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PURIFICATION AND CHARACTERIZATION OF STENOTROPHOMONAS SP.FZ L-ASPARAGINASE UNDER SOLID STATE FERMENTATION

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ABSTRACT: *L-asparaginase has emerged as one of the most important clinically used enzymes as it exhibits chemotherapeutic potential in treatment of acute lymphoblastic leukimia and lymphosacroma. A novel bacterium L-asparaginase producer was isolated from Lebanese soil and was identified as Stenotrophomonas sp.FZ using 16srRNA. The enzyme was produced under solid state fermentation using the wheat bran as carbon and nitrogen source The enzyme was partially purified by acetone precipitation with 73.32 % yield and a purification factor of 6.32 fold. Further purification includes gel filtration chromatoghraphy on Sephadex G-75 and C-25 ion exchange chromatogrraphy with final purification factor of 329.061 fold and 54.312 yield%. The total protein was reduced by 99.83% and the specific activity was increased to be 1.6124IU/mgX1000. The maximum enzyme activity was recorded at pH 7 and 35°C with a linear relationship concerning the increase in enzyme concentration. The effect of substrate concentration showed a progressive increase in the enzyme activity in a concentration dependent manner till it reaches a plateau where saturation was reached. The kinetics parameters(Km and Vmax) of Stenotrophomonassp L-asparaginase production were 96.71 mg/ml and 3333.33 umol/ml/min respectively. The theapeutic potential of this enzyme is well established.*

KEYWORDS: Purification, characterization, therapeutic potential, *Stenotrophomonas*sp, L-asparaginase.

INTRODUCTION

Asparaginase is an enzyme which converts L-asparagine to L-aspartic acid and ammonia (Hill et al., 1967). The therapeutic potential of this enzyme is well established, as it has remarkably induced remission in most patients suffering from acute lymphoblastic leukemia. It has also been used for treatment of cancer cells since they are not capable of producing asparaginase (Aguayo et al., 1999). With the development of its new functions, a great demand for L-asparaginase is expected in the coming years. The biochemical and enzyme kinetic properties vary with the microbial source (Sarquis et al.,2004).L-asparaginase production using microbial systems has attracted considerable owning to cost-effective and eco-friendly nature. L-asparaginase is produced throughout the world by submerged fermentation. This technique has many disadvantages, such as low concentration production and consequent handling, reduction and disposal of large volumes of water during the downstream processing. Therefore the submerged fermentation technique is cost intensive, highly problematic and poorly understood unit operation. Solid-State fermentation is a very effective technique as the yield of the product is many time higher when compared to that in submerged fermentation and it also offers many other advantages (Losane & Ghildyal, 1985). A wide range of microorganisms such as filamentous fungi, yeast and bacteria have proved to be beneficial sources of this enzyme (Sarquis & oliviera, 2004) . However, *Erwina* asparaginase had a shorter life than *E.coli*; are

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serologically different, but have similar beneficial effects (Swain et al., 1993). The aim of the present study is to isolate a bacterial strain with a potent L-asparaginase activity, and to study the enzyme production by purifying and characterising it.

MATERIALS AND METHODS

Chemicals

L-asparagine , Nessler reagent Sephadex C25 and Sephadex G-75-50 were obtained from sigma, Aldrich USA. Other used chemicals are of analytical grade were obtained from recognized chemical suppliers.

Isolation of Microorganisms

L-asparaginase producers were isolated from soil samples collected from different locations in Bekka Valley at 30 cm depth from soil surface under aseptic conditions.Serial dulitions of the soil sample (1/10, $1/10^2$, $1/10^3$, $1/10^4$ and $1/10^5$) were prepared in sterile distilled water and plated onModified ISP-5 Medium (Rapid Screening Method) cotained:(L-asparagine 1 g, Dipotassium phosphate 1 g, trace salts solution 1 ml, lactose 20 g, phenol red 1 ml, agar 20 g, pH 7.0) was sterilized (autoclaved at 120°C for 20 min), inoculated with 1 ml of collected soil samples and poured to the Petri plates. All the plates were incubated at room temperature including the control plates, which were without carbon source (lactose)media plates and then incubated at 35◦C ± 2 for 2 days.The isolated organisms were maintained on Modified ISP-5 Medium.

Preparation of seed culture

Seed cultures were prepared in 250 ml Erlenmeyer flasks containing 50 ml nutrient broth medium with 3 ml of bacterial suspension (prepared from 18 hrs old cultures) and then incubated at 30˚ C ± 2 on a rotary shaker (180 rpm) till reaches $O.D_{600} \leq 1$.these were used as standard inocula (3ml/flask) unless otherwise stated.

Enzyme productionUnder solid state fermentation technique (SSF) (Ramesh & lonsane,1987)

The modified fermentation medium was dispensed in 250ml Erlenmeyer flasks each containing 5 g waste materials with 5 ml 0.1 M phosphate buffer (pH7.4). The fermentation medium were sterilized by autoclaving for 20 min. an inoculated with 3 ml as standard inocula unless otherwise stated and incubated at 35˚ C ± 2 for 24 hrs under shaken conditions using incubator shaker (180 rpm).

Preparation of cell-free extractUnder solid state fermentation media

At the end of the fermentation period, 90 ml of 0.01 M phosphate buffer pH 7 was added to the SSF medium. The mixture was washed for 15 min using reciprocal shaker (150 rotation per minute), then subjected to cooling centrifuge for 20 min at 8000 rpm. The obtained cell free extract (filtrate) was used as crude enzyme.

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Estimation of Enzyme activity

L-asparaginase catalyzes the hydrolysis of L-aspargine to L-aspartate and ammonia. L-asparaginase activity was measured by direct nesslarization methods as explained by Wriston (1970). This method is based on the determination of ammonia liberated from L-asparagine by L-asparaginase enzyme in the nessler reaction. The reaction was started by adding 0.2 ml of supernatant into 1.8 ml 0.01 M L-asparagine prepared in 0.05M Tris HCl buffer (pH 8.6) and incubated at 30˚ C ± 2 for 30 min. The reaction was stopped by the addition of 0.5 ml of 1.5 M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined calorimetrically by adding 0.2 ml Nessler's reagent into tubes containing 0.1 ml supernatant and 3.75 ml distilled water and incubated at room temperature for 10 min and absorbance of the supernatant was read using UV –visible spectrophotometer at wavelength of 450 nm. The A_{480} values were measured against the blank which was not inoculated with a bacterial suspension.

One L-asparaginase unit of activity was defined as the amount of enzyme that liberates 1 µmol ammonia/ min at 30˚ C ± 2. The ammonia concentration produced in the reaction was determined on the bases of a standard curve.

Estimation of protein content

The protein content was monitored at 280 nm

Purification of *Stenotrophomonas* spFZ L-asparaginase

The cell free extract obtained from a 24 hrs old culture of *Stenotrophomonas* sp grown under optimal experimental conditions was subjected to the following purification steps according to the methods of Distasio et al. (1976) with some modifications. All the purification steps were carried out at 4◦C.

Partial purification using acetone fractionation

Fractionation was done using acetone which was added to the crude enzyme extract at 35 % saturation. The mixture was stirred for 5 min and then centrifuged for 10 min at 8000 rpm. Using cooling centrifuge. The pellet was discarded, and then acetone was added until 60 % saturation. Then the mixture was stirred for 5 min and centrifuge for 10 min at 8000 rpm. The supernatant was discarded since it contains no enzyme activity and the precipitate was collected. The precipitated protein was dissolved in 10 ml of 0.01m phosphate buffer (pH8.5) and extensively dialyzed against the same buffer in cellophane bag in a cold room, until the buffer outside the bag gave no precipitate with 1 % barium chloride solution, indicating that the enzyme solution inside the bag became free of sulphate. This was achieved by changing the phosphate buffer outside the bag several times. The protein content and L-asparaginase activity was measured in the dialyzed enzyme solution after concentration against solid sucrose.

Gel Filtration chromatography on Sephadex G-75-50

Preparation of Sephadex G-75-50

Ten grams of sephadex G-75-50 were allowed to swell in 400 ml of 0.01M phosphate buffer pH8.5 for 1 hr, and the mixture was filtered. The gel was washed with 1 liter 0.2 M HCl

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followed by elution of the phosphate buffer and stored for 3-4 days at 4◦C to ensure complete swelling. The excess eluent was removed by decantation and the swollen gel degassed under reduced pressure.

Column packing and equilibration

The gel suspension adjusted to form thick slurry was poured carefully into the column either down side of the chromatographic column (20 cm long X 2.7 cm diameter) or down a glass rod to avoid bubble formation. The column outlet was opened to allow the gel to settle into the column. The column was equilibrated with 2.5 M phosphate buffer pH8.5 at flow rate of 1.5 ml/3 min. once a column has been prepared, a layer of eluent should always be maintained above the column surface.

Sample application

The eluent above the gel surface was allowed to drain away, and then the dialyzed acetone fraction was carefully layered onto the top of the bed. The column outlet was then opened and the sample was allowed to drain into the bed surface; the column wall was washed into the bed with small amount of eluent. The proteins were eluted with 0.5 M potassium phosphate buffer, pH8.5. the flow rate was 1.5 ml/5 min. Samples of effluent fractions were subjected to protein estimation at 280 nm and L-asparaginage activity. The most active fractions were collected and stored at -30◦C or below.

Concentration of L-asparaginase

The pooled enzyme fractions were placed in cellophane/ dialysis bag and covered with cold sucrose. Water and low molecular weight substances were dialyzed against sucrose. The concentrated extract was dialyzed against 0.01 M phosphate buffer pH 8 at 4 ◦C.

Cation exchange chromatography on CM Sephadex C25

Preparation of Sephadex C25

Ten grams Cm Sephadex C25 were allowed to swell in excess 0.25 M potassium phosphate buffer, pH 8. The swollen gel was allowed to settle and the supernatant was removed and replaced with fresh buffer several times. Air bubbles were avoided by degassing the gel before packing.

Column packing and Equilibration

The gel suspension adjusted to form thick slurry was poured inside the column, which has been equilibrated with 0.25 M phosphate buffer pH 8. Once a column has been prepared a layer of eluent should always be maintained above the column surface.

Sample application

The eluent above the gel surface was allowed to drain away, and then the dialyzed gel filtration fraction was carefully layered onto the top of the bed in a chromatography column (20 cm long* 2.7 cm diameter). The protein samples were eluted with 0.1 M sodium borate buffer, pH 7 containing NaCl with linear gradient of 0.1-0.5 M at a flow rate of 1.5 ml/ 2 min. the collected fraction were monitored for protein content at 280 nm and for L-asparaginase activity.

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Characterization of the purified enzyme

Effect of enzyme concentration

Aliquots of 50, 100, 150, 200 and 250 µLenzyme were added to 0.2 ml of the substrate solution (0.2 %) . The enzyme was assayed as described before.

Effect of substrate concentration

0.2 ml of different concentrations (10, 15, 20, 25,30, 35, 40 , 45 and 50 mM) of the substrate solution (L-asparagine) was mixed with 0.2 ml of appropriate diluted enzyme and then the reaction mixture was assayed for the enzyme activity. The initial velocity of L-asparaginase was measured as a function of substrate concentration and plotted as double reciprocals with Lineweaver-Burk analysis.

Effect of incubation temperature

The enzyme assay was carried out using 0.2 ml substrate solution and 0.2 ml diluted enzyme. The reaction mixture was incubated at 20 min at different temperatures 10, 20, 25, 30, 35, 40, 50, 60, 70 and 80◦C. Then the enzyme activity was measured and activity was plotted against the different temperature values.

Effect of pH

The enzyme assay was carried out at 35◦C for 20 min using buffer with different pH values 3, 4, 5, 6, 7, 8, 9,10 and 11. The substrate 0.2 % L-asparaginase was dissolved in each of these buffers with the same concentrations and then the enzyme assay was carried. The activity was plotted against the different pH values.

The anti- proliferative effect of L- asparaginase under test

Aliquots of 10^4 cells were incubated with1 Umol/L Asp. in 100 ul liquid medium(RPMI-1640) in costar 96-well flat-bottomed plates in duplicates. Fifty micro liters of protective antigen in medium were added to each column to yield concentrations ranging from 100 to ----umol/L. then followed by 48-hour incubation at 37^o C/5% CO_2, 50 ul of XTT(2,3-Bis(2-Methoxy-4-Nitro-5-sulfophenyl)2H-Tetrazolium-5-Carboxanilide). cell proliferation reagent (Sigma Aldrich) was added to each well and the plates incubated for another 4 hours. Absorbance was then read at 450nm using micro plate reader (Thermo fisher Scientific, Waltham, MA) Hang et al.(2004).

RESULTS AND DISCUSSION

Bacterial Identification

The most promising bacterial isolate was identified by sequencing PCR amplified 16S rNDA. The obtained sequences were submitted to FASTA3 data base in order to find homologies with other 16S rDNA. The tested strain was affiliated to the genus *Stenotrophomonas* with 86% similarity to*Stenotrophomonas maltophilia* R551-3 . The phylogeny of the tested strain and closely related species was analyzed using multi sequence alignment program and the results are presented in phylogenetic tree The strain showed 86% Identity to *Stenotrophomonas*

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maltophilia R551-3 (ac: NR074875.1) consequently the bacterial strain was named as *Stenotrophomonas*sp FZ.(El-Meched et al.,2014).

Purification of Stenotrophomonas sp ZF L-asparaginase production

Partial purification of L-asparaginase production using acetone fractionation

Partial purification was performed using 60% acetone as precipitant. It was found that the supernatant possessed a low activity and most of the activity was preserved in the precipitate. The specific activity of L-asparaginase was increased than that of the crude preparation (0.0310 U/ mg proteins) while the total protein was decreased from 26710 to 3016.14 mg with a purification factors of 6.3261 folds (Table 1). However *S. Gulbargensis*partial purification was performed by using 60% ammonium sulfate as precipitant. The specific activity of L-asparaginase by *S. Gulbargensis* was increasedthan that of the crude precipitaion while the total protein was decreased from 128 to 36 mg (Amena et al., 2010) . Moreover *Vibrio* sp using 50% ammonium sulfate as precipitant shown 18 U/ mg of specific activity while the total protein was decreased from 2.7 to 1.7 mg (John, 1976).El-Bessoumy found that the specific activity of Pseudomonas aeruginosa was 93.7 (U/mg) with 80% of ammonium sulfate.

Gel filtration chromatography on Sephadex G-75

The acetone fraction was dialyzed overnight at 4° C against 0.01 M phosphate buffer pH8.5. The dialyzed fraction was centrifuged at 4° C and the filtrate was applied to a pre-equilibrated sephadex G-75 gel filtration column. Fig 1 showed the complete profile of the gel filtration step. It was found that the acetone fraction contained different protein molecules. The protein obtained from the column was separated into 2 components, the first components was recorded from the fractions 2 to 25, the second proteins was recovered from by fractions 29 to 40. The L-asparaginase activity was fractionated into a single peak (10-22). The highest enzyme activity was obtained from fraction 15. The L-asparaginase activity was enriched after Sephadex G-75 and the specific activity became 0.3405 IU/ mg with a purification factor of 69.489 fold and protein reduced from 3016.14 to 241.25 mg .

El-Bessoumy proved that *Pseudomonas Aeruginosa*the highest enzyme activity was obtained from fraction 39. The L-asparaginase activity was enriched after Sephadex G-100 and the specific activity became 497IU/ mg with a purification factor of 27.7 fold and protein reduced from 674 to 91 mg . Moreover, The L-asparaginase activity of *Streptomyces gulbargensis*was enriched after Sephacryl S-200 and the specific activity became 672.2 IU/ mg with a purification factor of 26.6 fold and protein reduced from 36 to 1.8 mg (Vishalakshi el al ., 2010).

Cation- Exchange chromatography on CM Sephadex 25

The collected supernatant was applied to pre-equilibrated cation exchange chromatography CM sephadex C25 with 0.25 M phosphate buffer pH 8.0. Figure 2shows the complete profile of the cation exchange step. The specific activity of L-asparaginase was further increased and became 1.6124 IU/ mg protein with a purification factor equal to 329.061 fold increases. On the other hand, the protein decreased through this step of purification from 26710 to 44.30 mg (Table 1). A low affinity of the enzyme to CM sephadex C-25 was noticed since it was eluted from the column with lower concentration of NaCl gradient. The active fractions were pooled

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out, concentrated and dialyzed against (0.01 M phosphate buffer pH 8.0), centrifuged and kept at -20°C for further analyses.

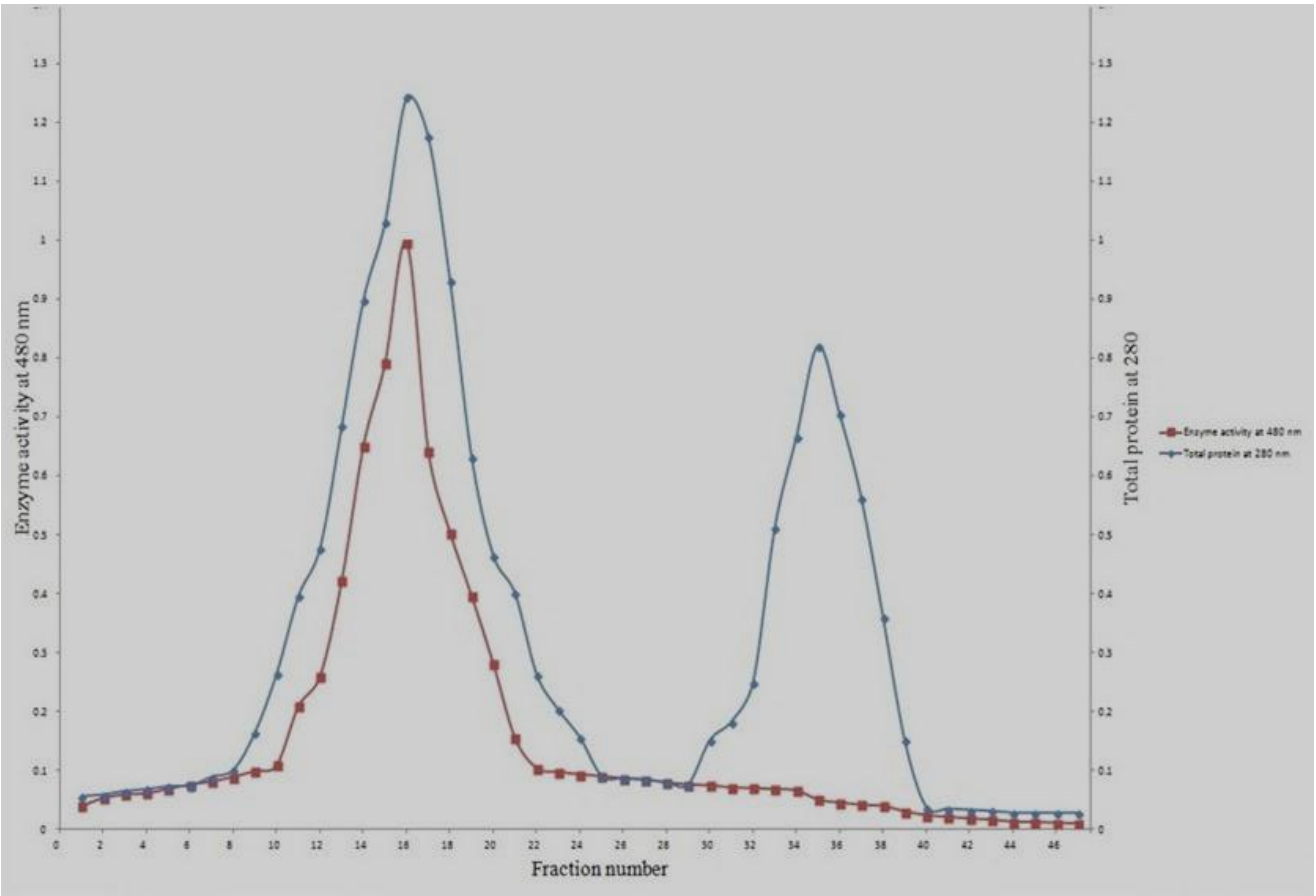

Fig 1 : Sephadex G-75-25 Chromatography of *Stenotrophomonas* sp FZ L-asparaginase. Total protein was monitored at 280 nm and the fraction were assayed for L-asparaginase activit

Table 1 : Purification Profile of L-asparaginase from *Stenotrophomonas* sp FZ

Purification steps	Total volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)X 1000	Purification fold	Yield %
Crude extract	1000	131.51	26710	0.0049	1.0000	100
Acetone precipitation	460	96.40	3016.14	0.0310	6.3261	73.325
Sephadex G-75-50 gel filtration column	235	82.16	241.25	0.3405	69.489	62.471

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CM Sephadex C25 Cation exchange column	135	71.43	44.30	1.6124	329.061	54.312

- Specific activity = Total activity /Total protein
- Purification factor = Specific activity of purified enxyme/Specificactivity of the crude enzyme
- Yield= Total activity of the purified enzyme /total activity of the crude enzyme

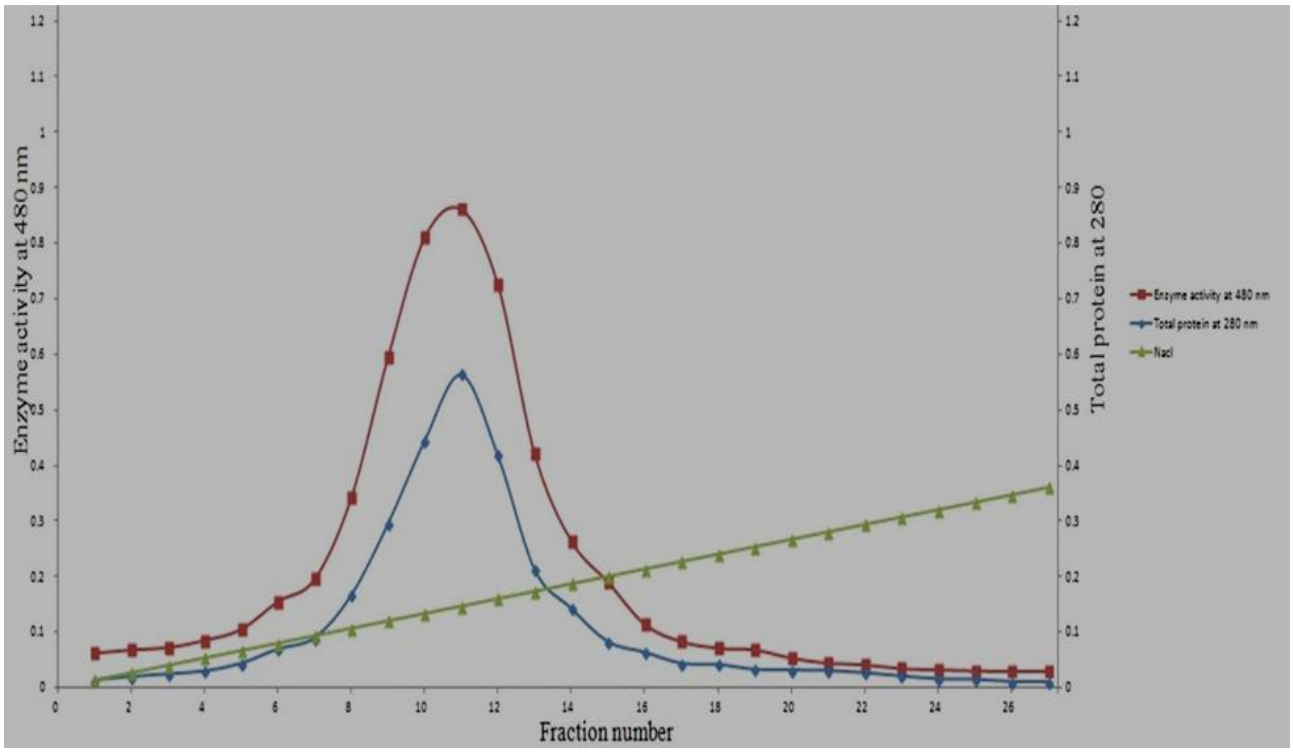

Fig 2: CM Sephadex C-25 chromatography of *Stenotrophomonas* sp FZ L-asparaginase. The first gel filtration C-75-50 collected fraction were applied to CM sephadex C-25. Protein was eluted with a linear gradient of 0.1-0.5M NaCl.Total protein was monitored at 280 nm

The L-asparaginase activity of *Streptomyces gulbargensis*was enriched after Sephadex C-50 and the specific activity became 2053 IU/mg with a purification factors of 82.12 fold and the total protein reduced from 128 to 0.5 mg (Vishalakshi el al ., 2010). El-Bessoumy noted that the L-asparaginase activity of *Pseudomonas Aeruginosa*after Sephadex C-50 was 1900 IU/mg with a purification factor 106 fold and the total protein was reduced to 17 mg.

Enzyme characterization

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In the present study of the work it was aimed to characterize the pure enzyme obtained after pooling and concentration of the active fractions recovered from gel filtration on Sephadex G-75 and C-25.

Effect of enzyme concentration

The effect of enzyme concentration on the activity of *Stenotrophomonas* sp FZ L-asparaginase production was investigated. Figure 3 showed that the enzyme activity increased proportionally with the increase in enzyme concentration.

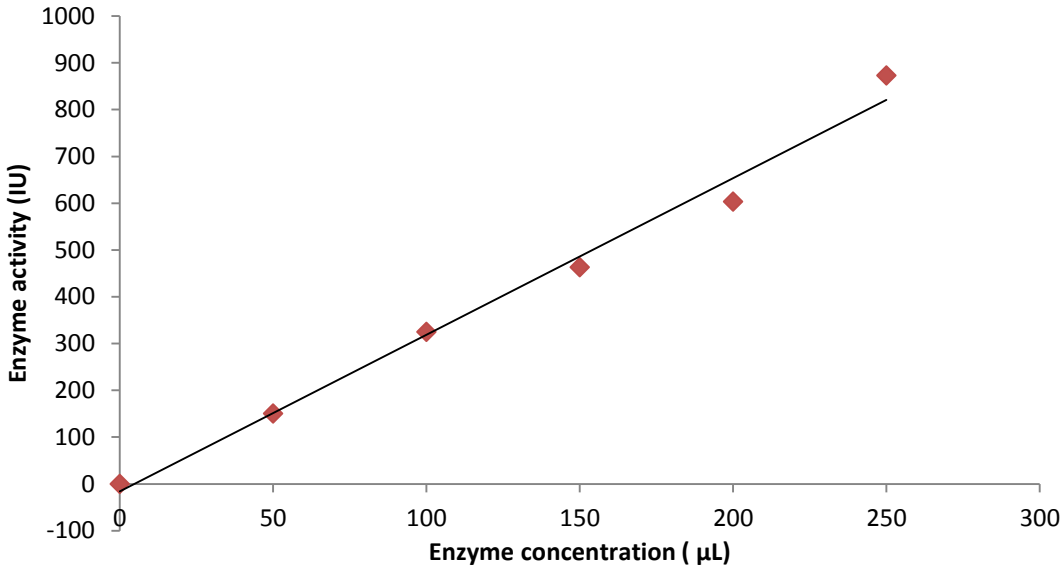

Fig 3: The effect of enzyme concentration on *Stenotrophomonas* sp ZF L-asparaginase activity.

Effect of substrate concentration

In order to establish the optimum substrate concentration, the relationship between the substrate concentration and the activity of purified L-asparaginase was studied (fig.4a). The results showed a gradual increase in the purified enzyme activity with the increase in substrate concentration 265 UI/ ml with 10 mg/ml substrate to 875 UI/ml with 35 mg/ml substrate. However, further increase in substrate concentration lead to slight decrease in enzyme activity to 822 UI/ml with 50 mg/ml substrate.The initial velocity of L-asparaginase was measured as a function of substrate concentration and plotted as double reciprocal in accordance with the lineweaver-Burk analysis (fig 4.b). the plot resulted in a Km value of 96.71 mg/ml and V max 3333.33 umol/ml/min . *C.glutamicum* pure enzyme showed apparent Km of 2.8 mg/ml for L-asparaginase (Resnik & Magasanik, 1976).

Effect of incubation temperature on L-asparaginase activity

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The effect of incubation temperature on purified L-asparaginase activity is shown in Fig.5. Low enzyme activity was attained at temperature ranging between 10-15°C. However, a sharp increase in enzyme activity took place with an increase in incubation temperature till 35°C recording the highest enzyme activity of 871 UI/ ml. at higher temperature the L-asparaginase activity declined sharply till almost 261 IU/ ml at 80°C.due to enzyme denaturation.

Effect of pH

L-asparaginase acitivity rate was studied as a function of pH in range between 3-11 (Fig.6). Data revealed that the enzyme activity increased gradually till pH 7 with maximum activity 869 IU. At higher pH's there was a decrease in enzyme activity.

The anti- proliferative effect of L- asparaginase under test

In a trial to test the effect of the enzyme under test on the treatment of lymphoblastic leukemia,fig.7 revealed that the most effective pure fractions of the enzyme: fractions from 9 to 15 selected after the second step of purification on CM-sephadex C25 chromatography were applied to cancer cells (Leukemia) it was revealed that the fractions numbers 10 and 11 (A2 and A3 respectively) showed positive activity and high selectivity to cancer cells by 0.2 nm each at a concentration of 105 and 100 ug/ul respectively , this indicated that the enzyme showed ant proliferative effect against the cancer cells and it kills the cancerous cells. These results illustrated high selectivity of L-asparaginase which indicate high specific toxicity of L-asparaginase to the cancer cells. Moreover the fractions numbers 9, 12, 13, 14 and 15 (A1, A3, A4, A5, A6 and A7 respectively) show a negative activity and low selectivity on cancer cells with 0.3, 0.6, 0.4, 0.7 and 0.6 nm respectively at a concentration of 100 ug/ul each.

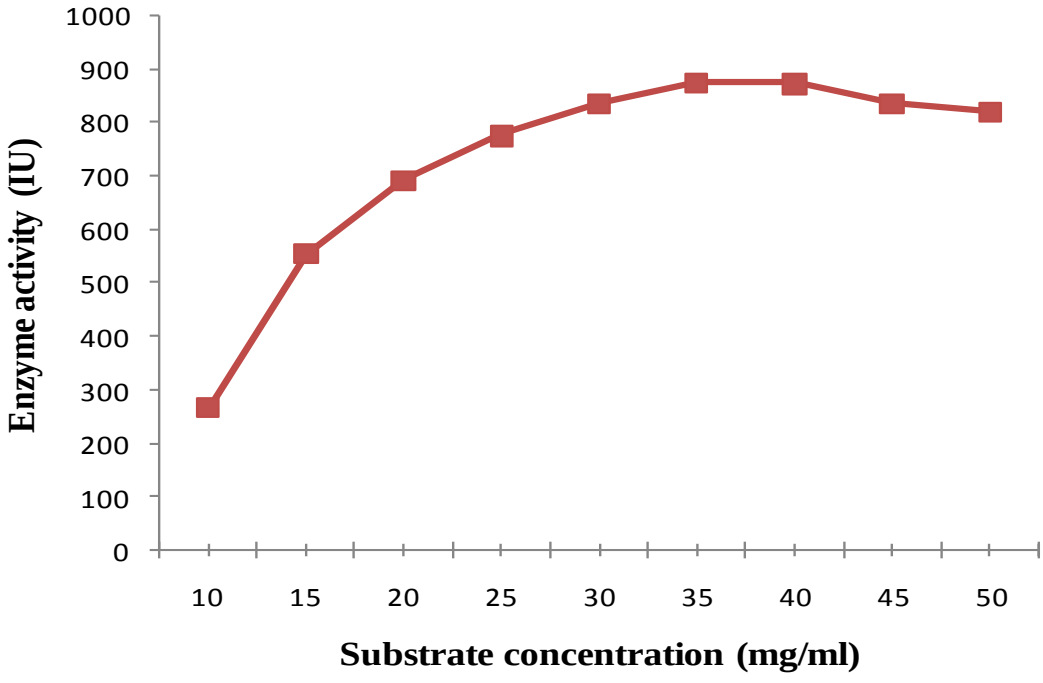

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Fig 4.a: The effect of substrate concentration on *Stenotrophomonas* sp FZ L-asparaginase activity.

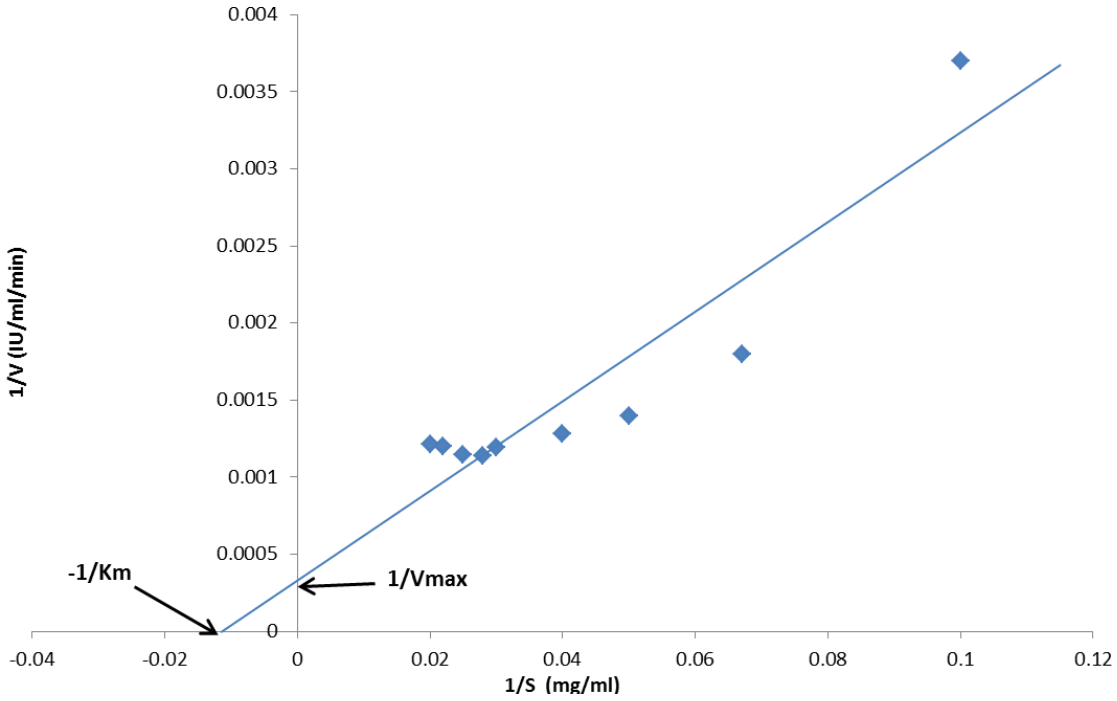

Fig 4.b: Lineweaver-Burk plot of *Stenotrophomonas* sp FZ L-asparaginase activity.

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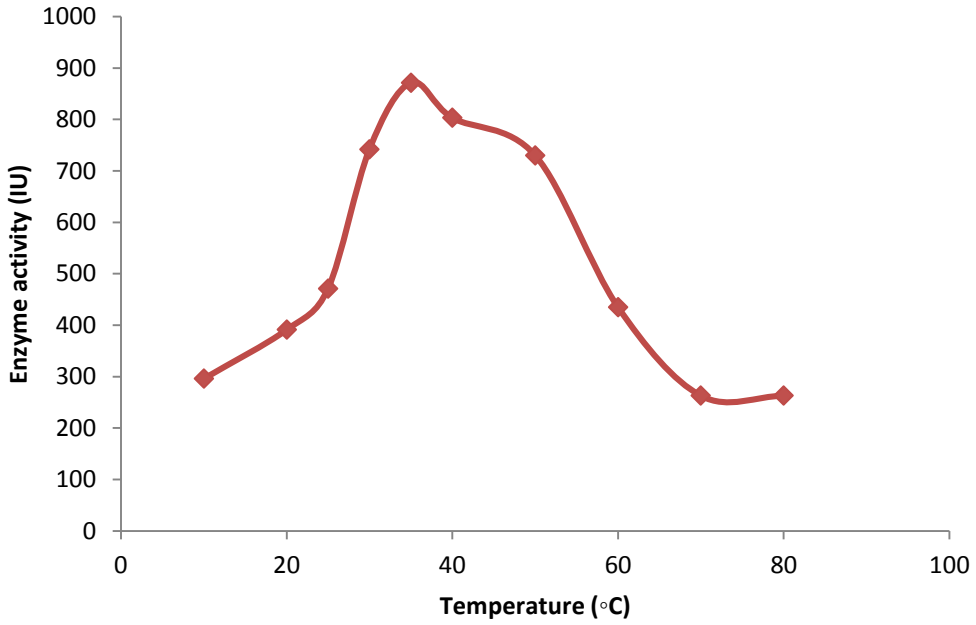

Fig 5: The effect of Incubation temperature on *Stenotrophomonas* sp FZ L-asparaginase activity.

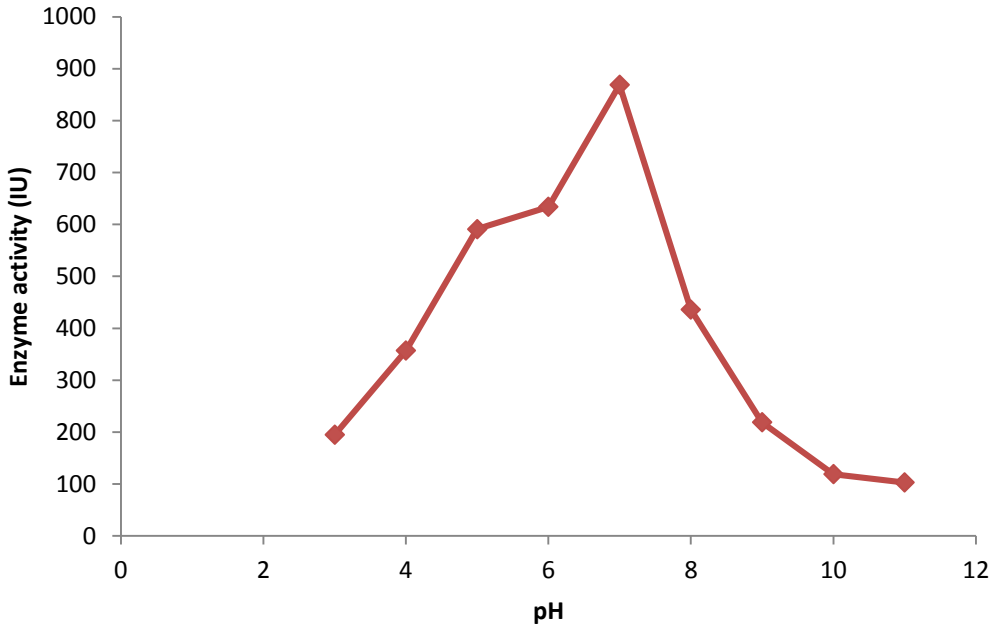

Fig 6 : The effect of pH on *Stenotrophomonas* sp FZ L-asparaginase activity.

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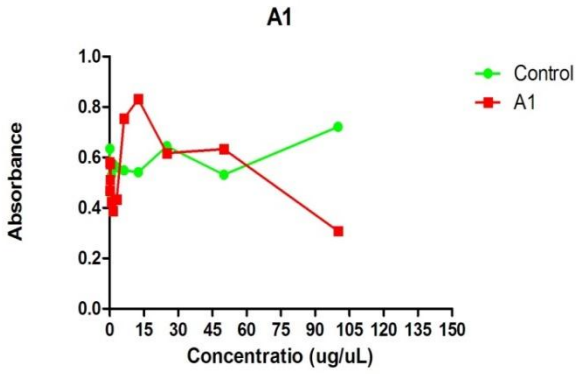
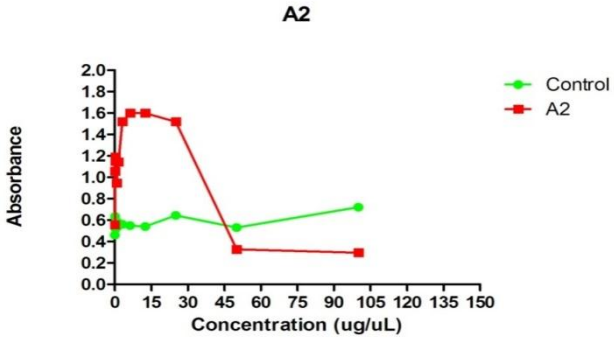
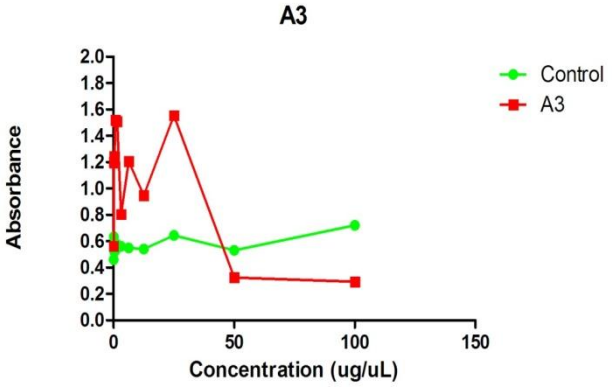


Control: P value= 0.0263 (significant)

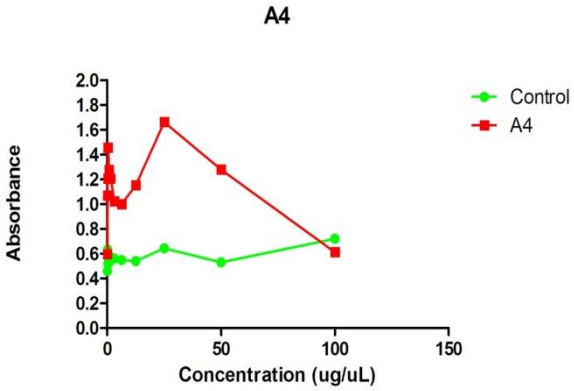
A1: P value = 0.418 (not significant)



A2: P value= 0.0403 (significant)



A3: P value= 0.0242 (significant)



A4: P value= 0.3319 (not significant)

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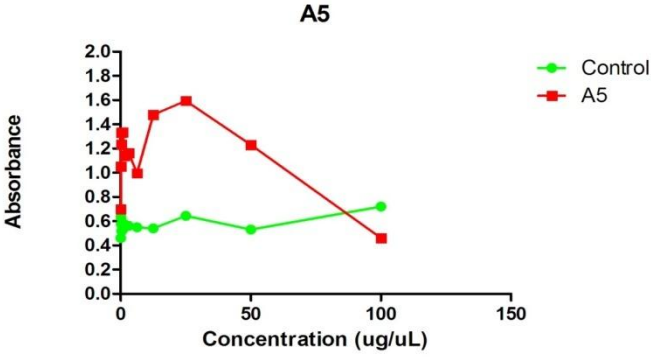
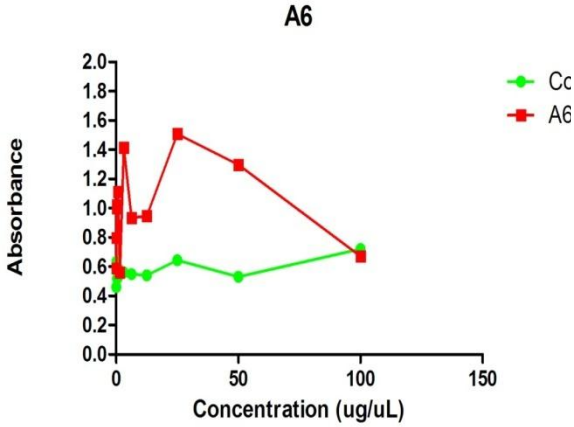
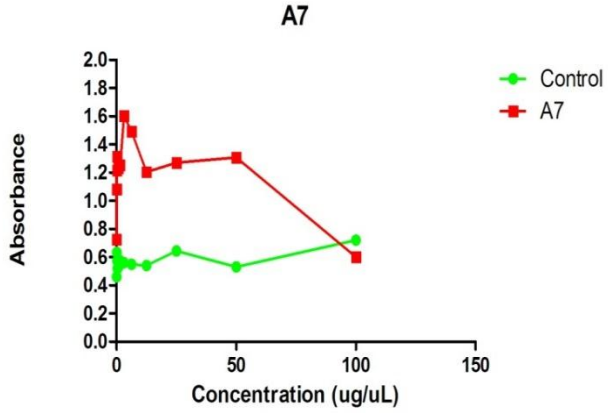


A5: P value = 0.1259 (not significant)



A6: P value= 0.9197 (not significant)



A7: P value= 0.0901 (not significant)

Fig. 7 Colorimetric reduction of AMLby *Stenotrophomonas* spFZ L-asparaginase

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