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Some properties of thermostable α-amylase of four isolates of *Bacillus licheniformis*

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Abstract. *Dawood ES, Ibrahim SA, El-Nagerabi SAF. 2015. Some properties of thermostable α-amylase of four isolates of* Bacillus licheniformis. *Nusantara Bioscience 7: 90-95.* Amylases are one of the most important enzymes in the food industry and biotechnology. The present investigation was concerned with the production, purification and partial characterization of extracellular amylase from endogenous Sudanese *Bacillus licheniformis* isolates namely SUDK1, SUDK2, SUDK4, and SUDO. The extracted enzyme was partially purified using DEAD Sephadex A-25 gel filtration, and the enzymatic reaction product was identified using thin layer chromatography. The results revealed that DEAE-Sephadex is an excellent and effective purification method and the activity of the partially purified enzymes (83.343-121.755 U/mg protein) compared to the crude extracts (4.626-7.250 U/mg protein) with increase of up to 16.8-18.3 folds. Amylase enzymes hydrolyze starch forming various maltooligosaccharides, such as maltose as major products and were identified as α- amylases. The enzyme's activity was optimum at 60-70°C and was active up to 90°C with residual activity of only 30-50%. All enzymes were stable between pH 6.0-9.0 with optimum activity at pH 7.0. The enzymes were stable and retained nearly all of their initial activities at -20°C at the end of 24 weeks and lost less than 60% of their initial activities at 4°C after 8 weeks. The Km values for this enzyme were 1.25-2.0 mg/mL which showed high affinity and needs only small amount of substrate to be saturated. Therefore, these α-amylases were thermostable at a wide range of temperature and pH values rendering them useful properties in food, feed, textiles, and relevant biotechnological industries.

Keywords: α-Amylases, *Bacillus licheniformis*, enzymes, DEAD Sephadex, partial purification, thermostable

INTRODUCTION

Microbial enzymes are widely used in industrial processes due to their low cost, large productivity, chemical stability, eco-friendly, plasticity, and vast availability. Enzyme kinetics and thermostability are the most important characteristics for their uses in food and feed production (Burhan et al. 2003; Mishra and Behera 2008; Deb et al. 2013). The emphasis was directed to the study of the thermotolerant microorganisms as an excellent source of novel thermostable enzymes. There is an evident increase in their uses in different food, beverage, textile, leather, paper industries, wastes treatment and biodegradation of organic materials to biofuel (Yandri et al. 2010). Of these enzymes, α-amylases (EC3.2.1.1, 1,4-a-D-glucan-glucanohydrolyase) are extracellular enzymes which hydrolyze starch into a range of products such as glucose and maltose or specific maltooligosaccharide or mixed maltooligosaccharides (Aiyer 2004; Hashim et al. 2004; Messaoud et al. 2004; Kathiresan and Manivannan 2006). Although α- amylases can be derived from several sources such as plants, human saliva, pancreatic juice, breast milk, blood serum, and liver, the enzymes from microbial sources are preferred (Kathiresan and Manivannan 2006; Enemchukwu et al. 2013).

Main properties of amylases from bacteria, yeast, and other fungi sources have been reported and described by many researchers (Gupta et al. 2008; Liu and Xu 2008; Chi et al. 2009). Although there are many microbial sources for amylases production, only few investigations were carried on *Bacillus subtilis, Bacillus licheniformis* and *Bacillus amyloliquefaciens* as commercial producers of these enzymes (Saxena et al. 2007). *Bacillus* strains were the most important bacteria producing about 60% of commercially available enzymes (Burhan et al. 2003). Therefore, production and yield improvement of α-amylases and consequent cost reduction depend mainly on the selection of the efficient strains, the optimization of the physicochemical factors, kinetic studies and the biochemical characterization at different temperature and pH levels (Saito 1973; Machaiah and Vakil 1981; Violet and Meunier 1989; Aiyer 2004; Dutta et al. 2006; Mohhmod et al. 2010; Enemchukwu et al. 2013; Miao et al. 2013). Besides, each application of α-amylase in different industry requires unique properties with respect to specificity and thermostability (Konsula and Liakopoulou-Kyriakides 2004). Although, the endogenous micro-organisms are the most important biological source for enzyme production, nonetheless, enzymes used in the local industries were commonly imported which represent economic burden for the country.

Therefore, this study was designed to develop proper technique of enzyme isolation, purification and characterization of purified extracellular α-amylase enzyme

from local *Bacillus licheniformis* isolates namely SUDKI, SUDK2, SUDK3, SUDK7, and SUDO. This will effectively contribute to the microbial food industry, textile, and related biotechnology.

MATERIALS AND METHODS

Inoculation of the bacterial isolates

The bacterial isolates of *B. licheniformis* used in this investigation were inoculated on broth containing 10 g of peptone (g/L) for all isolates except isolate SUDK2 which needs 10 g malt extract; dipotassium hydrogen phosphate 3 g, magnesium sulfate hydrated 1 g and 0.5 g soluble starch. The cultures were grown on a rotary shaker (200 rev./min) at 50˚C for 24 hours. The cultures were then centrifuged in a refrigerated centrifuge and the supernatants were collected and used for enzyme assay and study. A modified peptone-malt extract medium used for amylase production contained (g/L) peptone 10 g for all isolates except SUDK2 which needs 10 g of malt extract), dipotassium hydrogen phosphate 3 g, magnesium sulphate hydrated 1 g and starch 5 g (for all isolates except *B. licheniformis* SUDO which needs 2 g). The cultures were grown on a rotary shaker (200 rev/min) at 50˚C for 24 hours.

Amylase enzymes assay

Enzyme assay was estimated using dinitrosalicylic acid (DNSA) method (Miller 1959). The reaction mixtures consisted of 0.5 mL of substrate solution (1% soluble starch in 0.05 M phosphate buffer, pH7.0) and 0.5 mL of the cell-free extract. The reaction mixture was incubated at 30°C for 3 min and the reaction was terminated by the addition of 1 mL of dinitrosalicylic (DNSA) reagent. The mixture was heated at 100°C for 5 min, cooled, and the optical density was measured with spectrophotometer adjusted at 540 nm. One unit of amylase activity is expressed as one mg of maltose liberated per mL of culture supernatant at 30°C and pH 7.0. Protein content of the enzyme solution was assayed by Bradford method (Bradford 1976).

Purification of the enzyme

The crude enzyme preparations from the culture filtrates of *B. licheniformis* SUDK1, SUDK2, SUDK4, and SUDO were applied separately to 1.8×20 cm column of DEAE-Sephadex A-25 equilibrated with 0.05 M sodium phosphate buffer of pH 7.0, when the sample completely entered the resin, one bed volume of the equilibrating buffer was passed three times through the column until the unbound proteins were removed. The enzyme was eluted with a linear gradient of sodium chloride (0-0.4 M) in 200 mL of sodium phosphate buffer (0.05 M and pH 7.0) with the aid of gradient mixer. The flow rate was adjusted to 1 mL per minute and the 200 mL of eluents were collected into 40 tubes (1×7 cm) using an automatic circular fraction collector. The enzyme activity and protein concentration were similarly determined in each fraction as described above.

Identification of the enzyme digests

The products of starch hydrolysis by the partially purified enzyme were identified with thin layer chromatography using n-butanol:ethanol:water in a ratio of 4:2.2:2 as a solvent and maltose and glucose as standard sugars as described by Trevelyan et al. (1950).

Effect of temperature and pH on the enzyme activity

For testing the effects of temperature and pH on the enzyme activity, the test was carried out at different incubation temperature of between 40-90ºC and pH range of 4-12. The enzyme activity was measured at the end of incubation time.

Storability of the enzyme

For the evaluation of the effect of the storage conditions on the enzyme stability, six fractions of 5 mL each from partially purified enzyme were taken in sample bottles. Three of these were stored at 4ºC in a refrigerator and the rest were stored in a freezer at -20ºC. Every week the enzyme activity was assayed under both conditions. The Change in absorbance was measured using a spectrophotometer and the residual activity was calculated.

RESULTS AND DISCUSSION

Many bacterial isolates produce extracellular amylase enzymes as a result of starch fermentation (Aiyer 2004; Hashim et al. 2004; Messaoud et al. 2004; Kathiresan and Manivannan 2006). Various methods were adopted for purification of enzymes such as column chromatography, ion exchange chromatography, ammonium sulphate, starch absorption, DEAE-cellulose treatment, RESOURCE·s column, organic solvent fractionation, Sephadex G-1100 gel filtration, CM-Sephadex column chromatography, and CM-cellulose column chromatography (Saito 1973; Machaiah et al. 1981; Dey et al. 2002; Adeyanju et al. 2007; Ul-Haq et al. 2010). The enzyme from *B. licheniformis* NCIB 6346 was purified 30-fold by ion-exchange chromatography and was then characterized. It had an endo-action on starch yielding maltopentose as the major product, and was identified as an α-amylase (Morgan and Priest 2008). The activity of α-amylase enzyme from mutant *B. licheniformis* EMS-6 purified up to homogeneity level by ammonium sulfate and ion-exchange chromatography using fast protein liquid chromatography (FPLC) system was increased by 4-5 time while the yield was found to be 40.4% and the purification fold by RESOURCE-S was recorded to be 3.58 (Ul-Haq et al. 2010). The enzyme was purified 126-fold by starch absorption, DEAE-cellulose treatment, and CM-cellulose column chromatography (Saito 1973). In the present study, the degradation products of starch hydrolysis by partially purified amylase enzymes from three isolates of *B. licheniformis* (SUDK1, SUDK4, and SUDO) and identified by thin layer chromatography (TLC) were mainly maltooligosaccharides and maltose while SUDK2 isolate produced maltose and glucose (Figure 1) as reported in

Table 1. Effect of partial purification of amylase enzyme activity and total protein contents of different *B. licheniformis* isolates

Bacillus isolates	Step	Volume (mL)	Specific activity (U/mg protein)	Total protein (mg)	Total activity	Yield (%)	Purification (Fold)
SUDK1	Crude enzyme	300	7.250	116.4	843.9	100	1
	DEAE-Sephadex	30	121.755	4.7	566.2	67.1	16.8
SUDK2	Crude enzyme	200	4.626	94.0	434.8	100	1
	DEAE-Sephadex	30	83.343	4.2	350.0	80.5	18.0
SUDK4	Crude enzyme	300	6.973	115.8	795.9	100	1
	DEAE-Sephadex	30	120.842	4.8	581.3	73.0	17.3
SUDKO	Crude enzyme	300	5.238	124.5	652.1	100	1
	DEAE-Sephadex	25	95.883	5.2	493.8	75.7	18.3

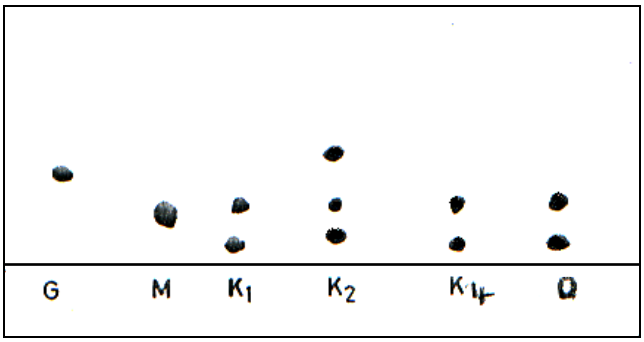

Figure 1. Thin layer chromatograms of the starch hydrolysis products by α-amylases from different *B. licheniformis* isolates. G: Glucose standard; M: Maltose standard; K-O: Samples

similar investigations (Kumar et al. 2010). The specific activity of partially purified amylases using DEAE-Sephadex from all isolated increased by 16.8-18.3 folds over the crude extract. The total protein content decreased from 94.0-124.5 mg for crude extract to 4.7-5.2 mg for partially purified extract, and the yield percentages of all amylases varied in the range of 67.1- 80.5% (Table 1). These investigations show that DEAE-Sephadex is an excellent exchanger for these enzymes and effectiveness purification method comparable to other methods as suggested in similar investigations (Al-Quadan et al. 2009).

Thermodynamic and kinetics parameters provide a detailed mechanism for many chemical and biological reactions (Tanaka and Hoshino 2003; Ul-Haq et al. 2010). The purified α-amylase from *B. licheniformis* NCIB 6346 strain was stable at pH 7.0 and 10.0 and was maximally active at 70-90°C and pH 7 as thermostable enzyme (Morgan and Priest 2008). Maximum enzyme production from *B. amyloliquefaciens* P-001 with initial pH 7 at 40°C, optimum pH 6.5 and 60°C and retained about 73% of its activity at 50°C (Deb et al. 2013). The pH 4.9 and 40°C were optimum for amylase from *Bacillus circulans* GRS 313 which was stable at 60°C and pH range of 5.0-8.0 (Dey et al. 2002). The temperature profile of α-amylase from *B. cereus* isolated from acidic soil showed a very broad temperature range of between 10-70°C and optimum at 50°C which is different from those known *Bacillus* amylases (Mahdavi et al. 2010). The optimum temperature range of purified α-amylase from *B. licheniformis* EMS-6 for hydrolysis of soluble starch was found to be at 60-70°C (Duy and Fitter 2005; Ul-Haq et al. 2010). Maximum activities of α-amylase from saliva samples of adult smokers and no smokers were obtained at an optimum temperature of 40°C (Enemchukwu et al. 2013). The enzyme was stable for at least six months at 25°C (Sampson et al. 1981). The enzyme activity in the serum of pancreatitis patients was very stable at 37-40°C (Mohhmod 2010; Amutha and Priya 2011) and optimum at 50°C (Kim et al. 1992). The enzyme activity from *Huai yam* powder was established for hydrolysis reaction under the temperature range of 40-70°C (Miao et al. 2013). The enzyme was stable at 25-60°C (Saito 1973). The deactivation mechanism involves the existence of a temperature-dependent intermediate form (Violet and Meunier 1989). Partially purified α-amylase enzyme from freshwater zooplankton *Heliodiaptomus viduus* (Gurney) showed activity up to 70°C and optimum at 30°C, and inactive at 60°C after 2 hrs and at 70°C after one hour (Dutta et al. 2006). In the present study, the optimum activity of the enzymes was recorded at 70°C except for SUDK2 isolate where the optimum activity occurred at 60°C and the enzyme was active up to 90°C with a residual activity of only 30-50% (Figure 2) as concluded by many researchers using different microorganism (Sampson et al. 1981; Violet and Meunier 1989; Duy and Fitter 2005; Dutta et al. 2006; Mohhmod 2010; Ul-Haq et al. 2010; Amutha and Priya 2011; Enemchukwu et al. 2013; Miao et al. 2013). The amylase enzymes isolated from four *B. licheniformis* isolates and stored at -20ºC retained more than 90% of the original activity during 24 weeks of storage, while at 4ºC all the enzymes showed gradual loss of their activities and lost 60% of their activities after 8 weeks (Figure 4-7) as reported in similar studies carried on *B. circulans* an *B. subtilis* (Adeyanju et al. 2007) and the enzyme showed broad temperature range of between 40-70°C (Mahdavi et al. 2010).

The amylase enzyme exhibited activity at a wide range of pH, optimum being pH 9.0 which is desirable characteristic for its application in detergents as additive and textile resizing (Aiyer 2004). Poor enzyme stability under standard conditions of pH and temperature evidently affect the yield of hydrolysis products (Riaz et al. 2007).

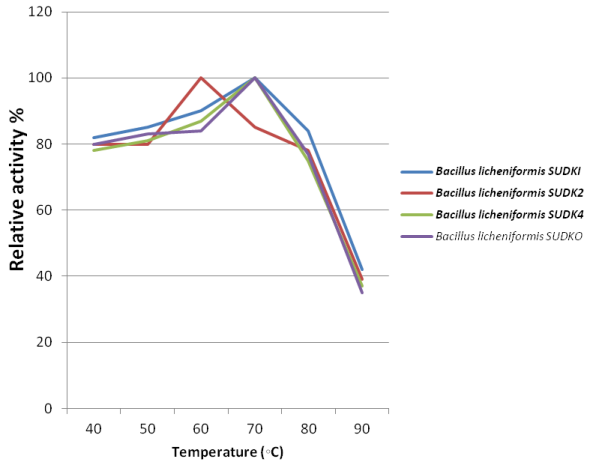

Figure 2. Effect of different temperature on α-amylase activity from different isolates of *Bacillus licheniformis*

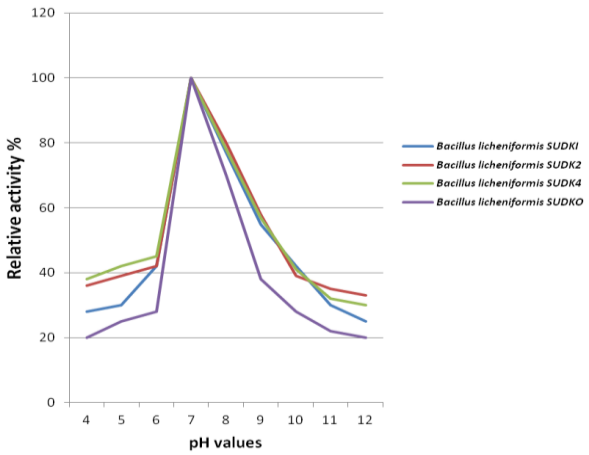

Figure 3. Effect of pH on α-amylase activity from different isolates of *Bacillus licheniformis*

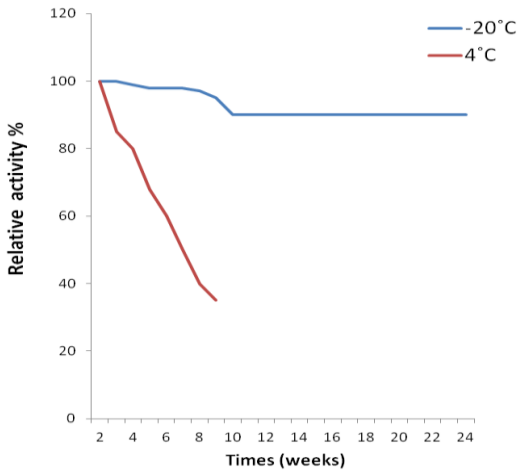

Figure 4. Effect of storage temperature on α-amylase activity from -amylase activity from *Bacillus licheniformis* SUDK1

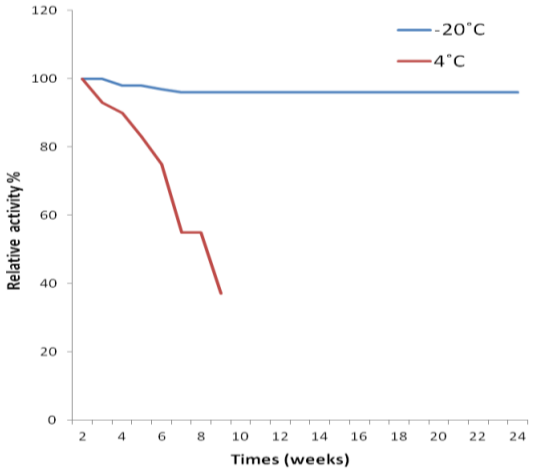

Figure 5. Effect of storage temperature on α-amylase activity from *Bacillus licheniformis* SUDK2

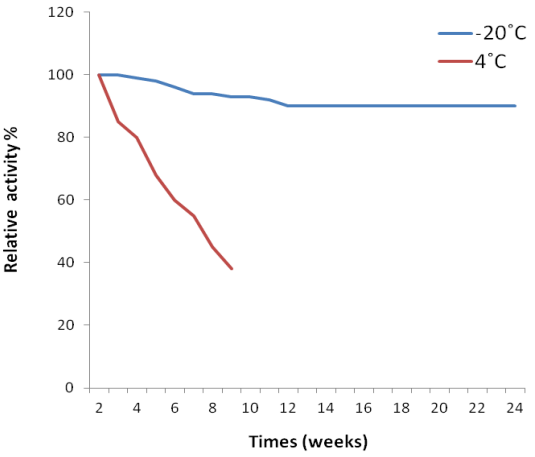

Figure 6. Effect of storage temperature on α-amylase activity from -amylase activity from *Bacillus licheniformis* SUDK4

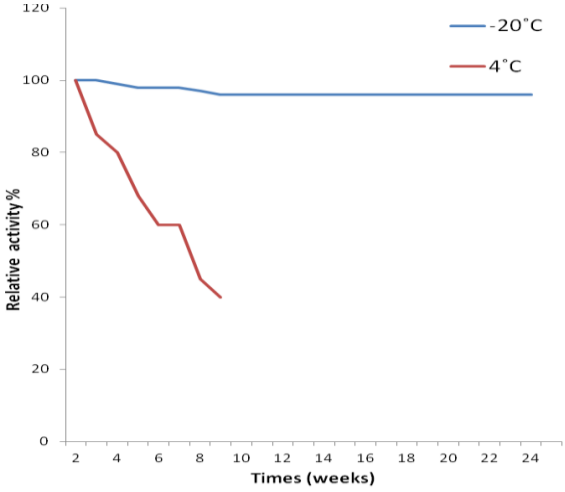

Figure 7. Effect of storage temperature on α-amylase activity from -amylase activity from *Bacillus licheniformis* SUDO

The amylase was stable over a wide pH range of between 4.5 and 9.0, and optimum at pH 7.0 (Ul-Haq et al. 2010). The enzyme activity in the serum of pancreatitis patients was very stable under pH 7.0-7.6 (Mohhmod 2010). Maximum activity for saliva amylase was obtained at an apparent pH of 7.0 (Enemchukwu et al. 2013). Enzyme from freshwater zooplankton, *Heliodiaptomus viduus* was active at pH 3.5-8.5 (Dutta et al. 2006). Either acidic or alkaline conditions significantly affect the growth and enzyme production by *B. subtilis* (Amutha and Priya 2011). The amylase was stable at pH 6.0 and 11.0 at 25°C, and below 60°C and pH 8.0 (Saito 1973). It was active over a pH range of 6.0-8.0 at 50°C (Kim et al. 1992). The α-amylase from irradiated wheat samples had a narrow range of pH optimum and was inactivated faster at alkaline conditions (Machaiah and Vakil 1981). Our results show that the highest activities for all amylase enzymes were observed at pH 7.0 and the enzymes were found to be stable over a wide range of pH (7.0-10.0) with residual activity of 20-30% at pH 4.0 and 12.0 (Fig 3) as reported by many authors in similar studies (Saito 1973; Machaiah and Vakil 1981; Kim et al. 1992; Dutta et al. 2006; Mohhmod 2010; Ul-Haq et al. 2010; Enemchukwu et al. 2013).

A high apparent Michaelis constant (km) and low maximum velocity Vmax for hydrolysis of soluble starch catalyzed by enzyme from radiated wheat, suggested some modifications in the formation of the substrate α-amylase complex (Machaiah and Vakil 1981). The Km was 6.66 mg/mL for α-amylase of *Huai yam* powder (Miao et al. 2013) and 11.6 mg/mL for enzyme isolated from *B. circulans* GRs 313 (Dey et al. 2002). The Michaelis constant values of 3.30×10^{-2} and 3.37×10^{-2} mg/mL was reported for the saliva samples of smokers and no smokers (Enemchukwu et al. 2013). In the present study, the kinetic parameters (Km) of substrate hydrolysis of the α-amylases from different *B. licheniformis* isolates were determined using different concentrations of soluble starch. In the present study, the Km values calculated from a typical Lineweaver-Burke plot, and were found to be in the range of between 1.25-2.0 mg/mL. This affinity of α-amylase activity was similar to that of thermophilic *B. acidocaldarius* (Km 0.8-1.6 mg/mL) as concludes by Carvalho et al. (2008).

In conclusion, the results suggest that the endogenous isolates of *B. licheniformis* produce different profiles of α-amylases which can easily be purified by DEAE-Sephadex and enzymatic reaction products were identified by thin layer chromatography. The enzyme's activity increased up to 16.8-18.3 folds and hydrolyzed starch to maltooligosaccharides such as maltose and glucose. The enzymes were active at 60-90℃ (60-70℃ optimum) and stable at pH 6.0-9.0 (pH 7.0 Optimum) maintaining their initial activity after long period of storage at 4℃, with low Km values (1.25-2.0 mg/mL). Therefore, these enzymes were thermostable under wide range of incubation temperature and pH levels. These findings stipulate their numerous uses in many industries.

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