Characterization of α-Amylase Produced by Different *Bacillus spp*

Ahmed, A. Alkando[1], Hassan Bashier Elamin[2] Hanan Moawia Ibrahim[1*]

[1]Central Lab. Ministry of Science and Technology, Sudan
[2] National Centre for Research, Ministry of Science and Technology
[*]Author correspondence
E-mail: hara111@yahoo.com Hp: +249-9-11171941 Fax: + 249-155183855

ABSTRACT

the amylase enzyme from different *Bacillus spp.* was carried out in culture The production of fermentation (SCF). Production of the amylase was carried out for the potential use of the enzyme for industrial purposes. The bacteria used were isolated and taxonomic characterized from Khartoum state. These bacteria were identified as *B megaterium, B. subtilis, B. licheniformis* and *B. amyloliquefaciens.* It was found that *Bacillus licheniformis* had 6.5 cm zone of clearance, .,*B megaterium* cm, *B. amyloliquefaciens* cm where as *B. subtilis* resulted in much lesser cm. Amylase produced by *Bacillus licheniformis B megaterium B. amyloliquefaciens* and *B. subtilis* was 42.64 mg/ml, 38.11 mg/ml, 35.14 mg/ml, and 37.93 mg/ml glucose, respectively.

Key words: α -amylase, thermophilic bacterium, thermostable enzymes, *Bacillus spp.*

INTRODUCTION

EC 3.2.1.1) hydrolyses α-1,4-glucosidic linkage in α-Amylase (1,4 α D-glucanohydrolase; starch and related molecules. It is one of several enzymes involved in starch degradation Starch is an abundant carbon source in nature.

Amylases constitute one of the most important groups of industrial enzymes being extensively studied because of it commercial applications in food additive, starch liquefaction to reduce the viscosity, production of maltose, high fructose syrup, saccharification of starch for alcohol production and brewing (Akhtar et al. 2001; Alhasan et al. 2010), sizing in textile industries, detergent manufacturing processes, as an additives to detergents for removing stains, distilling industries, sugar production and paper industries. The spectrum of amylase applications has expanded in several fields such as clinical, medicinal and analytical chemistry (Reddy et al 2003).

Most of the available amylases produced commercially are of microbial origin (Aboud-zeid 1997, Pandey et al., 2000, Alyer, 2005), and are produced by a variety of living organisms, ranging from bacteria to plants and humans. Bacteria and fungi secrete amylases to the outside of their cells to carry out extra cellular digestion. When they have broken down the insoluble starch, the soluble end products such as (glucose or maltose) are absorbed into their cell.

This enzyme is widespread among aerobes and anaerobes. Gram positive bacteria, particularly the genera *Bacillus* and *Clostridia* are prolific producers of amylases (Haddaoui *et al*., 1995; Shih and Labbe, 1995; Obineme *et al*., 2003; Swain and Ray, 2007). The bacterial enzyme is usually from either *Bacillus amyloliquefaciens or Bacillus licheniformis*, the latter now being of greater industrial importance (Declerck et al., 2000). Several species of *Bacillus* produces a wide range of extra cellular enzymes of which amylases and proteases are of significant industrial. The amylase activity has been shown to be influenced by temperature, pH and presence of some chemicals (Swain and Ray, 2007).

The production of enzyme is the major cost factor in the hydrolysis of starch to fermentable sugars. To reduce the cost of enzyme production, several ways can be used including using cheap raw materials as the production medium, genetically modified microbial strain with high enzyme productivity, or using suitable bioreactor systems. Recent research with thermostable α-amylase has concentrated on the enzymes of thermopiles and extreme thermopiles (Sadhukhan et al 1992, Arnesen et al 1998, Lin et al 1998),

Each application of α-amylase of microbial source requires unique properties with respect to specificity, stability, temperature and pH dependence (McTigue et al 1995). It can be produced in amount meeting all the demands of the market because diversity of enzyme available from microorganism is very great. Emphasis is still placed on the isolation of new strains and mutants of microbial producing more amylase with greater specific activity, operation stability and resistance to inhibition than those currently obtained (Gomes et al. 1991).

Despite the fact that many different α- amylases have been purified and characterized so far, and some of them have been used in biotechnological and industrial applications, the present known α- amylase are not sufficient to meet most of industrial demands. The aim of this research was to investigate the ability of *Bacillus spp.* as high α- amylase producer in comparison to the other different *Bacillus spp*.

MATERIAL AND METHODS

This research was conducted between 2006- 2009 at Central Lab. Ministry of Science and Technology. Sudan.

Screening and identification of bacteria strain

The locally soil isolate *Bacillus* strains were primary screened for α- amylase synthesis after incubation for 60˚C for 48 hr, the plates were flooded with a solution of 0.5% (w/v) I_2 and 5.0% (w/v)KI according to Thippeswamy et al 2006. Colonies exhibited halo starch hydrolysis were picked up. The clear zone surrounding the colony was measured in (cm) from the edge of the colony to the limit of clearing and also the diameter of colony was recorded. The relative amylolytic activity is expressed as an index of activity calculated according to the follow equation:

$$\frac{\text{Average diameter of microorganism - average diameter of colony}}{\text{Average diameter of colony}} \quad \text{equation (1)}$$

Identification of the selected *Bacillus* strain was identified on the basis of standard morphological and biochemical test according to the method described in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974). Subculture was prepared by a full loop of the suspension of vegetative cells was streaked aseptically onto nutrient agar (NA) several times until pure colonies were obtained. The pure isolates were maintained on NA slants and routinely sub-cultured every four weeks.

Fermentation process:

α- amylase fermentation was carried out by submerge fermentation. Ten ml from the bacterial inoculums' was inoculated to 500 ml shake-flasks containing 90 ml of a defined medium Horikoshi medium II, according to Horikoshi 1979 Soluble starch 1%(w/v), Peptone 0.5%(w/v),, Yeast extract 0.5%(w/v), $MgSO_4.7H_2O$ 0.02%(w/v), K_2HPO_4 0.1%(w/v), and $Na_2CO_3$1%. The flasks were incubated at 37˚C in an orbital shaker agitated at 150 rpm for 24-48 hr. Initial pH of 7.0.

Production of a crude α-amylase:

The culture of *Bacillus spp*. were transferred to sterile starch broth medium and incubated at 37 ˚C for 24 hr, after incubation the cell free filtrate (crude enzyme) was obtained by centrifugation at 1,000 rpm for 10 min. at 4 ˚C and stored at -20 ˚C

Enzyme Activity:

Hydrolysis activity: Iodine method was used according to Fuwa 1954. One unit of the activity is defined as the amount of enzyme that could produce a change of 0.01 absorbency at 700 nm in the standard assay.

Saccharification activity: Di-nitro-salsylic acid (DNS) method according to De-Moraes et al (1999) was tested. Glucose was used as standard. One unit of enzyme activity was defined as the amount of enzyme that formed 1mg of reducing sugar in 1 min.

Optimisation of α-amylase activity assay conditions (DNS)

The influence of pH, time of incubation, temperature and soluble starch concentration were measured to optimize the enzyme assay conditions. Optimum pH was determined at different pH ranged from 5-10 using 0.1M acetate buffer (4.5-5.5), 0.1M phosphate buffer range (6.5-8.5) and 0.2 M tris-HCl (pH 9.0-10.0). Reaction time was measured at different time interval, ranged from 5-60 min. The optimum temperature was measured at various temperature ranged from 20-100˚C. The range of substrate concentration from 0.5-3%.

Biomass Estimation

Dry cell weight was determined using Herbert et al (961).The pellets were washed with acetate buffer three times in order to remove the starchy material attached to the pellet. The suspension free from the starchy material were then filtered using a pre-weighted filter paper and dried in an oven at 60˚C for 1-2 days to a constant weight for the measurement of cell concentration (mg/ml).

The culture supernatant from batch culture fermentation was used to partially characterize the α-amylase

Thermal and storage stability of the crude enzyme

The partially purified enzyme was placed at different temperature ranged from 20 ˚C -90 ˚C for 0-30min. and after specific time period the activity was performed as residue activity. For storage stability, α-amylase was stored at different time interval ranged from 0-24 hr. aliquot from each storage time was taken out and the enzyme activity was determined. For all analysis that involved (HPLC), the conditions were set according to Kouame et al., (2004).

RESULTS

One hundred and twelve *Bacillus* species were isolated from soil sample and tested for α-amylase production. Four potential isolates of α- amylase producer were obtained during primary screening and secondary screening of these isolates. The biochemical and morphological characterization was carried out, and identified as *B. megaterium, B. subtilis, B. licheniformis* and *B. amyloliquefaciens* **(**Plate 1,2,3 and 4).

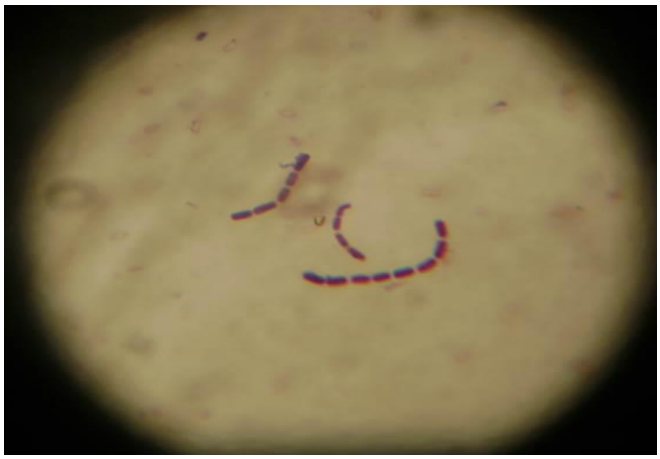


Plate (1): *B.megaterium* (Microscopically characteristics)

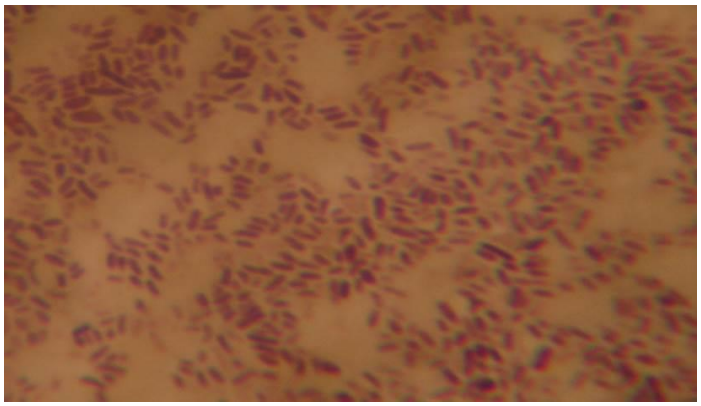


Plate (2): *B.licheniformis* (Microscopically characteristics)

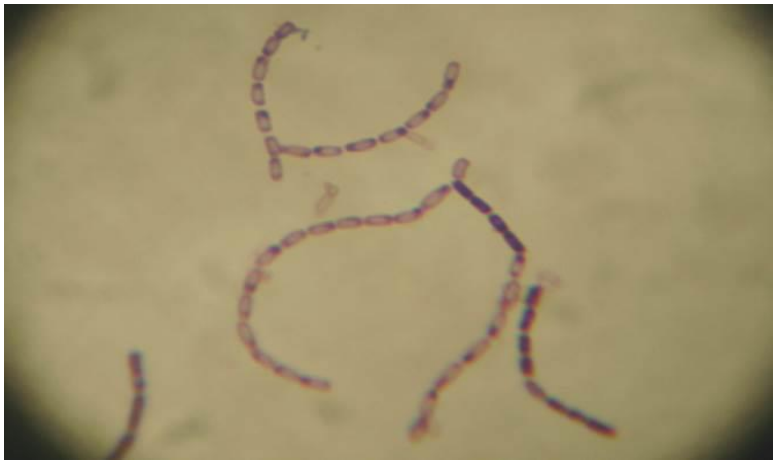


Plate (3): *B.Subtillis* (Microscopically characteristics)

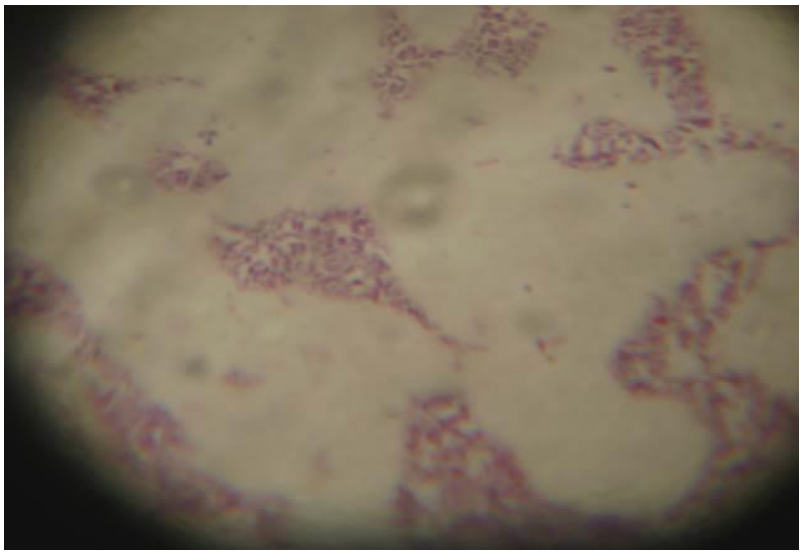


Plate (4): *B. amyloliquefaciens* (Microscopically characteristics)

Plate (5,6,7 and 8) showed the inhibition zone of each identified isolates. , *B. licheniformis* showed larger inhibition zone of about 1.5 cm. An organism was considered amylolytic if it produced a clear zone extending at least 1 mm from the edge of the colony by six days (Fryer et al 1967).

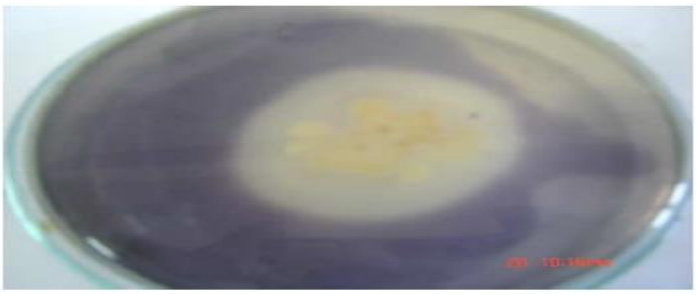


Plate (1): Inhibition Zone of *B. megaterium* which is 1.0mm

Plate (5): Inhibition Zone of B. *megaterium* which is 1.0 mm

Plate (6): Inhibition Zone of B. *licheniformis* which is 1.5 mm

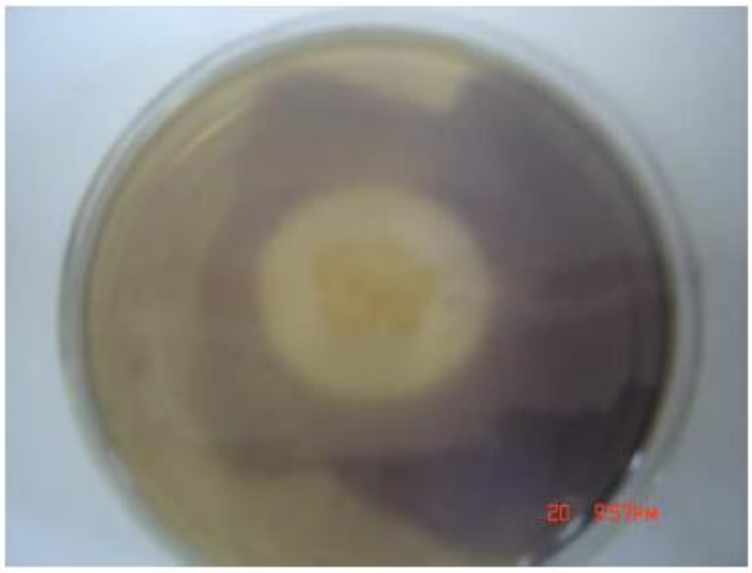


Plate (7): Inhibition Zone of B. *subtitles* which is 1.25 mm

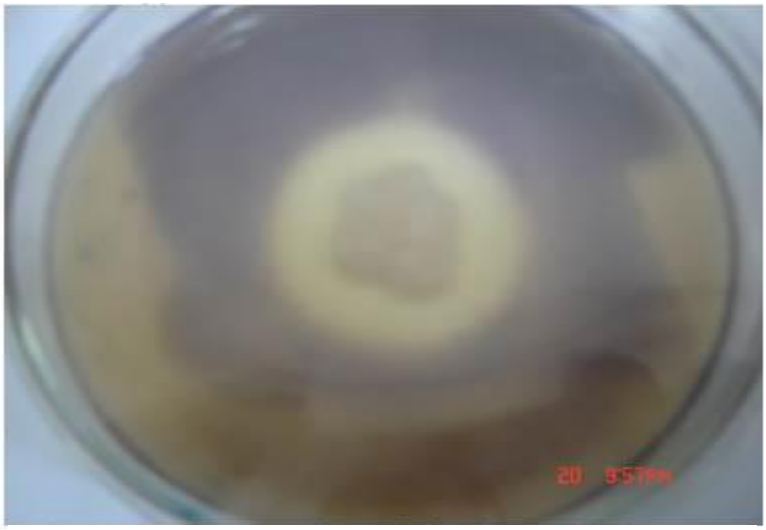


Plate (8): Inhibition Zone of B. *amyloliquefaciens* which is 1.25 mm

Table (1) showed the primary screening of these four species as hydrolysis method confirms the production of the α- amylase enzyme, The results also showed that the bacterial growth is concomitant with the enzyme activity.

Table (1) Primary screening of the 4 species

Species	Zone of inhibition (mm)	Relative amylolytic activity	Dry cell weight (mg/ml)
B *B.megaterium*	8.0	1.0	0.197
B.Subtillis	8.0	1.25	0.233
B.amyloliquefaciens	8.0	1.25	0.205
B.licheniformis	4.0	1.50	0.235

Effect of culture conditions on α- amylase activity assay

The optimal culture conditions on amylase activity assay in terms of temperature, pH, substrate concentration and incubation time, was studied (Table 2) *B.licheniformis* gave high temperature tolerance up to 90 ˚C and lower substrate concentration but long fermentation period of 72 hr. compared to the others species.

Table (2) Optimum conditions of α- amylase activity assay of the 4 species

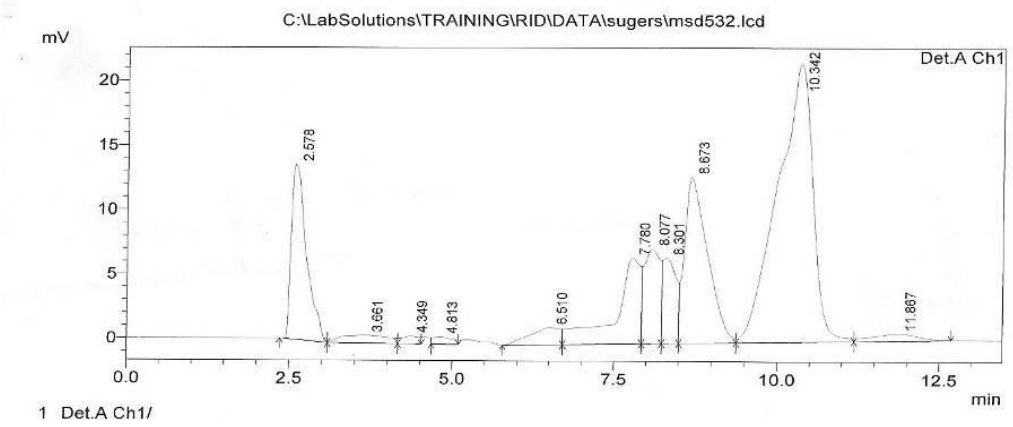
Species	Saccharifing activity (mg/ml/min)	pH	Temp (˚C)	Substrate concentration (%)	Incubation time (h)
B *B.megaterium*	1.524	7	80	1.5	72

B.Subtillis	2.511	8	80	1.5	60
Bamyloliquefaciens	3.506	7	80	1.5	60
B.licheniformis	4.535	5	90	1	72

Table (3) showed the concentration of the end product of the commercial enzyme and the four isolated species incubated with soluble starch in the optimum conditions that injected in HPLC. The sugar concentrations were calculated according to the formula: the concentration of the injected solution was propositional to the area (Fig. 1,2,3, and 4).

Table (3) The concentration of sugars produced by the commercial amylase and the 4 species using HPLC

Species	Glucose (mg/ml)	Maltose (mg/ml)
Standard amylase (Sigma)	36.26	3.92
B *B.megaterium*	42.64	4.06
B.Subtillis	38.11	166.78
B. amyloliquefaciens	35.14	126.44
B.licheniformis	37.93	3.93

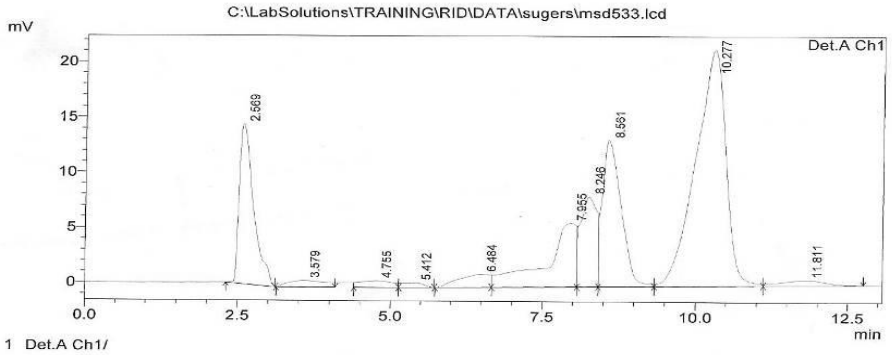


PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.578	218625	13595	11.680	18.839
2	3.661	30128	590	1.610	0.818
3	4.349	11160	588	0.596	0.814
4	4.813	11920	574	0.637	0.796
5	6.510	43606	1312	2.330	1.818
6	7.780	185030	6665	9.885	9.236
7	8.077	125275	7358	6.693	10.196
8	8.301	95014	6624	5.076	9.179
9	8.673	314755	12855	16.816	17.813
10	10.342	810408	21490	43.296	29.779
11	11.867	25850	513	1.381	0.711
Total		1871769	72163	100.000	100.000

Fig (1): The chromatogram of a crude amylase produced by *B.megaterium* incubated with soluble starch under the optimum conditions

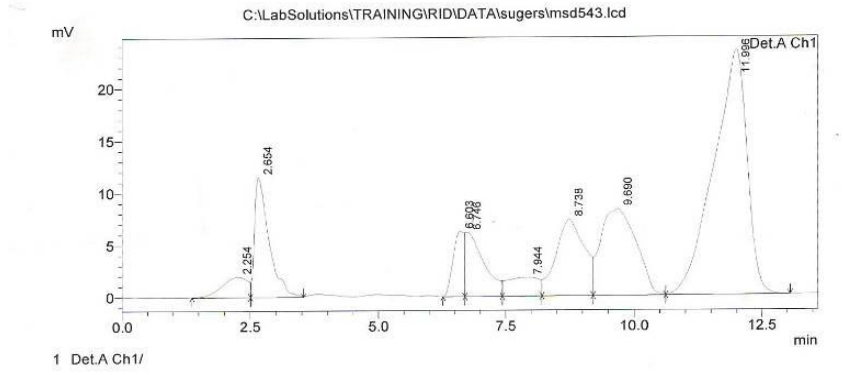


PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.569	233533	14479	13.530	21.883
2	3.579	24926	579	1.444	0.876
3	4.755	21833	581	1.265	0.878
4	5.412	10614	413	0.615	0.624
5	6.484	42275	1176	2.449	1.777
6	7.955	203997	5770	11.818	8.720
7	8.246	148376	8174	8.596	12.354
8	8.561	279965	13230	16.220	19.995
9	10.277	735520	21279	42.612	32.161
10	11.811	25050	484	1.451	0.732
Total		1726089	66165	100.000	100.000

Fig (2): The chromatogram of a crude amylase produced by *B.licheniformis* incubated with soluble starch under the optimum conditions

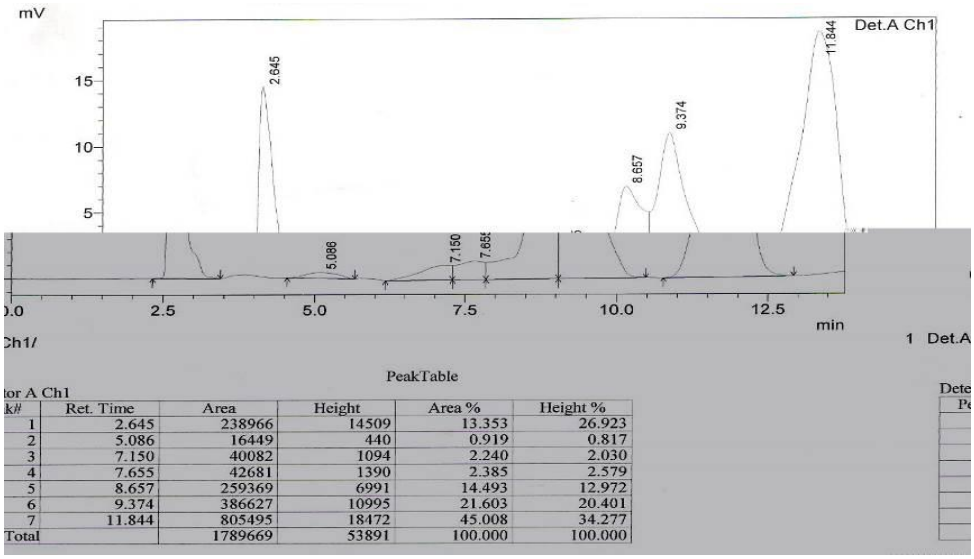


PeakTable

Detector A Ch1

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.254	67601	1944	2.866	2.919
2	2.654	223509	11541	9.477	17.334
3	6.603	95282	6246	4.040	9.380
4	6.746	156007	6124	6.615	9.198
5	7.944	77345	1776	3.280	2.668
6	8.738	281314	7310	11.928	10.979
7	9.690	394896	8283	16.744	12.440
8	11.996	1062464	23360	45.050	35.083
Total		2358418	66584	100.000	100.000

Fig (3) The chromatogram of a crude amylase produced by B. *subtilis* incubated with soluble starch under the optimum conditions



PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.645	238966	14509	13.353	26.923
2	5.086	16449	440	0.919	0.817
3	7.150	40082	1094	2.240	2.030
4	7.655	42681	1390	2.385	2.579
5	8.657	259369	6991	14.493	12.972
6	9.374	386627	10995	21.603	20.401
7	11.844	805495	18472	45.008	34.277
Total		1789669	53891	100.000	100.000

Fig (4): The chromatogram of a crude amylase produced by B. amyloliquefaciencs incubated with soluble starch under the optimum conditions

DISCUSSIONS

The current result of this investigation showed that temperature considerably affected the activity of the amylase enzyme. Other investigators working on alpha amylase have reported various temperature optima in this range. Obineme *et al*. (2003) and Shelby (1993) reported an optimal activity of α-amylase at 50°C for *Aspergillus oryzae* and *Streptococcus bovis* JBI respectively. The α-amylase of *B. subtilis* BS5 still retained 56.6 and 30.46% of its activity when heated for 10 min at 70 and 80°C, respectively. Aderibigbe, 1998 reported that temperature high than 70°C will denature the enzyme. The optimum pH for the α-amylase activity in this study was 6.0. This optimal α-amylase activity pH value was within the range of values reported for most starch degrading bacterial strain (Gupta *et al*., 2003). Mishra and Behera (2008) had also reported a pH of 6.0 and 7.0 for normal growth and enzyme activity for alpha amylase of *Bacillus* strains isolated from soil receiving kitchen waste. Similar values for bacterial amylases production by *Streptococcus bovis* JBI, new *Clostridium* isolate and *Clostridium acetobutylicum* SAI were

reported (Madi *et al*., 1987; Shelby, 1993). It was also noticed that amylolytic activity increased with substrate concentration. Among the physiological parameters, optimum temperature, substrate concentration and the pH of the growth medium plays an important role in production and activity of microbial enzymes. During growth of the microorganism in submerged culture fermentation in presence of starch as a carbon source, the pH of the medium decline from the original pH 5.5 to pH 3-4 as in Fig. 1. This change in the pH during the growth was probably due

to either the production of organic acids indirect relation to the carbohydrate consumed or release of ammonia.

It has been reported that the synthesis of carbohydrate-degrading enzymes in most species of the genus *Bacillus* is subject to catabolic repression by readily metabolizable substrates such as glucose (Lin et al 1998). Ours results are in good agreement with these findings. According to the results presented in this article, the α-amylase from the *Bacillus* sp isolated, is heat stable, although it showed optimum activity lower than that described for the α-amylase produced by *Bacillus* sp (Bajpai et al 1989, Lin et al 1998). Also the results presented in this study indicate *subtilis* B. amyloliquefaciencs, *B.licheniformis* , that the α-amylase produced by *B.* ***B.megaterium*** possesses properties of an industrial enzyme; productivity and thermo stability.

ACKNOWLEDGMENTS

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تصنيف إنزيم الأمايليز المنتج بواسطة أنواع مختلفة من جنس البكتيريا *Bacillus*

الملخص

إنتاج إنزيم الأمايليز تم بواسطة أنواع مختلفة من الجنس البكتيريا *Bacillus* spp وذلك بواسطة استخدام المزارع التخمرية. إنتاج الإنزيم كان بهدف استخدامه في الصناعة. أنواع البكتيريا المستخدمة تم عزلها من التربة بولاية الخرطوم وتم تصنيفها علي النحو **التالي:**

B. megaterium B. subtilis, B.licheniformis B.amyloliquefaciens. وجد أن المنطقة المثبتة كانت 8.0 لكل من البكتيريا *B.megaterium,* والبكتيريا *B. subtilis* والبكتيريا *B.*licheniformis في حين كانت للبكتيريا *B. amyloliquefaciens*أقل بكثير(4.0 سم). تركيز السكريات المنتجة بواسطة أنواع البكتيريا المختلفة كانت **كما يلي:** 42.64 جلكوز 4.06 ملتوز للبكتيريا *B megaterium* وللبكتيريا الثانية *B. subtilis* 38.11 و166.78 علي التوالي وللبكتيريا الثالثة *B. licheniformis* 35.14 و126.44 علي التوالي وللبكتيريا الرابعة *B. amyloliquefaciens* 37.39 و 3.93 علي التوالي.