

Some Properties Of Thermo-Stable α -Amylase Of Four Isolates Of *Bacillus Licheniformis*

Elham S. Dawood, Ibrahim S. A., Sohair A. Abdelwahab

Abstract: Four isolates of *Bacillus licheniformis* producing thermo-stable amylase enzymes were previously isolated from different Sudanese soils and designated as *B. licheniformis* SUDK1, SUDK2, SUDK4 and SUDO. The enzyme was partially purified using DEAE Sephadex A 25 gel filtration and then were identified using thin layer chromatography. Optimum pH and temperature for enzyme activity were determined. The effect of thermal stability was detected at two storage temperatures (-20°C and 4°C) for 24 weeks. The results showed that the activity of the partially purified enzymes increased up to 16-18 folds. The amylase enzymes were found to hydrolyze starch forming various maltooligosaccharides, such as dextrans and maltose as major products so they were identified as α -amylases. Optimum temperatures for enzyme activity was obtained at 60 and 70°C . All enzymes were stable between pH 6.0-9.0 with optimum pH 7.0. The enzymes were stable and retained nearly all of their initial activities at -20°C till the end of 24th week. At 4°C they lost less than 60% of their initial activities at the 8th week. The K_m values were 1.25 – 2.0 mg/ml.

Keywords: *Bacillus licheniformis*, thermo-stable amylase, α -Amylases, partial purification, DEAE Sephadex.

Introduction:

Thermostability is a characteristic of most of the enzymes available for bulk industrial usage. Thermotolerant microorganisms are of special interest as a source of novel thermo stable enzymes. Nowadays, the use of enzymes in industrial sector is increasing due to the increase of industries, especially in food, beverage, textile, leather and paper industries. Beside its uses in industry, it can also be used in the treatment of industrial waste, such as cellulase which is able to convert cellulose of wood and paper wastes to ethanol [1]. By this day, most of the enzymes used in industrial sector in Sudan especially for food industries are still imported enzymes. Economically, this situation is a loss, as Sudan is a country which is rich of natural resources, particularly the microbial as enzyme producer, including α -amylase enzyme. Based on this condition, it is important to develop a technique of enzyme isolation and purification in order to obtain purified and characterized enzyme such as extracellular α -amylase enzyme from local bacteria isolates *Bacillus licheniformis* SUD KI, SUDK2, SUDK3, SUDK7 and SUDO. α -Amylases (EC3.2.1.1, 1,4- α -D-glucan-glucanohydrolyase) are extracellular enzymes which hydrolyze starch into a range of products such as glucose and maltose or specific maltooligosaccharid or mixed maltooligosaccharides [2;3; and 4].

Although α -amylases can be derived from several sources, such as plants, animals and microorganisms, the enzymes from microbial sources are preferred in industrial sector and a large number of them are available commercially [5]. Sources of amylases in bacteria, yeast and other fungi have been reported and their properties described by [6; 7 and 8]. Although there are many microbial sources available for producing amylases, only a few such as *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus amyloliquifaciens* are recognized as commercial producers [9]. The capacity of *Bacillus* strains to produce large quantities of enzymes has placed them among the most important industrial enzyme producers. Indeed, they produce about 60% of commercially available enzymes [10]. Improvement of the yield of α -amylase and consequent cost reductions depend on the selection of strains, the optimization of the factors affecting biosynthesis, kinetic studies and the biochemical characterization of the enzyme. Each application of α -amylase requires unique properties with respect to specificity and stability [11]. This study reports partial purification, identification the type of amylase enzyme and characterization of α -amylase from four isolates of *Bacillus licheniformis* SUDKI, SUDK2, SUDK3, SUDK7 and SUDO isolated previously from different soil samples in Sudan.

Materials and Methods:-

Medium and Cultivation:-

Broth medium for amylase production was prepared as follows: (g/L distilled water) peptone 10g; (for all isolates except *B. licheniformis* SUDK2 which needed 10 g malt extract) dipotassium hydrogen phosphate 3g; magnesium sulphate $7\text{H}_2\text{O}$ 1g and 0.5g soluble starch for all isolates of *B. licheniformis* SUDO respectively. The cultures were grown on a rotary shaker (200rev./min) at 50°C for 24h. The cultures were then centrifuged in a refrigerated centrifuge and the supernatant collected for enzyme assay and study. The medium used for amylase production contained (per liter of distilled water) peptone 10 g; (for all isolated except *B. licheniformis* SUD-K2 which needed malt extract 10 g.) dipotassium hydrogen phosphate 3 g; magnesium sulphate $7\text{H}_2\text{O}$ 1g. and Starch 0,5g (for all isolates except *B. licheniformis* SUD-O which needed 2,0g). The cultures were grown on a rotary shaker (200rev/min) at 50°C for 24 h.

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- Department of life Science, Faculty of Education, Nile Valley university Associate Professor .Department of life Science Correspondence
E. mail elhamdawood@hotmail.com
Mobile 0024991893248
 - Department of Botany, Faculty of Science Khartoum university.
 - Department of biology, Faculty of Science Tahook University branch Duba- Saudi Arabia Assistant Professor in Microbiology (MSc. And PhD).

Enzyme Assay:-

Amylase assay: Enzyme assay was estimated by the dinitrosalicylic acid (DNSA) method of [12]. The reaction mixtures consist 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 for 3 min at 30°C. The reaction was terminated by the addition of 1 mL of dinitrosalicylic (DNSA) reagent. The mixture was heated at 100°C for 5 min and cooled. The optical density was read at 540 nm in a spectrophotometer (Jenway, 6305). One unit of amylase activity is expressed as one mg of maltose liberated per ml of culture supernatant at 60-70°C (pH 7.0). Protein content of the enzyme solution was assayed by the method of [13].

Enzyme Purification:-

The crude enzyme preparations of the culture filtrates (*B. licheniformis* SUDK₁, SUD-K₂, SUD-K₄, and SUD-O) were applied separately to 1.8x20 cm Column of DEAE – Sephadex A-25 equilibrated with 0.05 M sodium phosphate buffer pH 7.0. After all the sample had entered the resin, one bed volume of the equilibrating buffer was passed through 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 (1x7 cm) using an automatic circular fraction collector. Enzyme activity and protein concentration were determined in each fraction as in the assay method.

Identification of Enzyme Digests:-

The products of starch hydrolysis by the partially purified enzyme were identified by 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 [14].

Effect of temperature and pH on Enzyme activity:-

Enzyme assays were carried out at different temperatures (40-90°C) and pH values (4.0 – 12.0).

Storage Stability:-

Six fractions of 5 ml each from the pooled partially purified enzyme were taken in sample bottles; three of these were stored at 4°C in a refrigerator and the rest were stored in a freezer at -20°C. Every week the enzyme activity was assayed, both for the frozen enzyme and the enzyme in the refrigerator. The Change in absorbance was measured using a spectrophotometer and the residual activity was calculated.

Results

Partial Purification of Amylase Enzyme:-

Results of the column chromatography of the crude extract of the four bacilli are shown in table (1). It is seen that the specific activity of amylases from all bacilli was increased 16-18 folds over the crude extract, while the protein content decreased to about 4-5% of its original value. Also all

isolates gave a Single peak of amylase. At the same time the yield of all amylases varied in the range of 67- 80%.

Identification of Enzyme Product:-

The Products of starch hydrolysis by partially purified amylase enzymes of the four bacilli were identified by thin layer chromatography (TLC). The results are shown in Fig (1). The degradation Products of amylases produced by three isolates (*B. licheniformis* SUD-K₁, SUD-K₄ and SUD-O) were mainly Oligosaccharides and maltose while *B. licheniformis* -SUDK₂ produced Oligosaccharides, maltose and glucose.

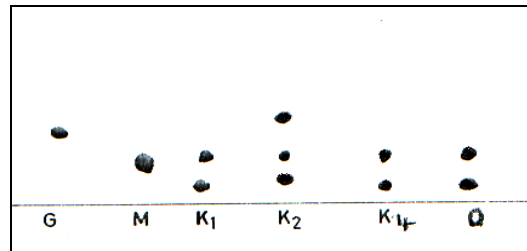


Fig.(1): Thin layer Chromatograms of the reaction products formed from Gelatinized starch by the action of amylases of different *Bacillus* isolates.

G = Glucose standard M = Maltose standard K-O = Samples

Table1: Partial Purification of Amylase Enzyme of Different isolates of Bacillus licheniformis

Bacillus isolates	Steps	Volume (ml)	Specific activity U/mg protein	Total protein (mg)	Total Activity (units)	Yield (%)	Purification (Fold)
B.licheniformis SUDK ₁	Crude enzyme DEAE-Sephadex	300 30	7.250 121.755	116.400 4.650	843.900 566.161	100 67.089	1 16.794
B. licheniformis SUDK ₂	Crude enzyme DEAE-Sephadex	200 30	4.626 83.343	94.000 4.200	434.844 350.041	100 80.498	1 18.016
B. licheniformis SUDK ₄	Crude enzyme DEAE-Sephadex	300 30	6.973 120.842	115.8 4.81	795.89 581.250	100 73.03	1 17.330
B. licheniformis SUDO	Crude enzyme DEAE-Sephadex	300 25	5.238 95.883	124.500 5.150	652.131 493.793	100 75.721	1 18.305

Effect of temperature and pH:-

Activity measurements of the partially purified preparations were carried out at pH 7.0 over the temperature range of 40 - 90°C. Optimum activity recorded at 70°C (except for SUD-K2 which occurred at 60°C) although was active up to 90 °C with a residual activity of only 30 -50% (Fig 2). Optimum values for all amylases were at pH 7.0 The enzymes were found to be stable over a wide range of pH (7.0 – 10.0) with residual activity 20 – 30% at pH 4.0 and 12.0 (Fig 3).

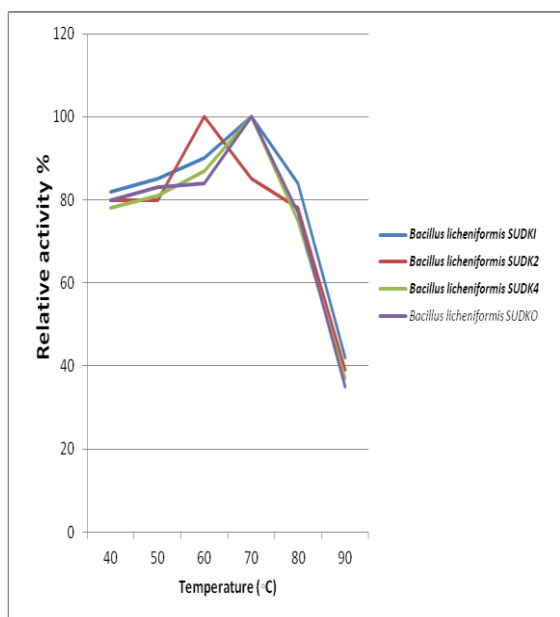


Figure 2: Effect of different temperature on amylase activity by different isolates of B. licheniformis.

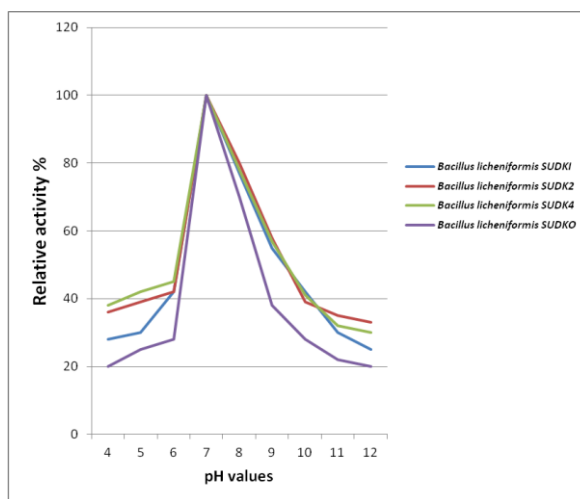


Figure 3: Effect of pH on amylotic production by different isolates of Bacillus spp.

Effect of Storage temperature:-

Two storage temperatures, namely 4°C and -20°C were evaluated with regard to their effect on enzyme stability. The results are presented in (Figs 4-7). All the enzymes stored at -20°C retained 90% or more of their original activity during 24 weeks. At 4°C all the enzymes showed gradual loss of their activity and by the eighth week 60% or more of their activity was lost.

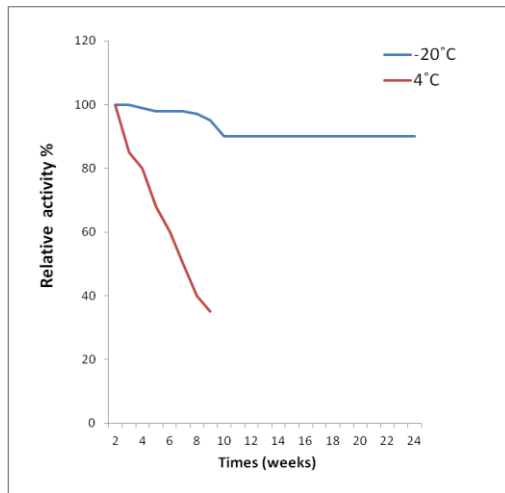


Figure 4: Effect of storage temperature on α -amylase activity from *Bacillus licheniformis* SUD-K1

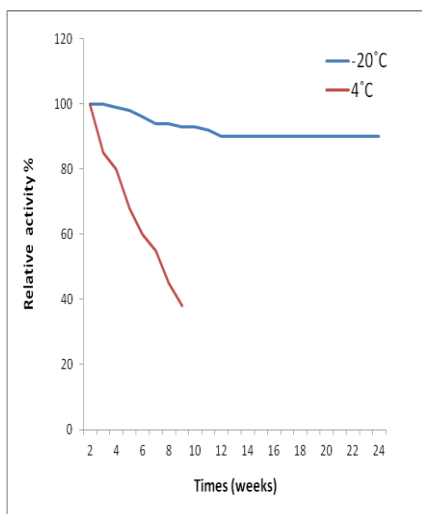


Figure 5: Effect of storage temperature on α -amylase activity from *Bacillus licheniformis* SUD- K4

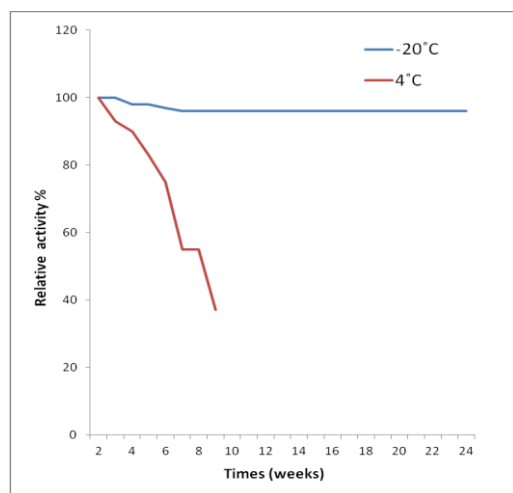


Figure 6: Effect of storage temperature on α -amylase activity from *Bacillus licheniformis* SUD-K2

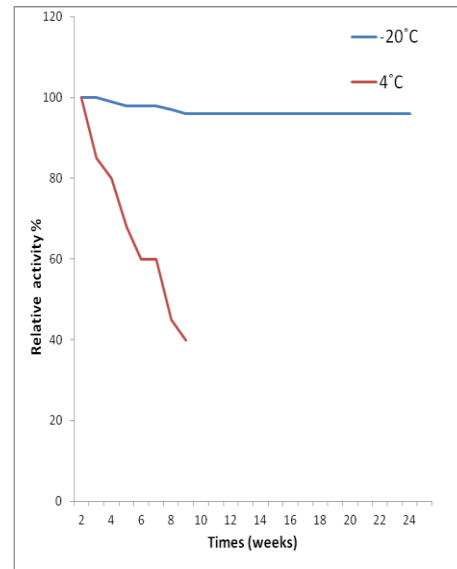


Figure 7: Effect of storage temperature on α -amylase activity from *Bacillus licheniformis* SUD-O

Kinetic parameters of substrate hydrolysis of the α -amylases of the different *Bacillus* isolates were determined using different concentrations of soluble Starch. The Km values were 1.25 – 2.0 mg/ml as calculated from a typical Lineweaver – Burke plot.

Discussion:

Many bacterial produced extracellular amylases during fermentation of starch. Purification of extracellular α – amylases obtained from different sources , including *Bacillus licheniformis* have been reported by [15]. The current result of this investigation showed that DEAE-Sephadex is an excellent exchanger for these enzymes, which proved the effectiveness of purification method applied in this research , Similar results was reported by [16]. using DEAE-Sephadex G100. The degradation product of the enzyme for all tested isolates gave results such as oligosaccharied ,maltose and glucose which confirmed that the enzymes produced was an α – amylases ,similar results was reported by [17]. Temperature was considerably affected the activity of the amylase enzyme. The optimum temperature for the α -amylase activity was 70°C for all isolates except SUDK2 (60°C). Other investigators working on alpha amylase have reported various temperature optima in this range. An optimal activity of α -amylase was reported at 50°C for *Alicyclobacillus acidocaldarius* and *Bacillus cereus* respectively by [17 and 18], while [19] reported 60°C as optimum temperature for *Bacillus amyloliquefaciens* P-001. The stability of enzymes activity were tested at two storage temperatures namely 4 °C and -20 °C for 24 weeks. It is clear that -20 °C was effective storage temperature because , α - amylases for all isolates retained 90% or more of their activities till the end of the 24th week. At 4°C they showed gradual loss of their activities and at the 8th week, 60% or more of their activities were lost. These results are similar to the finding of [15] who examined the α -amylase of *Bacillus circulans* and *B. subtilis*. The Km values of these -amylases were in the range of 1.25-2.0 mg/ml which were calculated from a typical Lineweaver-Burke plot . Affinity of these amylases is

similar to those reported by [20] using thermophilic *B. acidocaldarius* ($K_m=0.8-1.6\text{mg/ml}$).

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