss of activity. However, repeated freezing and thawing led to considerable loss of its activity (Iiori et al., 1997; Lin et al., 1998; Burhan, 2008;

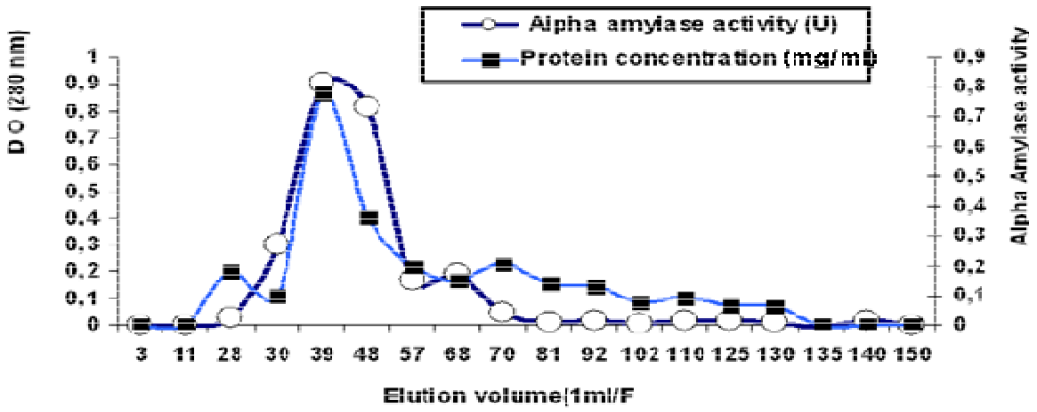

Figure 1. Purification profile of α-amylase produced by *P. camemberti* PL21.

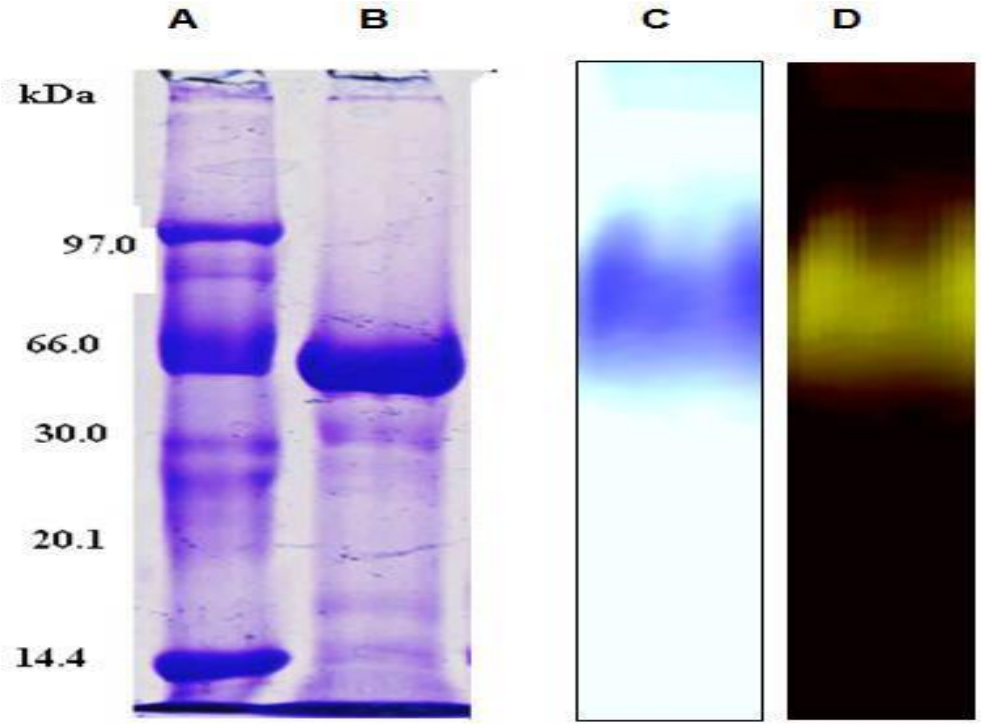

Figure 2. Polyacrylamide gel electrophoresis of the purified α-amylase from *P. Camemberti PL21.* SDS-PAGE, Lane (A); Molecular mass standard proteins: Phosphorylase B (97.0 kDa), SA (66.0 KDa), Carbonic anhydrase (30.0 KDa), soybean trypsin inhibitor (20.1 KDa) ,α-ctabumin (14,4 KD), Lane (B); the purified α-amylase from DEAE-Sepharose CL-6B column chromatography. Native-PAGE Lane (C); amylolytic activity on PAGE contained 1% starch. The protein of the purified α-amylase stained by coomassie Brilliant blue R-250, Lane (D); The purified α-amylase stained by KI/I_2 solution**.** Electrophoresis conditions: 80 V/2h.

Biljana et al., 2007; Yang et al., 2004).

Influence of pH on activity

The influence of pH on enzymatic activity is represented in (Figure 3). The α-amylase showed optimal activity (80% of the maximal activity 153 U) from pH 5.0 to 6.0 and this result was similar to the values reported for the most bacterial (Afifi et al., 2008, Mazen et al., 2002) and fungal (Ray et al., 2001) amylases.

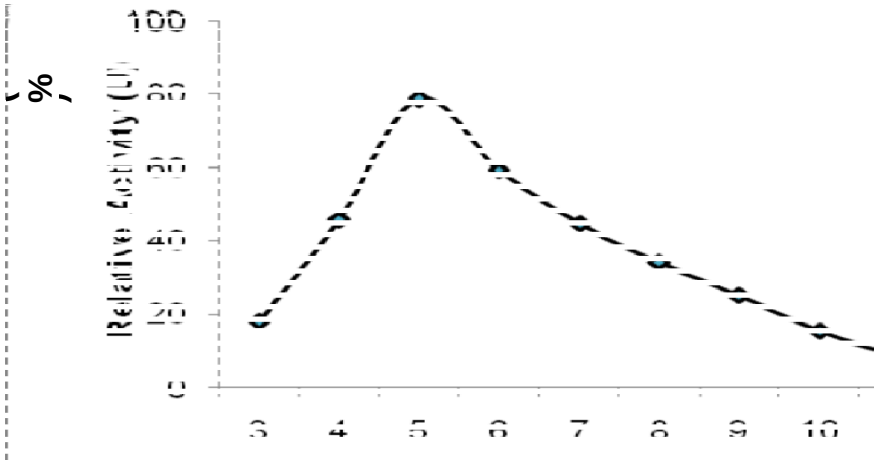

Figure 3. Effect of pH values on the specific activity of the purified alpha amylase from *P. camemberti PL 21.*

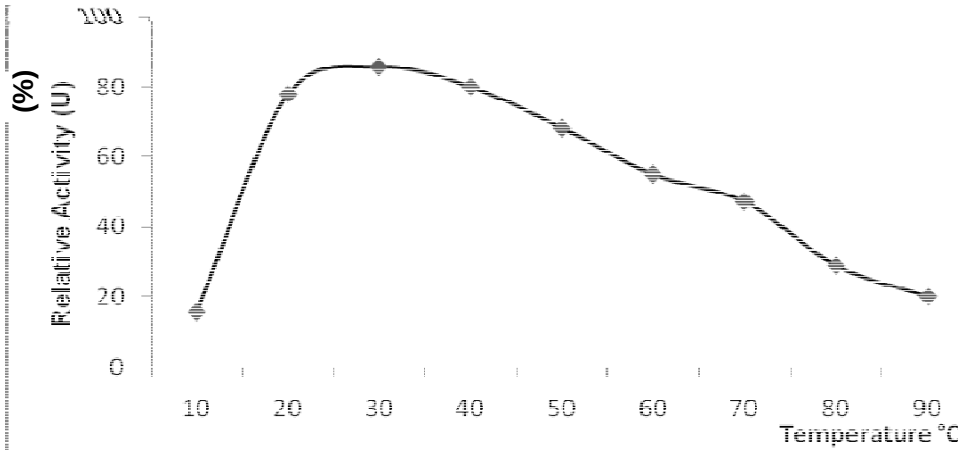

Figure 4. Effect of different temperatures on the specific activity of purified alpha amylase *P. camemberti PL 21.*

Effect of temperature and thermal stability on activity

The enzyme activity was determined at different temperatures (10 - 90°C) ,in order to estimate the optimum temperature. The enzyme has reflected a broad temperature range between (20 - 60°C) and the optimum temperature was observed around 30°C (Figure 4). The results accumulated on the thermal stability of this enzyme are represented in Figure 5. Apparently the activity of α-amylase was affected largely by exposing to temperature above 60°C. The enzyme lost about 50% of its activity at temperature ranging between 50 - 60°C for two hours (Afifi et al., 2008).

Kinetic determinations

With respect of the effect of different starch's concentrations on the enzyme activity, the results in Figure 6 showed that the activity of α-amylase increased with the increase of starch up to 1.00 mg/ml followed by slight decrease in the activity. From the Lineweaver- Burk plot of the reciprocal of initial velocities and substrate concentrations (Figure 7), The K_m and V_{max} values were 0.92 mg/ml and 38.5 µmole/min at 30°C and pH 6.0 with 0.1 M phosphate buffer. This is equal to Km value of α-amylase produced from *Bacillus sphaericus* (Al-Qodah et al., 2007).

Influence of different metal ions on activity

The study has showed a different influence of various metal ions on α-amylase activity (Table 2) summarized the results. Induction of the enzyme activity by Ca^{2+} (2mM) which seemed to be in accordance with what was reported in the literature concerning α- amylase from *A.*

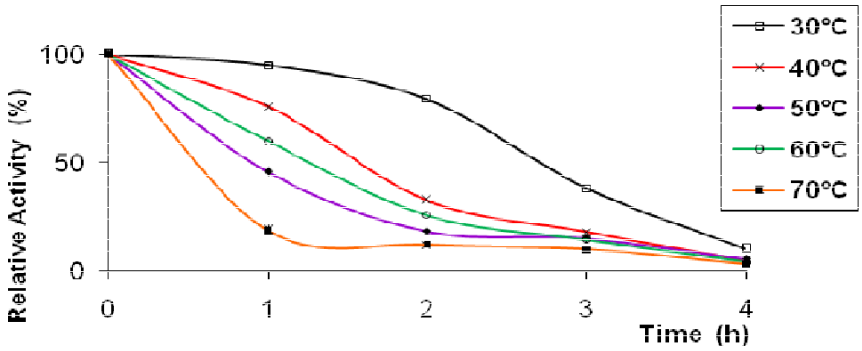

Figure 5. Thermal stability of the purified alpha amylase from *P. camemberti PL 21.*

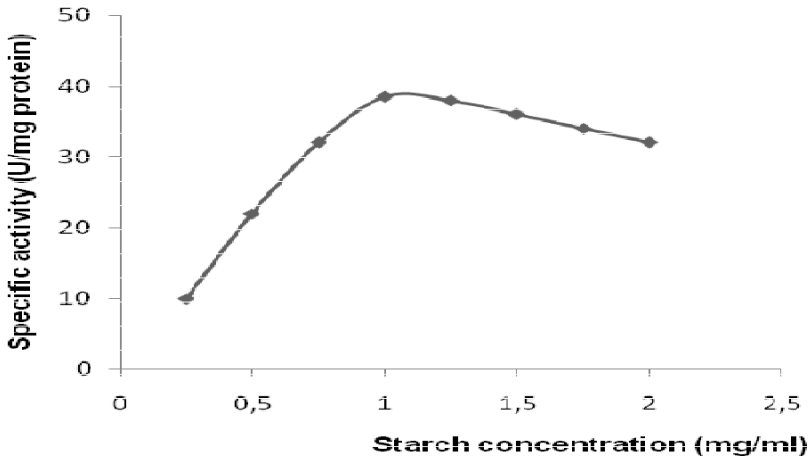

Figure 6. Effect of starch concentrations on the specific activities of purified α-amylase from *P. camemberti PL 21.*

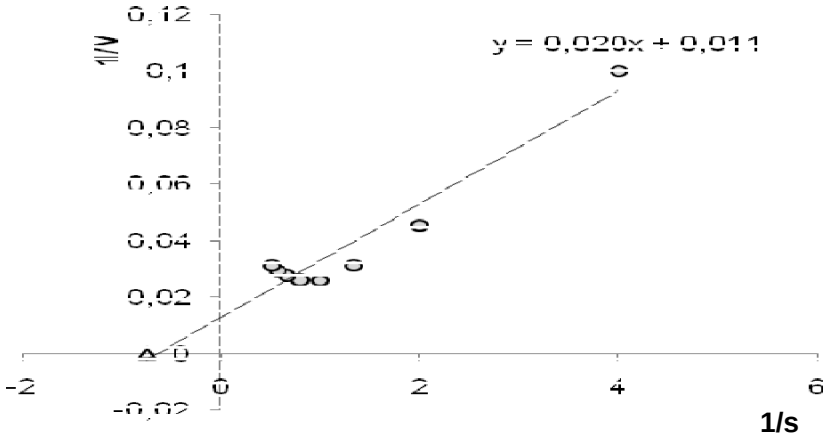

Figure 7. Lineweaver-Burk plot of the reciprocal of initial velocities and starch concentration.

oryzae EI212 (Bose and Das, 1996). However this induction was retraced when (1 mM) of Ca^{2+} was used. On the other hand, Mg^{2+} has decreased α- amylase activity by 10 % through Mg^{2+} ions were reported to inhibit slightly α- amylase activity (Chao-Hsun et al., 2004). Inhibition of α- amylase activity by Hg^{2+}, Ag^{+}

Table 2. Effect of some metal ions on the specific activity of α-amylase produced from *P. camemberti PL21.*

Metal ions	Concentration (mM)	Relative activity (%)
None	-	100
Ca^{2+}	2.0	108
Mg^{+}	1.0	90
Hg^{2+}	0.5	15
Ag^{+}	0.5	20
Cu^{2+}	1.0	75

(0.5 mM), Cu^{2+} (1 mM), respectively, which indicated by the involvement of the sulfhydryl groups in the catalytic activity of α- amylase.

Conclusion

The *P. camemberti PL21* extra-cellular α-amylase was purified by selective fractionation with ammonium sulfate, followed by two chromatography steps, which included gel filtration (Sephadex G-100 and Sepharose-CL-6B). The purification factor was (38.5-folds) and a recovery was (23.1%). α-amylase maximum activity was (154.2 U/ml.mg protein). The molecular mass was estimated to be around 60.5 Kda. The enzyme was active with pH and temperature optima at 6.0 and 30°C similar with the values reported for α-amylase from *Penicillium expansum* and *Aspergillus nedulans*. The α-amylase was inhibited by (0.5 Mm) $Hg2^{+}$ and Ag^{+} up to (85 and 80%) respectively. *P.camemberti PL21* is different from other fungal α-amylase several respects. In Cloning and characterization of the α-amylase gene from *P. camemberti PL21* will help to elucidate the structure and the regulation of this enzyme.

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