Sci. J. Fac. Sci. Menoufia Univ., 2014, Vol. XXVI, 101-130

L-ASPARAGINASE PRODUCTION BY *STREPTOMYCES HALSTEDII* ISOLATED FROM EGYPTIAN SOIL

Sabha Mahmoud Mabrouk El-Sabbagh

Botany Department, Faculty of Science, Menoufyia University, Menoufyia, Egypt

ABSTRACT

The discovery that L-asparaginase acts as an antitumor agent in children with lymphoblastic leukemia led to the extensive studies on the physicochemical properties of the enzyme as well as on clinical effect. In the present study thirty isolates of actinomycetes from Egyptian soil were screened for production of L-asparaginase enzyme using Batch fermentation. The most active producer was identified as *Streptomyces halstedii*. Optimal cultural factors affecting the production of L-asparaginase by *Streptomyces halstedii* on glycerol-asparagine broth were pH 7.0, 30 ºC for 5 days. The strain utilized glycerol and L-asparagine as the best carbon and nitrogen sources for L-asparaginase production. The enzyme was purified to homogeneity by ammonium sulfate precipitation, dialysis, and Sephadex G-200 gel filtration. The enzyme was purified to about 55.2 folds with a final specific activity of 2071.2 U/mg protein and 4.17% yield. The molecular weight of the enzyme was determined to be 100 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Key words: *Streptomyces*, Microorganisms, L-Asparaginase production, optimization.

Corresponding Author: Sabha Mahmoud El-Sabbagh, Botany Department, Faculty of Science, Menoufyia University, Cairo, Egypt.
E-mail: sabhaahmed63@hotmail.com

INTRODUCTION

Actinomycetes including *Streptomyces* are the dominant group of soil microorganisms. They are free living, saprophytic filamentous bacteria, and contribute a major source for production of antibiotics. They play a major role in recycling of organic matter, production of novel pharmaceuticals, cosmetics, enzymes, antitumor agents, enzyme inhibitors, immune-modifiers and vitamins. *Streptomyces* species produce about 80% of the total known antibiotic and many active secondary metabolites (Mohan and Ramasamy., 2007).

L-asparaginase (L-asparagine amido hydrolase, E.C.3.5.1.1) produced not only by certain animal organs (EL-Sayed *et al*., 2011; Kumar *et al*., 2012), but also by microorganisms like *Aspergillus tamari*, *Aspergillus terreus* (Sarquis *et al*., 2004; Siddalingeshwara and Lingappa., 2010), *E. coli* (Swain *et al*., 1993; Cornea *et al* ., 2000), *Erwinia aroideae* (Liu and Zaji., 1972), *Pseudomonas stutzeri* (Manna *et al*., 1995), *Pseudomonas aeruginosa* (Abdel-Fattah and Olama., 2002), *Serratia marcescens* (Radcliffe *et al*.,1979), and *Staphylococcus* sp. (Prakasham *et al*., 2007) as well as Streptomyces. *Streptomyces* also serve as a good source of L-asparaginase, an enzyme which converts L-asparagine to L-aspartic acid and ammonia, and has been used as a chemotherapeutic agent (Fisher and Wray, 2002). L-asparaginase has received increased attention in recent years for its anticarcinogenic potential (Manna *et al*., 1995), especially as an anti-neoplastic agent used in the chemotherapy of lymphoblastic leukaemia (Keating *et al*., 1993). The clinical action of this enzyme is attributed to reduction of L-asparagine to L-aspartic acid and ammonia, so that tumor cells cannot synthesize this amino acid and are selectively killed by L-asparagine deprivation. Several *Streptomyces* like *S. aurantiacus*, *S. karnatakensis*, *S. venezualae*, *S. longsporusflavus*, and *S. albidoflavus* were reported to produce detectable amounts of L-asparaginase (Gupta *et al*., 2007; Narayana *et al*., 2008), *Streptomyces* sp. PDK2 and PDK7 (Dhevagi and Poorani, 2006), and *Streptomyces* sp. S3, S4 and K8 (Basha *et al*., 2009).

The enzyme is produced throughout the world by both submerged and solid-state cultures. Extra-cellular asparaginases are more advantageous

than intracellular ones, since they can be produced abundantly in the culture broth under normal conditions, and purified economically. Considering the above facts, an attempt was made for the production, of an extra-cellular L-asparaginase, under Batch fermentation by a novel actinomycetes isolate, also characterization of L-asparaginase producing bacterial isolate using morphological and biochemical analysis, study factors affecting L-asparaginase activity, and determination of the molecular weight of the purified enzyme.

The biochemical characterizations of the purified enzyme that may be useful in its commercial applications and the therapeutic effect on living cells as an anti-tumor will be studied on the another paper.

MATERIALS AND METHODS

Isolation of actinomycetes

Soil samples were collected from different localities of Minoufiya Governorate, Egypt. A dilution plate method was used to isolate actinomycetes on starch-nitrate agar plates as reported by Waksman, (1962). The plates were incubated for 7 days at 30ºC, colonies were checked for purity by repeated sub-culturing and the pure colonies maintained on slants of the same medium and stored at 4ºC for further use.

Screening for L-asparaginase production

All actinomycetes isolates were screened for their ability to produce L-asparaginase according to Khamna *et al*. (2009). Each strain of actinomycetes were inoculated on glycerol asparagine agar containing pH indicator, where initial pH was adjusted to 7.0 and incubated at 30ºC for 7 days. Colonies with pink zones were considered as L-asparaginase-producing active strains. Two control glycerol asparagine agar plates were used one without dye while the other was without asparagine.

Fermentation procedure

The more potent strains were selected for fermentation process. Batch fermentation was carried out in 50 ml of glycerol asparagine broth, where each flask was inoculated by one ml of 3 days old spore suspension ($2.5x10^6$ spores /ml). After incubation at 30ºC for 5 days on a rotary shaker at 250 rpm, L- asparaginase activity was measured.

Determination of L-asparaginase activity

The activity of L-asparaginase was measured according to the method of Mashburn and Wriston (1963). A mixture of 0.1 ml of culture, 0.2 ml of 0.05M Tris-HCl buffer (pH 8.6), and 1.7 ml of 0.01M L-asparagine was incubated for 10 min at 37 ºC. The reaction was stopped by the addition of 0.5 ml of 1.5 M trichloroacetic acid and the ammonia released was determined colorimetrically at 480 nm. One unit of L-asparaginase enzyme was defined as the amount of enzyme that releases one micromole of ammonia per minute at the defined conditions.

Identification of the best strain of actinomycetes for production of L-asparaginase

For complete identification of the best strain of actinomycetes for production of L-asparaginase, several cultural, morphological and biochemical properties were examined.

Detailed observation of the mycelia and spore morphologies was performed using light microscope and scanning microscope (JEOL-JSM-5500 LV) on inorganic salt-starch; oat meal agar, yeast extract malt extract and glycerol asparagines agar after 14 days of incubation at 30^{o}C (Shirling and Gottlieb, 1966). The ability of the isolate under study to utilize different carbon sources were examined using carbon utilizing agar medium (Pridham and Gotlibe, 1948). Different carbon sources (Glucose, L-arabinose, sucrose, xylose, inositol, mannose, lactose, rhafinose, rhamnose, mannitol, galactose and starch were used at concentration of 1% (w/v) and utilization was determined by the method described by Williams et al. (1983a). The ability of strain no.5 to use different nitrogen sources was tested. Equimolecular weights of different nitrogen sources (KNO_3, L-asparagine, $NaNO_3$, L-cystein peptone, $(NH_4)_2NO_3$, and (NH_4) H_2PO_2 were added separately to starch nitrate medium. Growth was scored after 14 day by comparing test plates with both negative and positive controls (Williams *et al*., 1983b).

Melanin pigment production was determined after 4 days of incubation on peptone-yeast agar and tyrosine agar. The diffusible pigments were detected by allowing the organism to grow on glycerol-asparagine agar for 14 days at 30 °C (Shirling and Gottlieb, 1966).

Lipase activity was detected by using Sierra's medium (peptone, 10 g; NaCl, 5 g; $CaCl_2$. $2H_2O$, 0.1 g; tween 80, 10 g; 15 g and distilled water up to 1000 mL and pH 7.0). After incubation for 3, 7 and 14 days at 30 °C, the presence of white precipitate indicated positive results (Sierra's, 1957).

The degradation of starch (1 % w/v) was detected on Petri dishes containing starch nitrate medium and inoculated with the tested organism. After 7 days incubation at 30 °C the plates were floated with iodine solution (Cowan, 1974) for starch degradation.

Arbutin (Hydroquinone-B-D glcopyranoside) degradation was studied in tubes containing a medium of (g. L^{-1}): yeast extract (oxoid), 3; arbutin, 1; ferric ammonium citrate, 0.5 and agar, 7.5 (Kutzner, 1976). Controls without arbutin were also inoculated. After 21 days, a positive result was indicated by a brown-black pigment. Comparison with controls is essential to avoid confusion with melanin production.

Growth in presence of some inhibitors was carried out on Petri-dishes containing Bennett's agar with glycerol instead of glucose. The inhibitors were added separately to the medium before autoclaving. Sodium azide (0.01 % w/v), NaCl (3, 5, 7 and 10 % w/v) and phenol (0.1 % w/v) were used. After 14 days of incubation at 30 °C, growth was compared with that on the medium without inhibitor (Williams *et al*., 1983a).

Growth at 45^0C was determined in Petri- dishes using medium No. 2. Plates were incubated for 14 days at 45^0C and presence or absence of growth was noted.

Pectinase activity was determined according to the method of (Hankin *et al*., 1971) using complete medium for Pectinolytic activity was studied by spot-inoculation of the test organism on the plates. Hydrolysis zones were detected after 6 days by flooding the plates with a warmed solution of (1% w/v) hexadecyltrimethylammonium bromide and left for 1 hr. Excess reagent was decanted and hydrolysis zones appeared as clear areas around colonies.

Xanthine (0.4%) degradation was determined by inoculating organism as single streak onto plates containing Bennett's agar plates with glycerol instead of glucose. After 21 days incubation at 30°C, the appearance of clear zone around growth indicated a positive result (Jones, 1949).

Gelatin hydrolysis was studied. In this experimental procedure, nutrient gelatin deep tubes are used. The medium consists of nutrient broth supplemented with 12 percent gelatin. Following inoculation and incubation, the cultures are placed in a refrigerator at 4 °C for 30 minutes. Cultures that remain liquefied with acidified $MgCl_2$ solution produce gelatinase and demonstrate gelatin hydrolysis. Cultures that solidify on refrigeration lack gelatinase and give negative reactions (Frazier, 1926).

Resistance to the antibiotic rifampicine at a concentration of (50 μg ml^{-1}) and pencillin G were also determined on the basal medium no.1. Using the freez- dried filter paper discs method of Goodfellow and Orchard (1974), sterilized filter paper (5 mm diameter) (Whatman No. 1) were soaked in a filter sterilized antibiotics solution at the selected concentration for 2 min. These were then freez- dried for 90 min. and stored in sealed vials at 4^0C. Test strain inoculum (0.1 ml) was spread over the surface of plates of basal medium. Antibiotic discs were placed onto the plates using steril forcepes. Any definite inhibition zone was noted after 14- 30 h. at 30^0C; resistance to the antibiotic was recorded as positive

Antibiosis or the antagonistic activity of the studied actinomycetes was examined against the test organism (*Bacillus subtilus, E, coli, Candida albicans*, and *Aspergillus niger*) by the paper disc assay method as described by Pridham *et a*l. (1956). For assaying the antimicrobial spectrum of the isolated actinomycetes grown on starch-nitrate liquid medium in 50ml medium on a rotary shaker (350rpm) at 28^0C for 7 days, the content of each flasks was centrifuged at 6000 xg for 20min and the supernatant was sterilized through a 0.45nm bacterial filter. Three flasks were prepared for each strain. The resulting culture filtrates were tested for its antagonistic against the test organisms by using sterilized filter paper discs (5mm diamter), previously saturated with the culture filtrates of the actinomycetes strain. The discs were gently laid on the previously seeded plates with the test organisms and plates were incubated at 28^0C for 48, and 56 h for bacteria and fungi, respectively and the diameter of inhibition zones were measured.

Hydrogen sulphide production was studied. In tubes containing nutrient broth (oxide) supplemented with (g. L^{-1}): KNO3, agar 0.6 g and pH 7.0.

After inoculation and incubation for 14 days incubation at 30°C and inserting a strip of lead acetate paper into the mouth of each tube, browning or blackening of the paper indicated positive reaction (Kuster and Williams, 1964).

Nitrate reduction was determined by addition of 0.2 ml of Griess-Ilosvay reagent I (sulfonilic acid, 0.8 g in 100 mL 5 N acetic acid) followed by 0.2 ml of the reagent II (0.5 Ml dimethyl-α naphthylamine in 100 ml 5 N acetic acid) to culture in a sloppy medium composed of nutrient broth supplemented with KNO_3 (0.2 % w/v) and agar (0.6 %). After 7 and 14 days of incubation at 30 °C, the development of a red color over a period of 30 min indicated positive results (Williams *et al*., 1983a).

Acid fast stain and Gram stain examinations were carried out as described by (Williams *et al*., 1993; Benson, 1998).

To determine the mol. % G +C values of DNA, the isolation of DNA was carried out according to the method described by Colwell and Gigorova (1987), and the determination of the base composition by the absorbance ratios ($\varepsilon_{260}/\varepsilon_{280}$) at pH 3.0 was conducted according to method described by Fredericq *et al*. (1961).

The procedure of extraction of polar lipids is based on the methods of Bligh and Dyer (1959) modified by Card (1973). Briefly, 100 mg of dried cells were suspended in 2 ml of 0.3% saline. 20 ml of methanol were added and the mixture was boiled at 100 ^{o}C for 5 min in a screw-capped tube. After cooling, 10 ml chloroform and 6 ml of the saline were added to the tube. The mixture was shaken for three hours and the debris removed by filtration. 10 ml each of chloroform and saline are added to the mixture to separate the two layers. The chloroform layer is collected, concentrated to dryness, and re-dissolved in 100 µl of chloroform-methanol (2:1, v/v) and applied on Gas chromatography. This part of work was done in Al-Azhar University, The Regional Center for Mycology and Biotechnology, Cairo, Egypt.

2, 6 diamino pimelic acid was determined by thin layer chromatography according to Becker *et al*. (1964). DAP isomers appear as dark-green spots with R_f 0.29 (LL- isomer) and (meso- and DD-isomer). 3-Hydroxy-DAP appears lower than the meso- isomer (R_f = approximately 0.20). Spots of

other amino acids run faster than DAP (R_f = 0.37-0.80). The whole cell sugars of the lyophilized mycelia were determined according the method described by Staneck and Roberts (1974).

Optimization of culture conditions for the production of L-asparaginase enzyme

To optimize the cultural and nutritional requirements for L-asparaginase production by *S. halstedii*, several parameters were studied e.g. best medium, carbon, nitrogen, pH, incubation period and temperature. The used media were nutrient broth, glycerol asparagine, starch nitrate, Czapek′s Dox, malt extract yeast extract broth. The different carbon sources used were Glucose, sucrose, maltose, mannitol, lactose and sodium citrate in a concentration of 20 g/L. The different nitrogen sources used at equimolecular weights were potassium nitrate, urea, DL-phenyl alanine, DL-glutamic acid, L-aspartic acid and ammonium sulphate. The effect of incubation period on the production of L-asparaginase was considered. The organism grown in glycerol asparagine medium was followed by sample analysis every day for a period of 7 days.

To determine the optimal incubation temperature on the production of L-asparaginase the producer organism was grown on glycerol asparagine medium, and incubated at different temperatures (10, 20, 30, 35, 40, 45ºC) for five days.

The effect of pH on the production of L-asparaginase by the selected strain grown in buffered glycerol asparagine medium was determined. The following buffer systems were utilized: citrate – phosphate buffer pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. All culture media were inoculated and incubated at 30ºC and 200 rpm for 5 days.

Purification of L-asparaginase

Ammonium sulfate was added to the culture filtrate (culture grown on glycerol asparagines broth medium at 30ºC and 200 rpm) at 4°C to precipitate the L-asparaginase (Amena *et al*., 2010). The precipitate was collected by centrifugation at 10,000 rpm for 20 min, dissolved in 50 mM Tris-HCl buffer pH 8.6 and dialyzed against the same buffer. The concentrated enzyme solution was applied to the column of Sephadex G-200 (1.5×45 cm) (Pharmacia fine co., Uppsala, Sweden) that was pre-

equilibrated with 50 mM Tris-HCl buffer pH 8.6. The protein elution was done with the same buffer at a flow rate of 3 ml/30 min. The active fractions were collected, dialyzed and concentrated. The protein content was estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

SDS-Polyacrylamide gel electrophoresis

The molecular weight of the purified L-asparaginase sample was determined by using 10% SDS-PAGE according to the method of Laemmli (1970). SDS-PAGE was performed according to the method of Laemmli (8), with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250 and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5.

RESULTS

Only six isolates out of thirty actinomycetes producted L-asparaginase as indicated from the pink color on glycerol asparagine agar containing phenol red as indicator. Out of the six active strains grown in glycerol-asparagine broth, strain no. 5 showed the highest L-asparaginase activity about 8.643 U/ml followed by strain no. 3 (3.7 U/ml), strain no. 1(3.2 U/ml), strain no. 6 (2.1 U/ml), strain no. 2 (1.6 U/ml), and strain no. 4 (0.89 U/ml), respectively.

Optimization of culture conditions for production L-asparaginase by the selected strain

Effect of different media on L-asparaginase production

When the actinomycete strain no.5 was grown in nutrient broth, glycerol asparagine, starch nitrate, Czapek′ s Dox, malt extract yeast extract liquid media, the L-asparaginase production was maximum on glycerol asparagine liquid media. All other media showed little support for L-asparaginase production (Fig. 1).

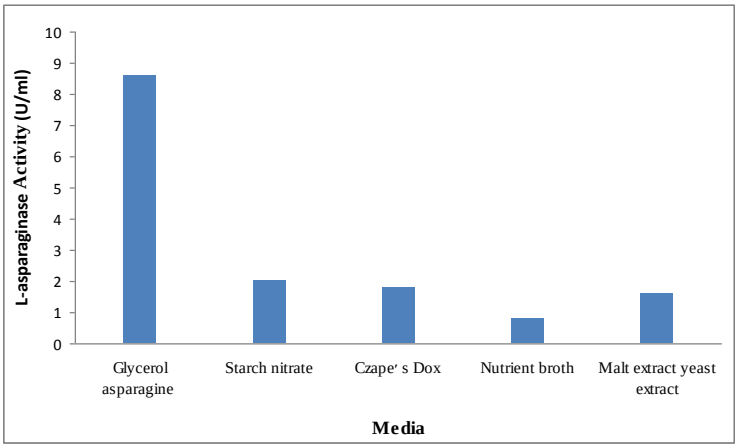

Fig. 1: Effect of different media on L-asparaginase production by the selected strain.

Effect of different carbon sources on L-asparaginase production

It is widely accepted in the literature that the nature of the carbon source of the medium greatly affect enzyme production. With strain no.5, maximum production of the enzyme was obtained on glycerol asparagine broth, followed by maltose, starch, mannitol, lactose, sodium citrate, glucose, and sucrose as shown in (Fig. 2).

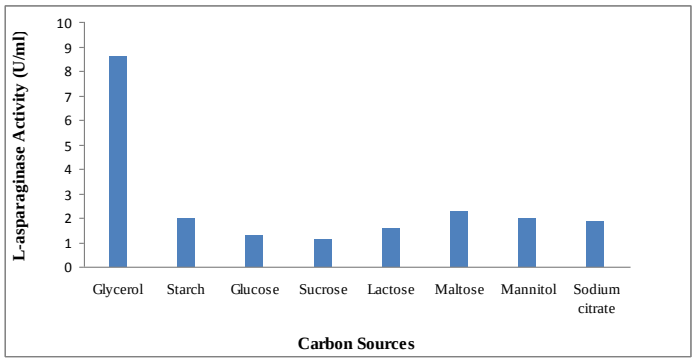

Fig. 2: Effect of different carbon sources on L-asparaginase production by the selected strain.

Effect of different nitrogen sources on L-asparaginase production

L-asparaginase production by the actinomycete strain no. 5 was greatly affected by the type of nitrogen source. L- asparagine produced the highest

value followed by potassium nitrate, ammmouim sulphate, DL- glutamic acid and urea, while DL.phenylalanine and L-aspartic acid completely inhibited L-asparaginase production (Fig 3).

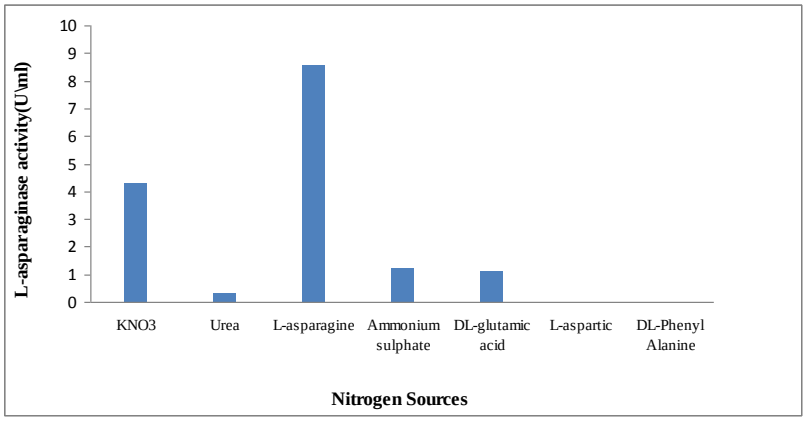

Fig. 3: Effect of different nitrogen sources on L-asparaginase production by the selected strain.

Effect of different incubation periods on L-asparaginase production

Results presented in Figure (4) illustrated that the selected strain reached its maximal value of L-asparaginase activity at the five day of incubation.

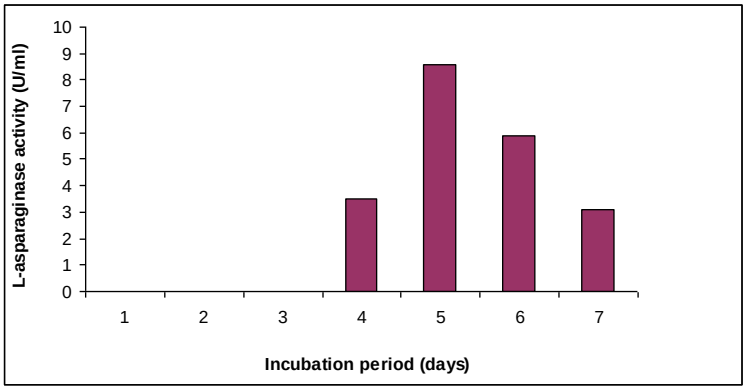

Fig.4: Effect of different incubation periods on L-asparaginase production by the selected strain.

Effect of different temperature on L-asparaginase production

The actinomycete strain was grown on glycerol asparagine medium and produced (8.6 U/ml) L-asparaginase at 30ºC (Fig. 5).

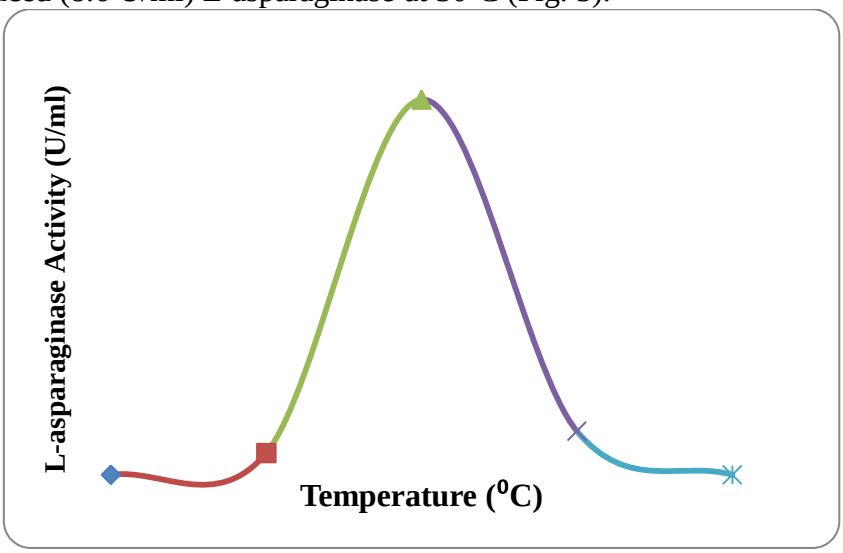

Fig. 5: Effect of different temperature on L-asparaginase production by the selected strain.

Effect of different pH values on L-asparaginase production

Data in (Fig. 6) shows that the L-asparaginase production by the actinomycete strain was greatly affected by the pH of the medium. Results also illustrate that pH 7.0 gave the highest value of L-asparaginase as compared to the other pH values.

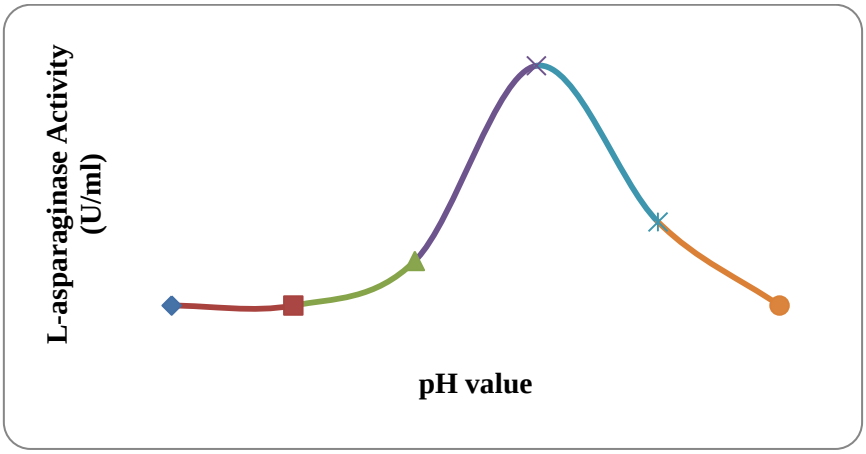

Fig. 6: Effect of different pH values on L-asparaginase production by the selected strain.

Optimization of ammonium sulfate precipitation

The results presented in Table (1) revealed that, as ammonium sulfate concentration increased, L-asparaginase activity and the specific activity in the precipitates increased. They reached their maximum value at 70% saturation, then, decreased by increasing ammonium sulfate concentration. Therefore, the 70% saturation was selected for the first step in the purification process of the enzyme.

Table 1: Effect of different concentrations of ammonium sulfate on L-asparaginase activity, protein content and specific activity

Concentration of $(NH_4)_2SO_4$%	Activity (U)	Protein content (mg)	Specific activity (U/mg protein)
40%	9.88 ±0.35	0.62 ±0.03	15.93 ±0.02
50%	19.10 ±0.26	0.76 ±0.03	25.13 ±0.35
60%	22.36 ±0.33	0.82 ±0.03	27.27 ±0.24
70%	40.97 ±1.08	1.87 ±0.04	44.05 ±0.16
80%	37.29 ±0.6	1.1 ±0.06	25.72 ±0.09
90%	33.47 ±1.01	0.93 ±0.02	17.9 ±0.4

Purification of L-asparaginase

L-asparaginase crude extract was precipitated by ammonium sulfate (70%) at this step the total protein decreased from 2.3 to 1.86 mg, and the specific activity increased from 37.5 to 228.7 IU/mg, at approximately 6 folds purity. Precipitated enzyme sample was further purified with Sephadex G-200 chromatography column an elution profile of L-asparaginase represented in figure (7). Fractions from 14 -18 showed L-asparaginase activity. Purification step using Sephadex G-200 chromatography column resulted in 2071.2 fold increase in specific activity of L-asparaginase produced by the selected strain and the purification fold was also increased to 55.2 fold (Table 2). All purification steps produced an increase in specific activity.

Table 2: Purification profile of L-asparaginase from the selected strain

Purification steps	Volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification folds	Yield (%)
Crude extract	100	86.4 ±1.41	2.3 ±0.28	37.56 ±0.28	1.0 ±0	100.0 ±0
Ammonium sulfate precipitation (70%)	10	425.4 ±1.64	1.86 ±0.04	228.71 ±1.04	6.02 ±0.11	4.92 ±1.9
Sephadex G-200 filtration	3	360.4 ±1.51	0.174 ±0.74	2071.26 ±0.35	55.14 ±1.49	4.17±1.75

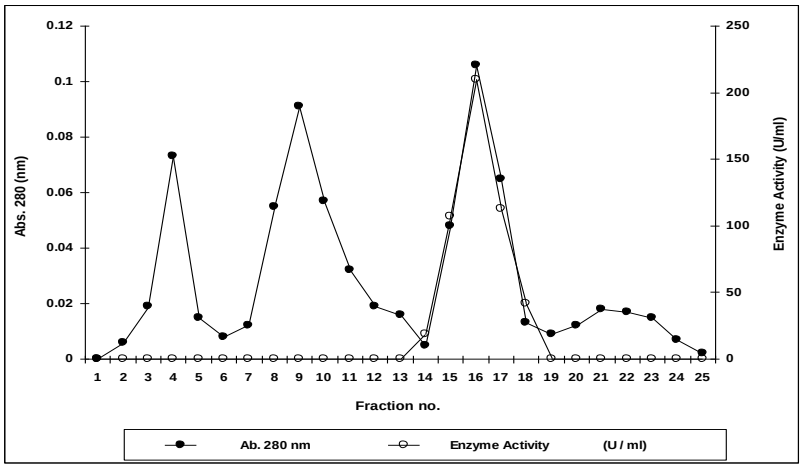

Fig. 7: Elution profile of L-asparaginase using Sephadex G-200 gel filtration chromatography. ● and ○ represent absorbance at 280 nm and L-asparaginase activity (units/ml) respectively.

Determination of protein molecular weight of the purified enzyme

Figure (8) shows the protein profile analysed by 10% SDS-PAGE. Analysis of the gel revealed that no detectable contamination as appeared as one distinct band with molecular weight of 100 KDa.

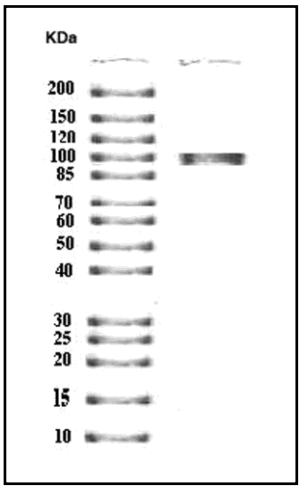

Fig. 8: SDS-Polyacrylamide gel electrophoresis of the purified purified L-asparaginase from *S. halstedii*.1.Protein marker of molecular molecular weight 200KDa (Pharmacia fine co., Uppsala, Sweden), 2. Purifed protein after final step of purification**.**

Identification of the best actinomycetes strain for production of L-asparaginase

From the taxonomical characteristics (Table 3, 4, 5 and 6); (Photos 1, and 2), and by following the Keys of Bergey's Mannual, 1989), the

isolated organism concluded to belong to the genus *Streptomyces* and closely resemble *S. halsetdii*.

Table (3): Cultural properties of the selected actinomycete strain on four different agar media.

Media used	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP_2)	Moderate	Grey	Yellowish brown	None
Inorganic salt starch agar (ISP_4)	Good	Grey	Light olive gray	None
Oat meal agar (ISP_3)	Good	Grey	Light grayish yellow	None
Glycerol-asparagine agar (ISP_5)	Moderate	Grey	Light yellowish brown	None

None : not detected under the experimental conditions

Table 4: physiological characteristics of the selected actinomycete strain

Test	Reaction	**Test**	Reaction
1-Melanin pigment.	-ve	12- Growth at 45°C	+ ve
2-Gelatin hydrolysis.	+ve	13-Hydrolysis of starch	+ ve
3-Starch hydrolysis.	+ve	14-Liquefaction of gelatin	+ ve
4-Nitrate reduction.	-ve	15-Growth with(%w/v)	
5-Pectin hydrolysis.	+ve	a-NaCl(7.0)	+ ve
6-Lipolysis.	+ve	b-Sodium azide (0.01)	+ ve
7-H_2S production.	+ve	c-Phenol (0.1)	+ ve
8-Xanthine degradation	+ve	16-Sensitivity to antibiotics:	- ve
9- Arbutin degradation	+ve	1-Rifampicin (50 Mg/ml)	- ve
10-Gram stain	+ve	2-Penicillin (10.I.U)	
11-Acid fast stain	-ve	17-Antibiosis against:	- ve
		a-*Bacillus subtilis*	- ve
		b-*Candida albicans*	- ve
		c-*Aspergillus niger*	- ve
		d-*E. coli*	

Table 5: Biochemical characteristics of the selected actinomycete strain cultured on starch-nitrate agar for 7 days

Tested material	Reaction
1.Diagnostic sugars.	-
2.Amino acids.	
a-Diaminopimelic acid	+ (L-form)
b-Glycine	+
c-Alanine	+
d-Glutamic acid	+
3. phospholipids.	
a-PE (phosphatidylethanolamine)	+
b-Pl (phosphatidylinositol)	-
c-PIM (phosphatidylinositolmonoside)	+
d-DPG(diphoshatidylgycerol)	-
e-PG (phosphatidylglycerol)	-
4. Fatty acids pattern.	
Dodecanoic acid (Lauric acid) (C12:0)	
Tetradecanoic acid (Myristic acid) (C14:0)	2.0 %
Pentadecenoic acid (C15:0)	19.2 %
Hexadecanoic acid (Palmitic acid) (C16:0)	2.8 %
Hexadecenoic acid (Palmitoleic acid) (C16:l)	40.1 %
Heptadecanoic acid (Margaric acid) (C17:0)	2.7 %
Octadecanoic acid (C18:0)	1.6 %
Octadecanoic acid (Stearic acid) (C18:1)	7.6 %
Octadecadienoic acid (Linoleic acid) (C18:2)	3.0 %
5. Mol.% G+C	0.0
	71.3

Table 6: Utilization of carbon and nitrogen sources of the selected actinomycete strain

Source	Utilization
D- glucose	++++
Mannose	+++
D- xylose	++++
Sucrose	-
L- arabinose	+++
Rhamonose	++
Raffinose	-
I-inositol	–
Mannitol	++
Starch	++++
Lactose	++++
Galactose	++++
Asparagine	++++
Na NO_3	++
L- valine	++
L-Cysteine	++
$(NH_4)H_2PO_2$	+
NH_4NO_3	+
Peptone	++++
NH_4Cl	++
KNO_3	++++
$NaNO_2$	+
$(NH_4)SO_4$	+

++++ ve : excellent growth
+++ ve : good growth.
++ ve : moderate growth.
+ ve : weak growth.
- ve : no growth.

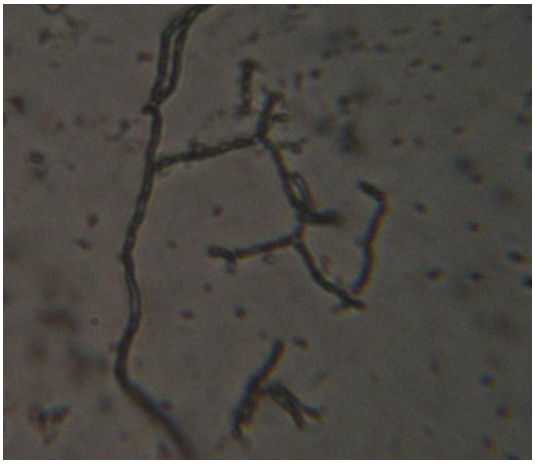

Photo 1: Mycelial growth and spore chains of the strain no.5 on oat-meal agar as examined by light microscopy (x 400).

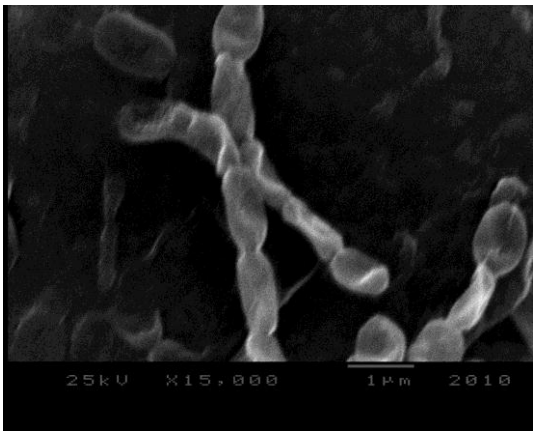

Photo (2): Spore view of the selected strain under scanning electron microscope from 14 days old culture on starch nitrate agar (× 15.000).

Photo (3): Strain no.5 grown at 50ºC.

DISCUSSION

In the present work, thirty actinomycetes strains were isolated from the Egyptian soil, only six of them gave pink color on glycerol asparagine agar containing phenol red as indicator to L-asparginase production. This is indication of the hydrolysis of L-asparagine into aspartic acid and ammonia by L-asparaginase synthesized by actinomycetes isolates. The release of ammonia changed the initial pH of growth media from acidic to basic as reported by De Jong., (1972) that L-asparginase production is accompanied by an increase in pH of the culture filtrate.

The selected isolate was subjected to further studies in order to be characterized and identified. It was form extensive branching substrate mycelia which are not able to fragment into bacillary or coccoid forms. Under light microscope strain no.5 showed well branched substrate mycelia and aerial mycelia bearing simple chains of spores with 20-30 spores. Spores were bacilli shaped. The aerial mass color of strain no. 5 was grey. It was found that the selected isolate can grow well on inorganic salt starch, and oat meal agar media, moderate growth in glycerol-asparagine and yeast extract malt extract media. Substrate mycelium was light grayish yellow on oat meal agar medium and light olive grey on inorganic salt starch medium. It was aerobic, Gram positive, non acid fast. No diffusible pigment was formed on all different media. The whole-cell hydrolysis contained LL-diamnopimelic acid, no diagnostic sugar in the whole cell. Fatty acid patterns showed hexadecanoic acid (Palmitic acid) (C16:0) and tetradecanoic acid (14:0), which were the predominant fatty acids. The mol % G+C of the DNA is 71.3. All of the previous characters confirmed that the selected isolate belong to the genus *Streptomyces*. Thereafter it was necessary to complete the identification to the level of species. Microscopic examination showed smooth spore surface and rectiflexibiles spore chain. This isolate was growing on sodium azide (0.01 % w⁄v), phenol (0.1 % w/v) and sodium chloride (3, 5, and 7 % w⁄v), and was susceptible to rifampicin (50 Mg/ml) and penicillin G (10 i.u). Decomposition of arbutine, , tween 80, xanthine and starch were positive. Gelatine hydrolysis and H_2S production was recorded. Nitrate reduction was positive. This isolate had not any

antimicrobial activity against *Bacillus subtilis, E. coli* and *Candida albicans*. Strain no.5 can utilize a large number of carbon source including glucose, mannose, starch, D-xylose, L-arabinose, rhamnose, mannitol, galactose and all added nitrogen sources. Following Bergey's manual of Systematic Bacteriology (1989*)* this isolate was considered to belong to *Streptomyces halsetdii* and closely resemble *S. halsetdii*.

All active strains were cultivated in glycerol-asparagine broth. Out of the six active strains no. 5 showed highest L-asparaginase activity (8.6 U/ml). The most active L-asparaginase producer isolate was identified as *S. halsetdii* according to morphological, physiological, and biochemical analysis, and by following the Keys of Bergey's Mannual, (1989). L-asparaginase by *Streptomyces tendae* isolated from laterite soil samples of Guntur region were investigated on glycerol-asparagine-salts (modified ISP-5) broth **(**Kavitha and Vijayalakshmi., 2010).

The selected strain was grown in nutrient broth, glycerol asparagine, starch nitrate, Czapek′ s Dox, malt extract yeast extract liquid media. The highest L-asparaginase production was achieved on glycerol asparagine liquid media. Similar results were obtained by Kavitha and Vijayalakshmi ., (2012) they showed that the maximal yields of L-asparaginase were recorded from 3-day-old culture grown in modified asparagine-glycerol salts broth with initial pH 7.0 at temperature 30°C by *Nocardia levis* MK-VL_113. In their study glycerol (2%) and yeast extract (1.5%) served as good carbon and nitrogen sources for L-asparaginase production, respectively

It is well known that the nature of the carbon source of the medium greatly affect enzyme production. In our study glycerol and L-asparagine were proved to be the best carbon and nitrogen sources for L-asparaginase production by *Streptomyces halstedii*. In contrast to our results yeast extract (2%) was served as good nitrogen source for the production of L-asparaginase by *Streptomyces albidoflavus* (Narayana *et al*., 2008).

Other investigation reported that L-aspartic inhibited growth and enzyme production, due to a feedback mechanism, and/or lowering the pH value. Both organisms *Streptomyces karnatakensis* and *Streptomyces venezuelae*

were stimulated to produce more enzyme with increasing concentrations of starch and L-asparagine, however, the optimum starch and L-asparagine concentration depended on the tolerance of the organism to low and high pH, respectively (Mostafa., 1979).

In our study the enzyme activity produced by the selected strain was optimum at pH 7.0 which was in accordance to Koshy *et al.* (1997) who found that maximum L-asparaginase activity of *Streptomyces plicatus* was obtained at pH 7.0 while Narayana *et al.* (2008) reported maximum L-asparaginase production of *S. albidoflavus* at pH 7.5.

In the present study the maximum L-asparaginase activity and growth of the selected strain were achieved at 30°C, extreme temperature did not favored L-asparaginase production by this strain. Other investigation reported that *S. collinus, Amycolatopsis kerataniphila* subsp. *kerataniphila* DSM 44409 produced high amount of L-asparaginase when grown at 28–30ºC and *S. albidoflavus* produced maximum amount of enzyme when cultured at 35ºC (Khamna *et al* .,2009 and Mostafa and Salama, 1979; Narayana *et al.*, 2008,).

Purification of the selected strain L- asparaginase crude extract was achieved using 70% ammonium sulfate saturation and sephadex G-200 gel filtration. The ammonium sulfate saturation (70%) reported by this study was comparatively higher than the result reported by Amena *et al*. (2010**)** which recorded 60% ammonium sulfate saturation and lower than those reported by Kumar and Selvam, (2011) by whom (80%) ammonium sulfate saturation was recorded.

In the present results, the specific activity of L-asparaginase increased from 37.56 to 2071.26 U/mg for the crude extract and the final preparation, respectively, which was approximately 55.2 folds purity. L-asparaginase from a marine *Streptomyces* sp. PDK2 has been purified 85-fold with 2.18% recovery in the final Sephadex G-200 purification step (Dhevagi and Poorani, 2006).

Our final purified enzyme was examined using SDS-PAGE, and turned to has an electrophoretic migration close to 100 kDa. However other researcher had reported variable molecular weights for the same enzyme

from other microbes such as those from *Streptomyces noursei* MTCC 10469 that showed molecular weight of 102 kDa (Dharmaraj., 2011), while *Streptomyces* sp. PDK2 *and Streptomyces* sp AFP47 were with molecular weight of 140 kDa (Ali *et al*., 2013 ; Dharmaraj and Dhevendaran, 2010), *S. albidoflavus*, with molecular weight of 112 kDa(Dharmaraj *et al*., 2009b), and *S. gulbargensis* with molecular weight of 85 kDa (Dharmaraj and Sumantha., 2009).

Generally, actinomycetes had been known to possess the ability to produce bioactivities such as pesticides, herbicides, antibiotics (Prapagdee *et al*., 2008; Boroujeni *et al*., 2012) and enzymes including asparaginase. Genus *Streptomyces* is a good source of L-asparaginase production when compared to bacteria and fungi (Sahu *et al*., 2007).

REFERENCES

Abdel F, Yasser R, Olama ZA. 2002. L-asparaginase production by *Pseudomonas aeruginosa* in solid-state culture: Evaluation and optimization of culture conditions using factorial designs. Process Biochemistr, 38:115-122.

Aly MA, Jastaniah S, Kuchari GM. 2013. L- asparaginase from *Streptomyces* sp. isolated from the rhizosphere of a palm tree, its separation, purification and antitumor activity. Int J Pharm Bio Sci., 4(2): (B) 12 – 21.

Amena S, Vishalakshi N, Prabhakar M, Dayanand A, Lingappa K. 2010. Production, purification and characterization of Lasparaginase from *Streptomyces gulbargensis*. Brazil J. Microbiol., 41: 173-178.

Basha SN, Rekha R, Komala1 M, Ruby S. 2009. Production of extracellular anti-leukaemic enzyme L-asparaginase from marine actinomycetes by solidstate and submerged fermentation: Purification and characterization. Tropical J. Pharmaceutical Res., 8: 353-360.

Becker B, Lechevalier MP, Gordon RE, Lechevalier HA. 1964. Rapid differentiation between *Nocardia* and *Streptomyces* by paper

chromatography of whole cell hydrolysates. Applied Microbiology., 12: 421-423.

Benson HJ 1998. In Microbiological Applications 7th edn. Laboratory McGrow-Hill. Manual in General microbiology, Seventh Edition, pp. 63.

Bergey's Manual of Systematic Bacteriology 1989. Edited by Williams, S. T., Sharp E. and Holt, J. G. VOL. 4: 2452-2492. Baltimore, Hongkong, Sydney. William and Wilkins.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37: 911- 917.

Boroujeni ME, Das A, Prashanthi K, Suryan S, Bhattacharya S. 2012. Enzymatic screening and random amplified polymorphic DNA fingerprinting of soil Streptomycetes isolated from Wayanad District in Kerala, India. Journal of Biological Sciences, 12: 43-50.

Card GL. 1973. Metabolism of phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin of *Bacillus stearothermophilus*. J. Bacteriol., 114 (3): 1125-1137.

Colwell RR, Grigorova R. 1987. Identification of microorganisms by rapid DNA-DNA hybridization (Tour ova, T. P, and Antonov, A S., eds). In Methods in Microbiology Academic press Limited. pp 335-336.

Cornea CP, Lupescu I, Vatafu I, Savoiu VG, Campeanu GH. 2000. Isolation and characterization of some L-asparaginase producing recombinant *Escherichia coli* strains. Roum. Biotechnol. Lett., 5: 471- 478.

Cowan ST. 1974. Cowan and Steel's Manual for the Identification of Medical Bacteria, 2nd edn. Cambridge University Press, London.

De Jong PJ. 1972. L-Asparaginase production by *Streptomyces griseus.* Appl. Microbiol., 23(6): 1163-1164.

Dharmaraj S, Ashokkumar B, Dhevendaran K. 2009b. Fermentative production of pigments from marine actinomycetes. Iran J. Microbiol. 1: 36-41.

Dharmaraj S, Sumantha A. 2009. Bioactive potential of *Streptomyces* isolated from marine sponges. World J. Microbiol Biotechnol., 25: 1971-1979.

Dharmaraj S. 2011. Study of L-asparaginase production by *Streptomyces noursei* MTCC 10469, isolated from marine sponge *Callyspongia diffusa*. Iran J Microbiol., 9(2): 102-198.

Dhevagi P, Poorani E. 2006. Isolation and characterization of L Asparaginase from marine actinomycetes. Ind. J. Biotech., 5: 514-520.

EL-Sayed M, El-Sayed STE, Wafaa, GS, Abeer NS, Shimaa SH. 2011. Purification, Characterization and Antitumor Activity of L-asparaginase from Chicken liver. J. of American Science, 7(1): 439-449.

Frazier N. 1926. A method for the production of changes in gelatin due to bacteria. J. Infect. Dis., 9: 302-309.

Fisher SH, Wray JV 2002. *Bacillus subtilis* 168 contains two differentially regulated genes encoding L-asparaginase. J. Bacteriol., 184 (3):2148-2154.

Fredericq E, Oth A, Fontaine F. 1961. The ultraviolet spectrum of deoxyribonucleic acids and their constituents. J. Mol. Biol., 3: 11-17.

Goodfellow M, Orchard VA. 1974. Antibiotic sensitivity of some nocardioform bacteria and its value as a criterion for taxonomy. J. General of Microbiolog, 83: 375-387.

Gupta N, Mishra S, Basak UC. 2007. Occurrence of *Streptomyces aurantiacus* in mangroves of Bhitarkanika. Malaysian J. Microbiol., 3: 7-1.

Hankin L, Zucker M, Sands DC. 1971. Improved solid medium for the detection and enumeration of pectolytic bacteria. Applied of Microbiology, 22: 205-209.

Jones KL. 1949. Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. J. Bacteriol, 57: 141-145.

Koshy A, Dhevendaran K, Georgekutty MI, Natarajan P. 1997. L-Asparaginase in *Streptomyces plicatus* isolated from the alimentary canal of the fish, Gerres filamentosus (Cuvier). J. Mar. Biotechnol., 5:181-185.

Kavitha A, Vijayalakshmi M. 2010. Optimization and purification of L-asparaginase produced by *Streptomyces tendae* TK-VL_333. Z. Naturforsch C., 65(7-8): 528-31.

Kavitha A, Vijayalakshmi M. 2012. A Study on L-Asparaginase of *Nocardia levis* MK-VL_113. Scientific World Journal. 2012:160434. doi: 10.1100/2012/160434

Keating MJ, Holmes R, Lerner SH. 1993. L-asparaginase and PEG asparaginase past, present and future. Leuk. Lympmoma., 10: 153-157.

Khamna S, Yokota A, Lumyong S. 2009. L-Asparaginase production by actinomycetes isolated from some Thai medicinal plant rhizosphere soils. International J. of Integrative.

Kumar B, Selvam MS, Singaravel K. 2012. Statistical Assessment of Medium Components by Factorial Design and Surface Methodology of L-Asparaginase Production by Isolated *Streptomyces radiopugnans* MS1 in Submerged Fermentation using Tapioca Effluent. Asian J Applied Sciences, 5(5): 252-265.

Küster E, Williams ST. 1964. Production of hydrogen sulphide by *streptomyces* and methode for its detection. Apllied Microbiology, 12: 46-52.

Kutzner HJ. 1976. Mothoder zur unterschung von streptomyceten und einigen anderen actinomyceten. Teilsammulung Darmstadt an Institute Fur Mikrobiologie der Technischen Hochscule, Darmstadt.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680-685.

Liu FS and Zajic JE. 1972. L-asparaginase synthesis by *Erwinia aroideae*. Appl. Microbiol., 23:667–668.

Lowery OH, Rosebrough JN, Farr AL, Randall RL. 1951. Protein Measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-273.

Manna S, Sinha A, Sadhukhan R, Chakrabaty SL. 1995. Purification, characterization and antitumor activity of L-

asparaginase isolated from *pseudomonas stutzeri* MB-405. Curr. Microbiol., 30(5): 291-298.

Mashburn L, Wriston J. 1963. Tumor inhibitory effect of L-asparaginase. Biochem. Biophy. Res. Commun., 12: 50.

Mohan R, Ramasamy, V. 2007. Isolation and characterization of marine antagonistic actinomycetes from west coast of India. Medicine and Biology., 15(1):13 – 19.

Mostafa SA. 1979. Production of L-asparaginase by *Streptomyces karnatakensis* and *Streptomyces venezuelae*. Zentralbl Bakteriol Naturwiss., 134(5): 429-36.

Mostafa SA, Salma MS. 1979. L-asparaginase producing *Streptomyces* from soil of Kuwait. Zentralbl Bakterol Natuurwiss., 134 (4): 325-334.

Narayana KJ, Kumar KG, Vijayalakshmi M. 2008. L-asparaginase production by *Streptomyces albidoflavus*. Ind.. J Microbiol., 48: 331-336.

Prakasham RS, Subba RC, Rao RS, Lakshmi GS, Sarma PN 2007. L-asparaginase production by isolated *Staphylococcus* sp.– 6A: design of experiment considering interaction effect for process parameter optimization. J. Appl. Microbiol., 102: 1382-1391

Prapagdee B, Kuekulvong C, Mongkolsuk S. 2008. Antifungal potential of extracellular metabolites produced by *Streptomyces hygroscopicus* against phytopathogenic fungi. International J. Biological Science, 4(5): 330-337.

Pridham T, Gottlieb D. 1948. The utilization of carbon compounds by some *Actinomycetales* as aid for species determination. J. Bacteriol., 56: 107-114.

Pridham, T.G., Lindenfelser, L. A., Shotwell, O. L., Stodola, F. H., Benedict, R.G., Florey, C., Jackson, R. W., Zaumeyer, W. J., Preston, W. H. & Mitchell R W. 1956. Antibiotics against plant disease I. Laboratory and greenhouse survey Phytopath., 46: 568-574.

Radcliffe CW, Kafkewitz D, Abuchowski A. 1979. Asparaginase production by human clinical isolates of *Vibrio succinogenes*. Appl. Environ. Microbiol., 38: 761-762.

Sahu MK, Poorani ES, Thangaradjou T, Kannan L. 2007. Studies on Lasparaginase enzyme of actinomycetes isolated from estuarine fishes. J. of Environmental Biology., 28 (2): 465-474.

Sarquis MI, Oliveira EM, Santos AS, Costa GL. 2004. Production of L-asparaginase by filamentous fungi. Mem Inst Oswaldo Cruz , 99: 489-492.

Shirling EB, Gottlieb D. 1966. Methods for characterization of *streptomyces* species. International Journal of Systematic Bacteriology., 16: 313-340.

Sierra G 1957. A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek J. Microbiol. Serol, 23: 15-22.

Siddalingeshwara KG, Lingappa K. 2010. Key fermentation factors for the synthesis of L-Asparaginase - an Anti tumour agent through Solid state fermentation methodology. An International J. of pharmaceutical sciences, 1(1): 55-60.

Stanek, JL and Roberts, GD. 1974. Simplified approach to identifcation of aerobic actinomycetes by thin layer chromatography. Applied Microbiology, 28: 226-231.

Swain AL, Jaskolski M, Housset D, Mohana Rao JK, Wlodawer A. 1993. Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. Proc. Natl. Acad. Sci. USA., 90: 1474-1478.

Szabo IM, Csortos CS. 1975. A study of the nutrient content of canopy through fall in an Oak forest *(Quercetum petraeae-cerris)* measured for one year. Acta. Bot. Acad. Sci. Hung., 21: 419-432.

Szabo I, Marton M, Buti M, Fernandez C. 1975. A diagnostic key for the identification of "species" of the *Streptomyces* and *Streptoverticillium* included in the international *Streptomyces* Project. Acta Bot. Acad. Sci. Hung., 21: 387-418.

Waksman SA. 1962. "The actinomycetes" Vol. III, Antibiotics of actinomycetes. The Williams and Wilkins Company, U.S.A.

Williams ST, Goodfellow M, Alderson G, Wellington EMH, Sneath PHA, Sackin MJ 1983a. Numerical classification of *Streptomyces* and related genera. J. Gen. Microbiol., 129: 1743-1813.

Williams ST, Goodfellow M, Wellington EMH, Vickers JG, Alderson G, Sneath PHA, Sackin MJ, Mortimer AM 1983b. A probability matrix for identification of some streptomycetes. Journal of General Microbiology., 129: 1815-1830.

William ST, Locci R, Beswick A, Kurtboke DI, Kuznetsov VD, Lemonnior F J, Long PF 1993. Detection and identification of novel actinomycetes. Res Microbiol, 144(8): 653-656.

Williams S, Goodfellow M, Alderson G. 1989. Genus Streptomyces, Waksman and Henrici. (1943), In: Bergey's Manual of systematic Bacteriology. Edited by: Williams, S. T., Sharp, M. E., and Holt, J. G. Baltimore Hongkong Sydney. William and Wilkins, 4. pp. 2452-2492.

انتاج انزيم الأسبارجينيز بواسطة الاستربتوميسس هاليستديي المعزول من التربة المصرية

صابحه محمود مبروك الصباغ

قسم النبات- كلية العلوم- جامعة المنوفية

يحفز انزيم الأسبارجينيز تميؤ الحمض الأميني الأسبارجين الى حمض الأسبارتيك والأمونيا ولذلك فهو يستخدم لعلاج لوكيميا الخلايا الليمفاوية الحادة وقد أجريت هذه الدراسة بهدف استخلاص و تنقية انزيم الأسبارجينيز من أفضل السلالات البكتيرية المنتجة التي تم الحصول عليها من التربة كمصدر رخيص ومتوافر و دراسة أفضل ظروف لانتاج الانزيم وكذلك تنقيتة٠ أوضحت الدراسة أن ستة سلالات من الأكتينوميستات من التربة لهم القدرة على افرازانزيم الأسبارجينيز بدرجات متفاوته وقد كانت العزلة (رقم٥) هى الاكثر انتاجا و بالتعرف عليها عن طريق الصفات المورفولوجية والبيوكيميائية تبين أنها تنتمى الى سلالة *S. halstedii* . و قد تم دراسة الظروف الغذائية و البيئية المختلفة المؤثرة على انتاج الانزيم فوجد أن الجليسرول هو أفضل مصدر للكربون في بيئة النمو لانتاج الانزيم وجد أيضا أن أفضل مصدر نيتروجيني لانتاج الانزيم من السلالة موضع الدراسة هو ل- أسبارجين. وبتنمية هذه السلالات على بيئات مختلفة تبين ان بيئة الجليسرول مدعمة بالأسبارجين هى الاكثر ملائمة لانتاج الانزيم. أوضحت الدراسة أن أقصى انتاج للانزيم في البيئة السائلة بعد فترة نمو خمسة أيام٠ كان نشاط الانزيمي ثابت عند درجات حرارة(٣٠°م). وتبين أيضا أن السلالة *S. halstedii* ثابتة حراريا اذ أنها استطاعت النمو عند درجة حرارة حتى ٥٠ درجة مئوية. لوحظ ايضا أن أقصى نشاط لانزيم البروتييز كان عند أس هيدروجيني ٧. وعند تنقية الانزيم الناتج بواسطة ٧٠ % من كبريتات الأمونيوم ثم بواسطة كروماتوجرافيا السائل HPLC وصلت درجة النشاط التخصصي الي ٢٠٧١ وحدة لكل ملجم بروتين كما وصلت درجة نقاوة الانزيم الى ٥٥ضعف على التوالي.