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

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**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.

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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 tamarii, 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-asparaginase deprivation. Several Streptomyces like S. aurantiacus, S. karnatakensis, S. venezuelae, S. longsporusflavus, and S. albidaflavus 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 30°C, colonies were checked for purity by repeated sub-culturing and the pure colonies maintained on slants of the same medium and stored at 4°C 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 30°C 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 30°C 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 °C. 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°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 Gottliebe, 1948). Different carbon sources (Glucose, L-arabinose, sucrose, xylose, inositol, mannose, lactose, rhamnose, 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 (KNO₃, L-asparagine, NaNO₃, L-cystein peptone, (NH₄)₂NO₃, and (NH₄) H₂PO₄ 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; CaCl₂, 2H₂O, 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 glucopyranoside) 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°C was determined in Petri-dishes using medium No. 2. Plates were incubated for 14 days at 45°C 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 MgCl₂ 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 penicillin G were also determined on the basal medium no.1. Using the freeze-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 freeze-dried for 90 min. and stored in sealed vials at 4°C. 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 forceps. Any definite inhibition zone was noted after 14-30 h. at 30°C; resistance to the antibiotic was recorded as positive.

Antibiosis or the antagonistic activity of the studied actinomycetes was examined against the test organism (Bacillus subtilis, E. coli, Candida albicans, and Aspergillus niger) by the paper disc assay method as described by Pridham et al. (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°C for 7 days, the content of each flask 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 diameter), 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°C 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 KNO₃ (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 (ε₂₆₀/ε₂₈₀) 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 °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 Rf 0.29 (LL- isomer) and (meso- and DD-isomer). 3-Hydroxy-DAP appears lower than the meso- isomer (Rf = approximately 0.20). Spots of
other amino acids run faster than DAP (Rf = 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, 45°C) 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 30°C 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 30°C 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 produced 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).
L-ASPARAGINASE PRODUCTION BY STREPTOMYCES HALSTEDII ISOLATED....

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).

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, ammonium 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.
**L-ASPARAGINASE PRODUCTION BY STREPTOMYCES HALSTEDII ISOLATED....**

**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 30°C (Fig. 5).

![Graph showing the effect of different temperatures on L-asparaginase activity](image)

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**

<table>
<thead>
<tr>
<th>Concentration of (NH₄)₂SO₄ (%)</th>
<th>Activity (U)</th>
<th>Protein content (mg)</th>
<th>Specific activity (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40%</td>
<td>9.88 ±0.35</td>
<td>0.62 ±0.03</td>
<td>15.93 ±0.02</td>
</tr>
<tr>
<td>50%</td>
<td>19.10 ±0.26</td>
<td>0.76 ±0.03</td>
<td>25.13 ±0.35</td>
</tr>
<tr>
<td>60%</td>
<td>22.36 ±0.33</td>
<td>0.82 ±0.03</td>
<td>27.27 ±0.24</td>
</tr>
<tr>
<td>70%</td>
<td>40.97 ±1.08</td>
<td>1.87 ±0.04</td>
<td>44.05 ±0.16</td>
</tr>
<tr>
<td>80%</td>
<td>37.29 ±0.6</td>
<td>1.1 ±0.06</td>
<td>25.72 ±0.09</td>
</tr>
<tr>
<td>90%</td>
<td>33.47 ±1.01</td>
<td>0.93 ±0.02</td>
<td>17.9 ±0.4</td>
</tr>
</tbody>
</table>
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

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

L-ASPARAGINASE PRODUCTION BY STREPTOMYCES HALSTEDII ISOLATED....
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. Purified 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 Manual, 1989), the
isolated organism concluded to belong to the genus *Streptomyces* and closely resemble *S. halstedii*.

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

<table>
<thead>
<tr>
<th>Media used</th>
<th>Growth</th>
<th>Aerial mycelium</th>
<th>Substrate mycelium</th>
<th>Soluble pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract-malt extract agar (ISP₂)</td>
<td>Moderate</td>
<td>Grey</td>
<td>Yellowish brown</td>
<td>None</td>
</tr>
<tr>
<td>Inorganic salt starch agar (ISP₃)</td>
<td>Good</td>
<td>Grey</td>
<td>Light olive gray</td>
<td>None</td>
</tr>
<tr>
<td>Oat meal agar (ISP₃)</td>
<td>Good</td>
<td>Grey</td>
<td>Light grayish yellow</td>
<td>None</td>
</tr>
<tr>
<td>Glycerol-asparagine agar (ISP₃)</td>
<td>Moderate</td>
<td>Grey</td>
<td>Light yellowish brown</td>
<td>None</td>
</tr>
</tbody>
</table>

None : not detected under the experimental conditions

**Table 4: physiological characteristics of the selected actinomycete strain**

<table>
<thead>
<tr>
<th>Test</th>
<th>Reaction</th>
<th>Test</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Melanin pigment</td>
<td>-ve</td>
<td>12- Growth at 45°C</td>
<td>+ ve</td>
</tr>
<tr>
<td>2-Gelatin hydrolysis.</td>
<td>+ve</td>
<td>13-Hydrolysis of starch</td>
<td>+ ve</td>
</tr>
<tr>
<td>3-Starch hydrolysis.</td>
<td>+ve</td>
<td>14-Liquefaction of gelatin</td>
<td>+ ve</td>
</tr>
<tr>
<td>4-Nitrate reduction.</td>
<td>-ve</td>
<td>15-Growth with(%w/v)</td>
<td></td>
</tr>
<tr>
<td>5-Pectin hydrolysis.</td>
<td>+ve</td>
<td>a-NaCl(7.0)</td>
<td>+ ve</td>
</tr>
<tr>
<td>6-Lipolysis.</td>
<td>+ve</td>
<td>b-Sodium azide (0.01)</td>
<td>+ ve</td>
</tr>
<tr>
<td>7-H₂S production.</td>
<td>+ve</td>
<td>c-Phenol (0.1)</td>
<td>+ ve</td>
</tr>
<tr>
<td>8-Xanthine degradation</td>
<td>+ve</td>
<td>16-Sensitivity to antibiotics:</td>
<td>- ve</td>
</tr>
<tr>
<td>9- Arbutin degradation</td>
<td>+ve</td>
<td>1-Rifampicin (50 Mg/ml)</td>
<td>- ve</td>
</tr>
<tr>
<td>10-Gram stain</td>
<td>+ve</td>
<td>2-Penicillin (10.I.U)</td>
<td></td>
</tr>
<tr>
<td>11-Acid fast stain</td>
<td>-ve</td>
<td>17-Antibiosis against:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>a-<em>Bacillus subtilis</em></td>
<td>- ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b-<em>Candida albicans</em></td>
<td>- ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c-<em>Aspergillus niger</em></td>
<td>- ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d-<em>E. coli</em></td>
<td></td>
</tr>
</tbody>
</table>
Table 5: Biochemical characteristics of the selected actinomycete strain cultured on starch-nitrate agar for 7 days

<table>
<thead>
<tr>
<th>Tested material</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diagnostic sugars.</td>
<td>-</td>
</tr>
<tr>
<td>2. Amino acids.</td>
<td></td>
</tr>
<tr>
<td>a-Diaminopimelic acid</td>
<td>+ (L-form)</td>
</tr>
<tr>
<td>b-Glycine</td>
<td>+</td>
</tr>
<tr>
<td>c-Alanine</td>
<td>+</td>
</tr>
<tr>
<td>d-GLutamic acid</td>
<td>+</td>
</tr>
<tr>
<td>3. phospholipids.</td>
<td></td>
</tr>
<tr>
<td>a-PE (phosphatidylethanolamine)</td>
<td>+</td>
</tr>
<tr>
<td>b-PI (phosphatidylinositol)</td>
<td>-</td>
</tr>
<tr>
<td>c-PIM (phosphatidylinositolmonoside)</td>
<td>+</td>
</tr>
<tr>
<td>d-DPG (diphosphatidyglycerol)</td>
<td>-</td>
</tr>
<tr>
<td>e-PG (phosphatidylglycerol)</td>
<td>-</td>
</tr>
<tr>
<td>4. Fatty acids pattern.</td>
<td></td>
</tr>
<tr>
<td>Dodecanoic acid (Lauric acid) (C12:0)</td>
<td></td>
</tr>
<tr>
<td>Tetradecanoic acid (Myristic acid) (C14:0)</td>
<td>2.0%</td>
</tr>
<tr>
<td>Pentadecanoic acid (C15:0)</td>
<td>19.2%</td>
</tr>
<tr>
<td>Hexadecanoic acid (Palmitic acid) (C16:0)</td>
<td>2.8%</td>
</tr>
<tr>
<td>Hexadecenoic acid (Palmitoleic acid) (C16:1)</td>
<td>40.1%</td>
</tr>
<tr>
<td>Heptadecanoic acid (Margaric acid) (C17:0)</td>
<td>2.7%</td>
</tr>
<tr>
<td>Octadeanoic acid (C18:0)</td>
<td>1.6%</td>
</tr>
<tr>
<td>Octadecanoic acid (Stearic acid) (C18:1)</td>
<td>7.6%</td>
</tr>
<tr>
<td>Octadecadienoic acid (Linoleic acid) (C18:2)</td>
<td>3.0%</td>
</tr>
<tr>
<td>5. Mol.% G+C</td>
<td></td>
</tr>
</tbody>
</table>

| G+C | 71.3 |
Table 6: Utilization of carbon and nitrogen sources of the selected actinomycete strain

<table>
<thead>
<tr>
<th>Source</th>
<th>Utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>D- glucose</td>
<td>++++</td>
</tr>
<tr>
<td>Mannose</td>
<td>+++</td>
</tr>
<tr>
<td>D- xylose</td>
<td>++++</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
</tr>
<tr>
<td>L- arabinose</td>
<td>+++</td>
</tr>
<tr>
<td>Rhamonose</td>
<td>++</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
</tr>
<tr>
<td>L- inositol</td>
<td>_</td>
</tr>
<tr>
<td>Mannitol</td>
<td>++</td>
</tr>
<tr>
<td>Starch</td>
<td>++++</td>
</tr>
<tr>
<td>Lactose</td>
<td>++++</td>
</tr>
<tr>
<td>Galactose</td>
<td>++++</td>
</tr>
<tr>
<td>Asparagine</td>
<td>++++</td>
</tr>
<tr>
<td>Na NO₃</td>
<td>++</td>
</tr>
<tr>
<td>L- valine</td>
<td>++</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>++</td>
</tr>
<tr>
<td>(NH₄)H₂PO₄</td>
<td>+</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>+</td>
</tr>
<tr>
<td>Peptone</td>
<td>++++</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>++</td>
</tr>
<tr>
<td>KNO₃</td>
<td>++++</td>
</tr>
<tr>
<td>NaNO₂</td>
<td>+</td>
</tr>
<tr>
<td>(NH₄)SO₄</td>
<td>+</td>
</tr>
</tbody>
</table>

+++] 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 50°C.
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-asparaginase 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-asparaginase 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 L- 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 rectiflexibles 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 H2S 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 halstedii* and closely resemble *S. halstedii*.

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. halstedii* according to morphological, physiological, and biochemical analysis, and by following the Keys of Bergey's Manual, (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 Koshiy 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 28–30°C and S. albidoflavus produced maximum amount of enzyme when cultured at 35°C (Khamma 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 Amen 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).

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L-ASPARAGINASE PRODUCTION BY STREPTOMYCES HALSTEDII ISOLATED....


إنتاج الأنزيم الأسبارجينيس بواسطة الاستربتوهيسس هاليستديي المعزول من النوبة المصرية

صاحب محعم مبروك الصباغ

قسم النباتات، كلية العلوم، جامعة المنوفية

يعتبر إنزيم الأسبارجينيس تميز الحمض الأميني الأسبارجيلا إلى حمض الأسبارتيك والأمونيا ولذلك فهو يستخدم لعلاج لوكيميا الخلايا الليفية الحادة وقد أجربت هذه الدراسة بهدف استخلاص وتنقية إنزيم الأسبارجينيس من أفضل السلالات البكتيرية المنتجة التي تم الحصول عليها من النوبة كمصدر رخيص ومتوفر، ودراسة أفضل ظروف لإنتاج الإنزيم وكذلك تكنولوجيا، أوضحت الدراسة أن ستة سلالات من الأكسيوميستات من النوبة لهم القدرة على إنتاج الإنزيم الأسبارجينيس بدرجات متفاوتة وقد كانت النزيلة (رقم 5) هي الأكثر انتاجاً وتعتبر عليها عن طريق الصفات المورفولوجية والبيوكيميائية تبين أنها تنتمي إلى سلالة S. halstedii. وقد تم دراسة الظروف الغذائية والبيئية المختلفة المؤثرة على إنتاج الإنزيم فوجد أن الجلبرول هو أفضل مصدر للكربون في بيئة النمو لإنتاج الإنزيم وجد أيضاً أن أفضل مصدر نتروجيني لإنتاج الإنزيم من السلاسل موضع الدراسة هو لأسبارجن، وتتمح هذه السلالات على بيئات مختلفة تبين أن بيئة الجلبرول مدعمة بالأسبارجينيس هي الأكثر ملاءمة لإنتاج الإنزيم. أوضحت الدراسة أن أقصى إنتاج للإنزيم في البيئة السائلة بعد فترة نمو خمسة أيام، كان نشاط الإنزيم تثبت عند درجات حرارة (30م). وتبين أن ثابتة حرارية إذا أنها استطاعت النمو عند درجة حرارة حتى 50 درجة مئوية. لوحظ أيضاً أن أقصى نشاط للإنزيم البروتيني كان عند أس هيدروجيني 7. وزمن تقيية الإنزيم الناتج بواسطة HPLC 70% من كبريتات الأمونيوم ثم بواسطة كروماتوجرافيا السائلة ووصلت درجة النشاط التخصصي إلى 2071 وحدة لكل ملجم بروتين كما وصلت درجة نقاوة الإنزيم إلى 55 ضعف على التوالي.