Original Article

Purification and Characterization of L-asparaginase II Production from pseudomonas aeruginosa

Esraa Raheem Sheltagh[1] , Entesar Hussain Ali [2]

[1,2]Department of Applied Science, University of Technology, Baghdad, Iraq

Email esraaraheem83@gmail.com

Abstract

From twenty clinical isolates of pseudomonas aeruginosa were collected from Al-Yarmouk Teaching Hospital in Baghdad, Iraq, were screened for findings of L-asparaginase-producing bacterial isolate with high yield, and were cultivated in a Modified M9 medium, which contained (L-asparagine 1%) was used to produce the L–asparaginase. After 2 days of incubation at 37 ° C, pH 7.5, with 250 rpm shaking, the highest L-asparaginase productivity was obtained. The extracellular enzyme was then extracted using a cooling centrifugation process to obtain the filtrate that represents the crude enzyme. Enzyme activity was then measured at every stage of the purification process, beginning with dialysis by sucrose and followed by ion-exchange chromatography with The specific activity of 4.2unit/mg protein, with a fold of purification of 5.2, and next by Sephadex G-200 column increases enzyme purity by 10.5-fold with specific activity 8.37 unit/mg protein after using gel filtration, making gel filtration the optimum stage in the purification process due to the high specific activity of the enzyme. Purified Lasparaginase had a molecular weight of 120 kDa by SDS- PAGE. The maximum activity of the enzyme was detected at 37°C and pH 8.0 after 30 min. Besides, the enzyme had stability at 7.0-8.0 with thermal stability at 30-40 °C. L-asparaginase is activated in the presence of metal ions such as K+, and Ca 2+, and chelating agent EDTA and strongly inhibited in the presence of, Hg 2+.

Keywords: L-asparaginase, Enzyme activity, optimization, purification, molecular weight

1. Introduction

L- asparaginase (E.C.3.5.l.l) amidohydrolase catalyzes asparagine hydrolysis to yield L-aspartate and ammonia that occurs in the presence of water. The action of asparaginase plays a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes. 1

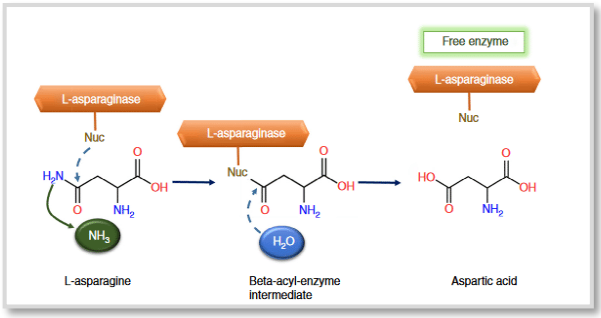

Figure 1: The general mechanism of L-asparaginase catalyzed reactions 2

L-ASNase is commonly used as an anticancer medication in the treatment of acute lymphoblastic leukemia (ALL). It also aids in the treatment of other hematological and non-hematological conditions such as pancreatic cancer, lymphosarcoma, Hodgkin's illness, and acute myeloid leukemia. L-ASNase played a crucial role in the hydrolysis of exogenous L-asparagine that exists in the bloodstream thus causing depletion of asparagine which is an essential amino acid for cancerous cell proliferation finally causing apoptotic death. 3.

Address for correspondence: Esraa Raheem Sheltagh, Department of Applied Science, University of Technology, Baghdad, Iraq, Email esraaraheem83@gmail.com

Received date: 15 August 2022 Accepted: 17 September, 2022

Published: 10 October, 2022

How to cite this article: Sheltagh R E, Hussain Ali E, Purification and Characterization of L-asparaginase II Production from pseudomonas aeruginosa, J Pharm Negative Results 2022;13(4):35-45

Access this article online

Quick Response Code:

Website: www.pnrjournal.com

DOI: 10.47750/pnr.2022.13.04.005

Besides its clinical applications, L-asparaginase is utilized in the food industry to drastically cut down on acrylamide, a powerful carcinogen produced in foods via the Millard process, 4. Furthermore, employs L-asparaginase in the design of a biosensor to measure asparagine levels in the food industry or leukemia. 5.

L-ASNase is produced by a wide range of species, including bacteria, fungus, algae, and plants with different physiochemical characters and kinetic parameters, and it is currently manufactured from two main sources of bacteria: recombinant Escherichia coli and Erwinia chrysanthemi. They are employed in therapeutics but raise concerns due to several adverse effects, including thrombosis and pancreatitis, L-ASNase production consequently required alternative sources. 6 The present study has been undertaken to isolate, purify, and characterize L-asparaginase from p. aeruginosa culture filtrate.

2. Material And Methods

2.1 Identification of bacterial isolate with the highest l-asparaginase productivity

2.2.1 Semi-quantitative screening

A screening method based on the premise of incorporating the pH indicator phenol red. In acidic conditions, it is yellow, and in alkaline conditions, it turns pink.

This method depends on the appearance of a pink zone around l-asparaginase producing colonies on a modified M9 agar medium containing (KH2PO4 0.4g,

L-asparagine 1 g, MgSO4.7H2O 0.2g, CaCl.2H2O 0.2 g, Glucose 0.6 g, Agar 2g, and Few drops of Phenol red as an indicator) in 100 ml of de-ionized water.

Fifty ml of modified M9 broth medium was taken in conical flasks, inoculated 1 ml of 24-h-aged bacterial culture suspension, and incubated for 24 h at 37 °C. After that, 30 min centrifugation for the culture broth was carried out at 4 °C and 5000 rpm. For each isolate, cell-free supernatant (100 µl) was poured into a well (8 mm diameter) in a modified M9 agar plate. At 4 °C for 12 h, the inoculated plate was left to diffuse the filtrate into the medium and then incubated for 24 h at 37 °C. Diameters measuring the pink area with a yellow background around the hole (mm) stating L-asparaginase activity. The cultures with high enzyme production were selected for further studies. 7

2.2.2. Quantitative assay of L –asparaginase

Pseudomonas aeruginosa isolates were grown in modified M9 broth (with L-asparagine 1%) (50 ml) and incubated at 37°C with shaking (250 rpm) for 48 h. Centrifugation of the bacterial culture was carried out at 8000 rpm for 20 min. Since the enzyme is extracellular, the filtrate is used to estimate L- asparaginase activity. 8

2.3. Eestimation of L-asparaginase enzyme activity in culture filtrates

Nesslerization determined the culture filtrate enzyme activity. From the cell-free supernatant (crude enzyme), (0.25) ml was combined with (1) ml 0.05M Potassium phosphate buffer (pH 8.0), and then (1) ml 0.2 M L-asparagine substrate was added. After incubating at 37°C for 30 minutes, the reaction was terminated with (0.5) ml of tri-chloro-acetic acid (TCA) 1.5 M. and then centrifuged at 8000 rpm for 10 minutes. The concentration of ammonia was determined for each sample by mixing (3) ml of distilled water with (0.5) ml of supernatant and (0.5)ml of Nessler reagent. The mixture was shaken well and incubated at 37ºC for 30 minutes. After 30 min of incubation at 37 °C, the absorbance was measured at 450 nm. A blank tube contained (3.5) ml of distilled water with (0.5) ml of Nessler reagent. Under the assay conditions, one unit of enzyme activity is described as the amount of enzyme required to generate 1 µmol of ammonia per minute per min. A typical ammonium-sulfate graph at a series of concentrations (1.5- 11.8 µg / ml) was used for evaluating the liberated ammonia. 9.

$$\text{Enzyme activity (U/ml)} = \frac{\textbf{concentration of librated Ammonia}}{\boldsymbol{T \times V}}$$

C: Concentration of Ammonia

T: Incubation time (min.)

V: Volume of the enzyme

2.4. Estimation of Protein Concentration in the Sample

The protein concentration has been calculated according to the Classics Lowry, Rosebrough 9 method. The total protein content of the L-asparaginase was estimated by a spectrophotometer at 600 nm. Bovine serum albumin was used as the standard protein. 10

2.5. optimization of P. aeruginosa L-asparaginase production 11

2.5.1 Determination of Optimum Temperature for L-asparaginase Production

To determine the optimal temperature for enzyme activity, bacterial cells were cultivated in the production medium and incubated at (25, 30, 35, 40, and 42°C) for 48 hours. Afterward, the activity of the enzyme produced by bacteria was evaluated after extraction to find the optimum temperature for production.

2.5.2 Determination of Optimum pH for L-asparaginase Production

To determine the optimum pH The production medium with different pH (5, 5.5, 6, 6.5, 7, 7.5, and 8) were inoculated with bacterial cells and incubated at 37°C for 48 hours. After that, the activity of the enzyme produced by bacteria was measured after extraction to determine the optimum pH for production.

2.7 Purification of L-Asparaginase Crude enzyme preparation

P.aeruginosa was grown in L-asparagine broth under optimal conditions. Afterward, the cells were precipitated in a cooling centrifuge at 8000 rpm for 20 min to extract the enzyme. Since the enzyme is extracellular, the filtrate is separated from the sediment for the purification process 12

2.7.1 Dialysis by Sucrose

The supernatant was taken and placed in a dialysis bag with sucrose to concentrate from (75) ml to (20) ml. after that the enzyme was taken to measure the enzyme activity and protein concentration respectively. The concentrated enzyme was used as a crude asparaginase enzyme for further purification steps. 13

2.7.2 Asparaginase purification using ion exchange Column by Diethylaminoethyl Cellulose

According to Whitaker and Bernard in 1972, ion exchange chromatography was used for L-asparaginase purification. After equilibration with phosphate buffer (pH 8, 0.05 M) and packing in a 3×13 cm column, 10 ml of the enzyme was slowly put on the walls of the ion exchanger using a dropper. The separated fraction was then collected in the appropriate tubes at a rate of 5 ml/tube, and a phosphate buffer (pH 8, 0.05 M) was used for the washing process Following that, the elution stage was carried out by using a linear gradient NaCl concentrations (0.15-1 M NaCl); finally, the absorbance of each fraction was measured at 280 nm wavelength for. To identify the fractions with L-asparaginase activity. 14

2.7.3. Purification by Gel Filtration Column Using Sephadex G200

Gel filtering material was made following Pharmacia Fine Chemicals guidelines. Then, the gel was boxed softly in a glass column with dimensions of 21× 1 mm column. A phosphate buffer solution was then used to titrate the column. After the column was equilibrated with 0.05M potassium phosphate buffer (pH= 8,0), 5 ml of the purified enzyme was put onto the column. At a flow rate of about 5 ml/fraction, potassium phosphate buffer was used to elute the samples; a UV spectrophotometer was used to evaluate each sample at 280 nm, and the enzyme activity was determined by measuring the height of the peaks. 15

2.8 Characterization of L-asparaginase

2.8.1 SDS

PAGE protein electrophoresis SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Brunelle, et al, (2014). using a 12 % separating gel and 5% stacking gel containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250. Then disdained with methanol, acetic acid, and water in the ratio of 4:1:5. 16

2.8.2 The Optimum pH and Temperature of L-ASNase Stability

The stability was examined at different pH values, and the enzyme was incubated in buffers ranging from (5.0 - 9.0) for one hour at 37ºC. For pH (5.0, 6.0), sodium acetate buffer (0.1 M) was used, for pH (7.0,8.0, and 9.0) potassium phosphate buffer (0.1 M) was used, and immediately transferred it was placed in ice. the remaining activity (%) of L-ASNase was plotted against the PH.

Thermal stability was carried out by incubating equal volumes of the enzyme in a water bath at (25,30,35,40,45, and 50) °C for one hour, then placing them in an ice bath to stop the reaction. A graph of remaining activity (%) was plotted against the temperature. 17

2.8.3. The Optimum pH and Temperature of L-ASNase activity

The activity was examined at different pH values, and the enzyme was incubated in buffers ranging from (5.0 - 9.0) for 30 min at 37ºC. For pH (5.0, 6.0), sodium acetate buffer (0.1 M) was used, for pH (7.0,8.0, and 9.0) potassium phosphate buffer (0.1 M) was used, and L-ASNase activity was evaluated.

The optimal temperature was also identified. After incubating the reaction mixture for 30 minutes at various temperatures ranging from 25 to 45oC, the enzyme activity was measured. 18

2.8.3. Effect of Various Effectors on L- ASNase stability

One volume of the purified L-ASNase was mixed with the same volume of either (10) mM of HgCl2, CuCl2, MgCl2, KCl, EDTA, and Tween80. The mixtures were incubated at (37) C for (30) min. The enzymatic remaining activity was measured under standard assay conditions. The control was the enzyme solution without any of these compounds. 19

3- RESULTS AND DISCUSSION

3.1. Screening of P. aeruginosa for the production of L-asparaginase

The L-asparaginase-producing potential of all P. aeruginosa was tested in a screening process using a modified M9 medium containing a single source of nitrogen (asparagine). When the media turns from yellow to pink, it means that enzymes are being produced. According to the plate culture assay, Four of the bacterial isolates demonstrated L-

asparaginase synthesis at varying zone diameters as shown below in figure 2. The efficiency of L-asparaginase was evaluated using spectrophotometry. L-asparaginase activity, measured in U/ml, and the pink zone diameters in mm. L-asparaginase activity of the isolates was observed to range from 0.21 to 1.64 U / ml and the diameter to range from 8 to 18 mm. The results are summarized in Table 1. 20.

Figure 2. presents (A) positive L-Asparaginase isolate,(B) negative L-Asparaginase isolate, (C) On L-Asparagine agar, the pink zone (Agar well diffusion assay), and (D)Control

Table 1. screening of paeruginosa isolates for the production of L-asparaginase

p.aeruginosa isolates no.	p.aeruginosa isolates source	Pink zone (mm)	Enzymatic activity (U/ml)
P 3	burn	18	1.64
P 7	burn	11.5	0.72
P 21	burn	10	0.58
P 24	burn	8	0.21

*out of four isolates, isolate no 3 was selected as the most L-asparaginase producer.

3.3 Effect of incubation temperatures on L-production

The optimal incubation temperature for L-asparaginase was investigated by testing a range of temperatures (25, 30, 37,40, and 42°C). The results showed that after 48 hours of growth. The maximum L-asparaginase activity exhibited at 37° was 2 U/ml (Figure 3). Any deviation from the optimal temperature for the enzyme's activity will be minimized. 21

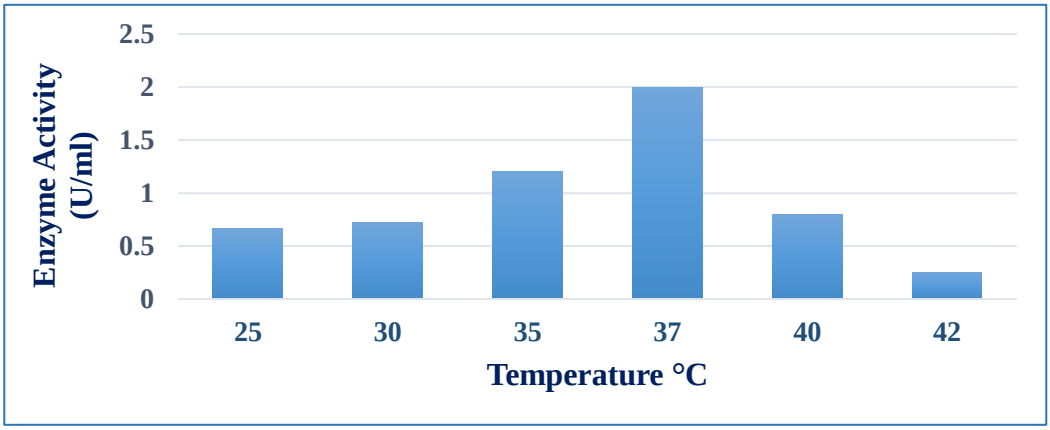

Figure 3. Effect of incubation temperatures on the production of L- asparaginase extracted from p.aeruginosa 3, incubated at 37°C for 48 hr

3-4 Effect of optimal pH on L-Asparaginase production

p..aeruginosa isolate no.3 was incubated in a medium with pH values (5,5.5,6,6.5,7,7.5, and 8) for optimum pH selection. Results showed that the optimum pH for L-asparaginase production was 7.5, at this value, the enzymatic activity was recorded at 1.8 U/ml. Furthermore, pH has an impact on the ionization of the nutritional substances, which in turn affects the solubility of the substances, and the impact it has on the stability of the synthesized enzyme These results are consistent with Mahajan et al.(2014) 22. As shown in figure (4)

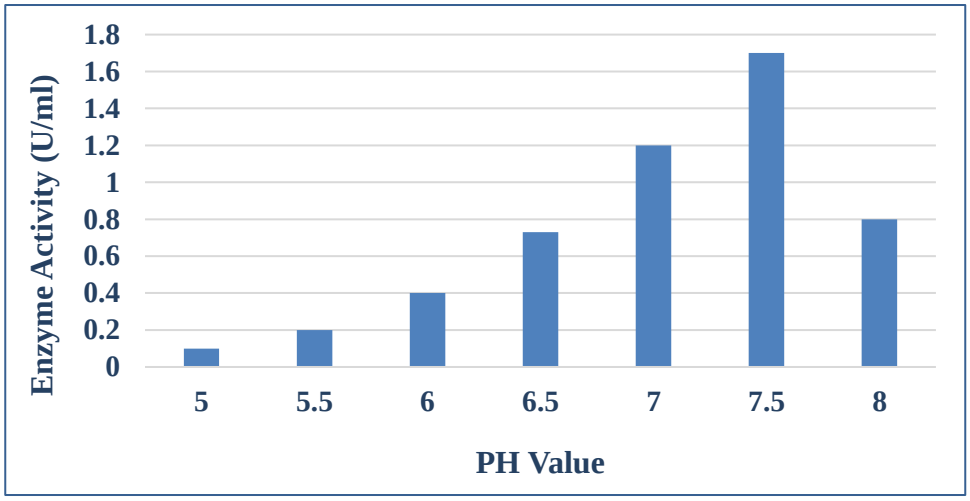

Figure 4. Effect of PH Value on the production of local L- asparaginase extracted from p. aeruginosa 3, incubated at 37°C for 48 hr

3-5 Purification of L- Asparaginase

3-5-1 purification by Ion-exchange Chromatography

The rough crude enzyme was poured through a DEAE-C ion-exchange column that had been pre-conditioned with phosphate buffer (pH 8.0, 0.05 M). The absorbance at a wavelength of 280 nm was determined for the proteins that were washed (which are positively charged). The binding protein (negative proteins) was eluted with phosphate buffer (PH8.0) supplement with NaCl (0.15-1M). only one distinctive protein band in the elution showed L-asparaginase activity (Figure 4). Results illustrated in Table 2 revealed that the final specific activity of 4.2 units/mg protein, the total activity was 42 Units, with a purification fold of 5.2 and 77.1% of the overall yield. 23. As shown in figure (5)

3-5-2 Purification by Gel Filtration Chromatography

There was only one unique protein peak that indicated L-asparaginase activity after purification by gel filtration column and that one peak was recorded from (16-20) (Figure 5), with a specific activity of 8.37 units/mg protein, with total activity of 16.75 units, exhibited purification fold of 10.5 and an enzyme yield of 30.7%, thus making gel filtration the optimum stage in the purification process due to the high specific activity of the enzyme 24 As shown in figure (6)

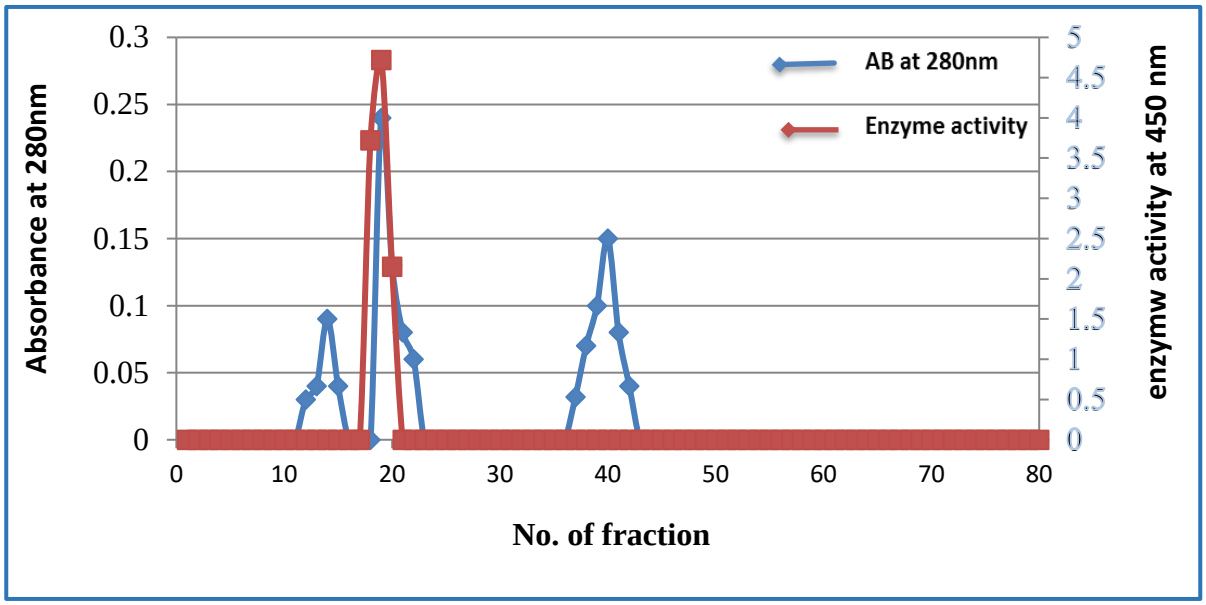

Figure 5. Ion exchange chromatography for L-asparaginase purification from P. aeruginosa using DEAE-Column (3×13 cm) equilibrated with a potassium phosphate buffer (pH 8.0, 0.05 M), eluted with a potassium phosphate buffer with NaCl gradient (0.15-1) M in potassium phosphate buffer.

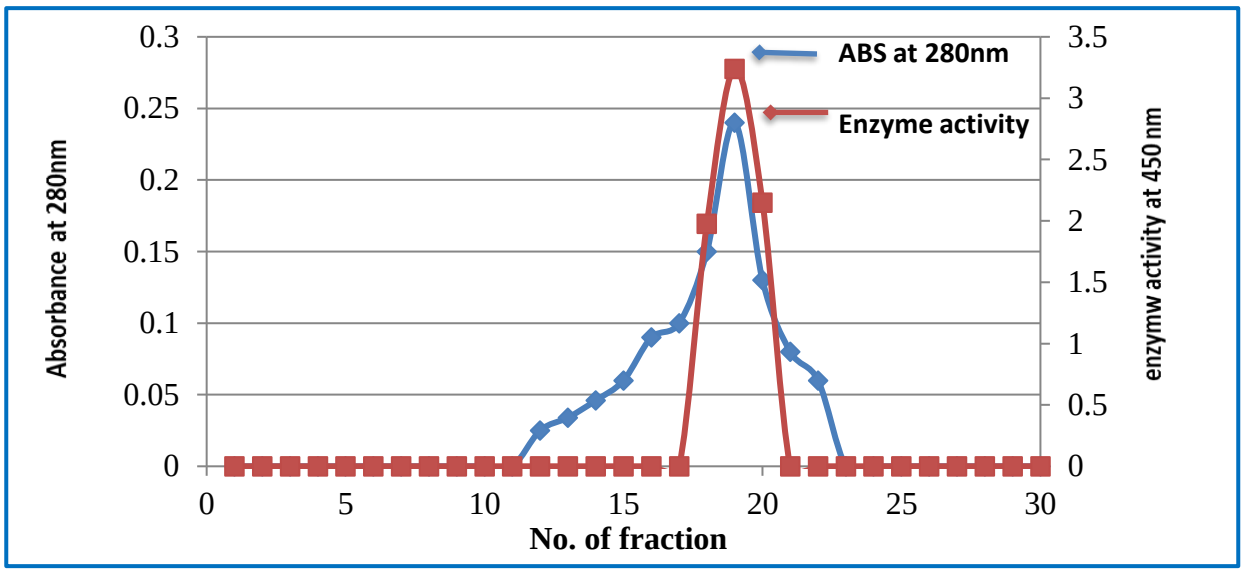

Figure 6. Gel filtration chromatography for L-asparaginase purification from P. aeruginosa using Sephadex G200 (21×1) equilibrated with Potassium phosphate buffer (PH 8.0, 0.05 M).

Table 2. profile of L-asparaginase production

Steps	Volume (ml)	Enzyme Activity (U/ml)	Protein Concentration (mg/ml)	Specific Activity (U/mg)	Total Activity (units)	Fold of Purification	Yield (%)
Concentrated supernatant	15	3.63	4.9	0.79	54.45	1	100
Ion exchange chromatography by DEAE-C	10	4.72	1.13	4.2	42	5.2	77.1
Gel filtration By Sephadex G200	5	3.35	0.41	8.37	16.75	10.5	30.7

*DEAE: diethylaminoethyl cellulose

3-6 Characterization of Purified L-asparaginase II

3-6-1 molecular weight of the Purified L-asparaginase II

The molecular weight of the enzyme was determined, and its purity was demonstrated. The molecular weight of isolated L-asparaginase from Pseudomonas aeruginosa was 120 KD shown in (Figure 5).

Each genus and species of organism has a unique L-asparaginase size distribution. The size of this protein drops to 33 kDa 25. Also, Amena et al.,(2009) established that L-asparaginase produced from S. gulbargensis had an apparent molecular weight of 85 kDa 26 According to reports, L-asparaginase from P. aeruginosa is a 160 KD a monomer 27. Thus, the subunits of L-asparaginase from the abovementioned bacteria display a wide range of structural diversity.

3.6.2 The Optimum pH and Temperature of L-ASNase activity

The activity was evaluated over a range of pH values. The maximal activity of the L-asparaginase was calculated to be 4.72 U/ml at a pH of 8.0 (Figure 8). L-asparaginase activity was shown to be highest at a slightly alkaline or natural pH compared to a nearly acidic pH. 28

Enzyme reactions were performed at several temperatures (25-50 C) to determine the optimal temperature for pure enzyme activity. As can be seen in Figure 9, enzyme activity increased with increasing temperature, reaching a high of 4.70 U/ml at 37 C, before beginning to fall with decreasing temperature, eventually reaching 0.7 U/ml at 50 C.29

3.6.3 The Optimum pH and Temperature of L-ASNase Stability

The remaining activity was measured to confirm pH stability. As can be seen in Figure 10, the optimal pH range for L-asparaginase stability is between 7.0 and 8.0, where 100% of the enzyme's original activity is still present. The enzyme maintained 40% and 60% of its activity at pH 5 and 6, but this stability dropped off dramatically at extremely acidic pH, respectively. 30

2.8.4. Effect of Various Effectors on L- ASNase stability

The results show an observable rise when chelating agent EDTA was applied, but HgCl2 and MgCl2 inhibited enzyme activity to 30.6 %, and 74.5% lost respectively. Some of the metal ions (K+, and Ca+2) led to increased activity of the enzyme, increase the stabilization of enzyme structure, and required protection against thermal denaturation. As shown in figure .11 31

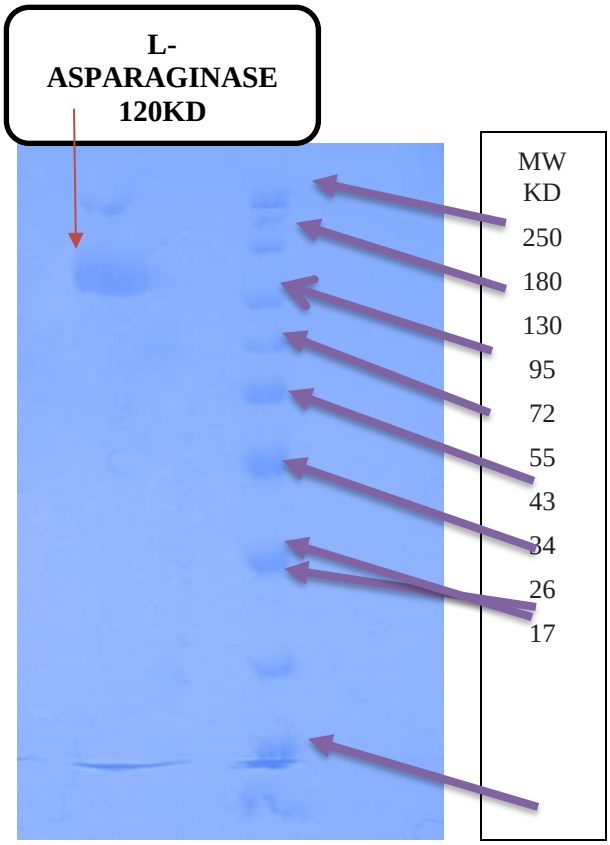

Figure 7. SDS- PAGE of locally purified L-asparaginase from P. aeruginosa

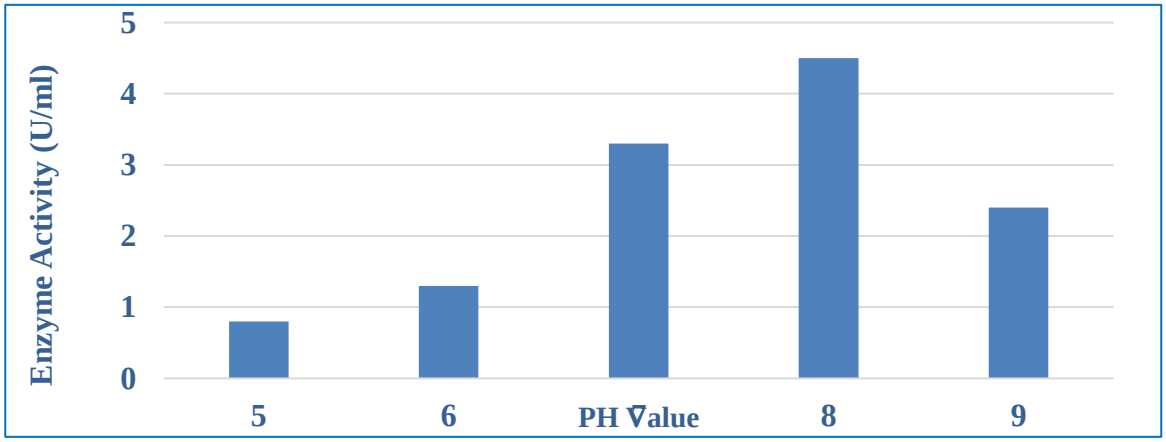

Figure 8. Effect of different pH values (5.0-9.0) on locally purified L-asparaginase activity from P. aeruginosa

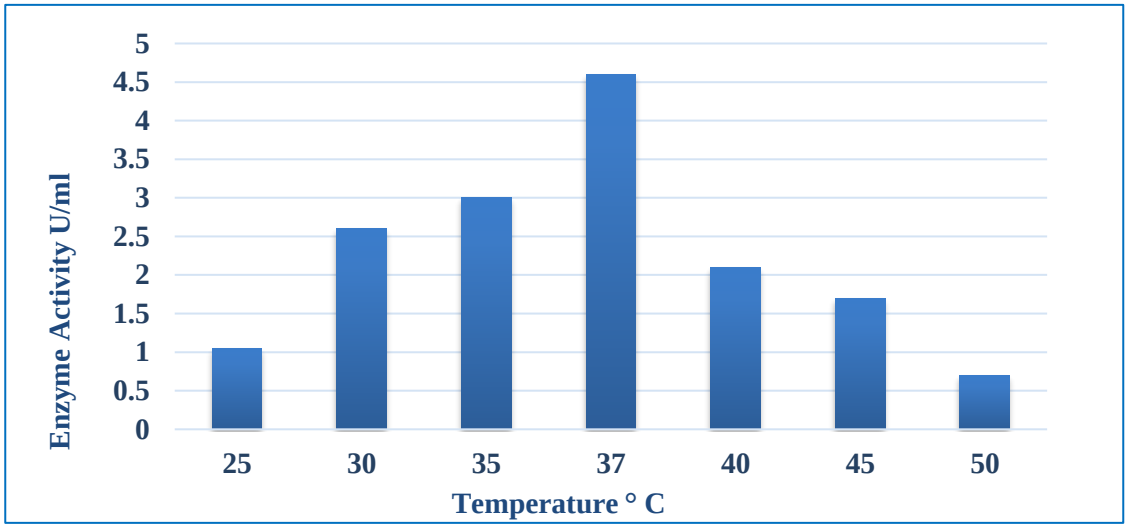

Figure 9. Effect of different Temperature (25-50)°C on locally purified L-asparaginase activity from P. aeruginosa

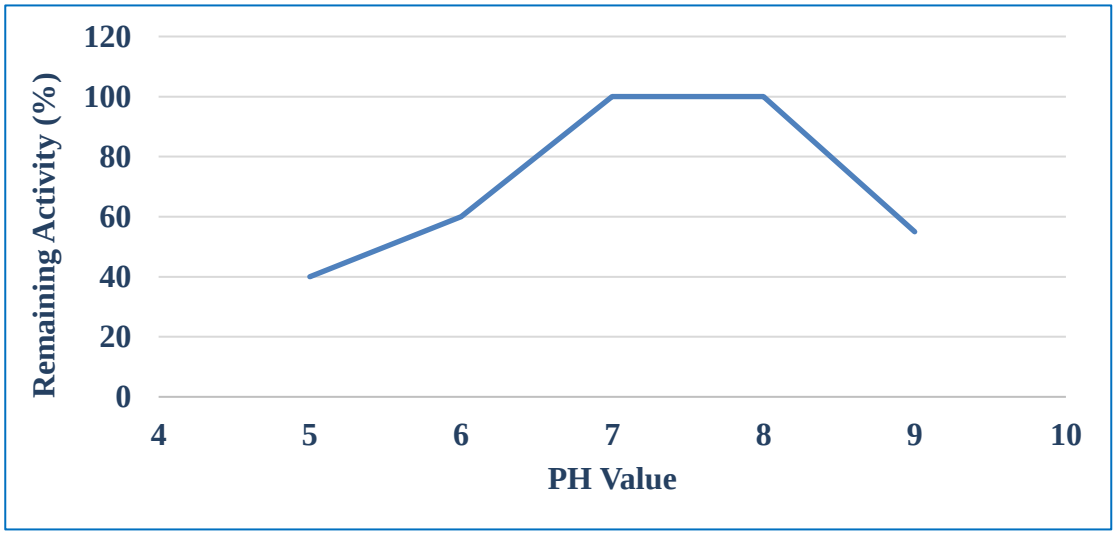

Figure 10. Effect of different pH values (5.0-9.0) on the stability of locally purified L-asparaginase from P. aeruginosa

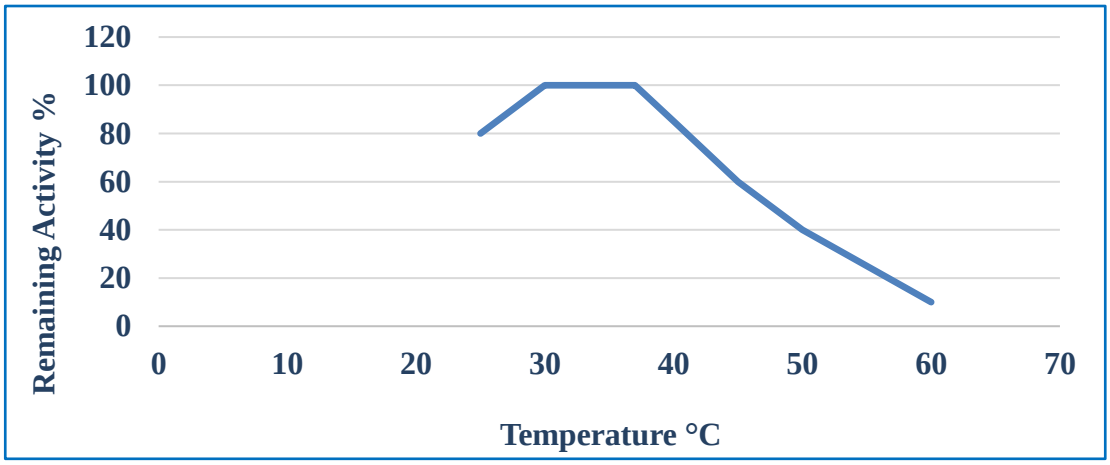

Figure 11. Effect of different Temperatures (25-60) °C on the stability of locally purified L-asparaginase from P. aeruginosa.

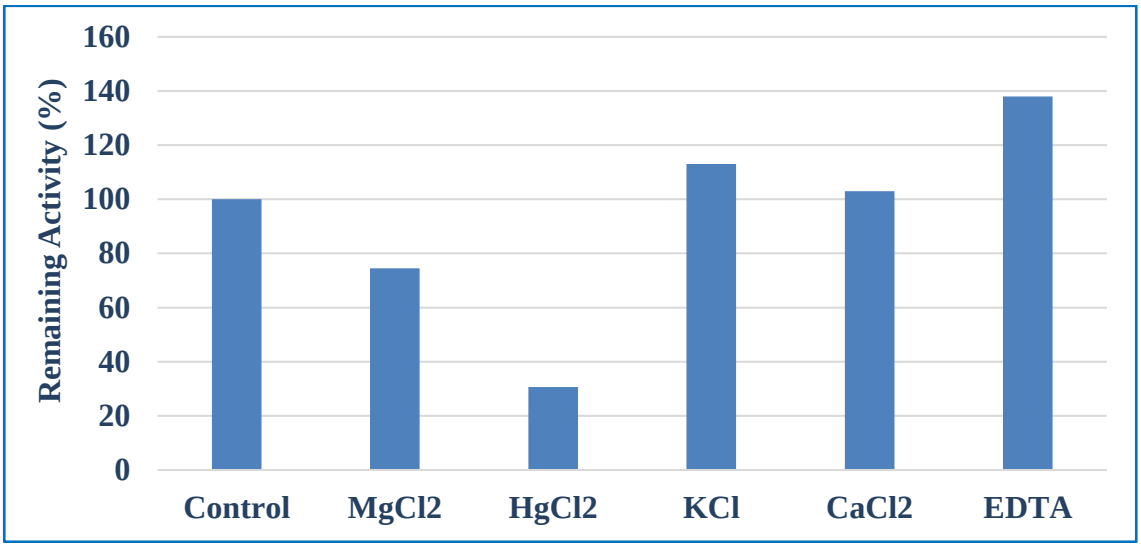

Figure 12: Effect of some metal ions, and EDTA on The stability of locally purified L-asparaginase from P. aeruginosa.

Compliance with Ethical Standards statements

Ethical approval :Department of Applied Science, University of Technology, Baghdad, Iraq / Iraq / certifies the ethical approval, Funding details (In case of Funding) :I am responsible for paying the financing, Conflict of interest : There is no conflict of interest, Informed Consent: Department of Applied Science, University of Technology, Baghdad, Iraq \ Agreed

References

Borek, D.; Michalska, K.; Brzezinski, K.; Kisiel, A.; Podkowinski, J.; Bonthron, D. T.; Krowarsch, D.; Otlewski, J. and Jaskolski, M., (2004). Expression, Purification and Catalytic activity of Lupinus luteus asparagine β-amidohydrolase and its Escherichia coli homolog. Eur. J. of Biochem., 271:3215-3226

Cachumba, J. J. M., Antunes, F. A. F., Peres, G. F. D., Brumano, L. P., Santos, J. C. D., & Silva, S. S. D. (2016). Current applications and different approaches for microbial L-asparaginase production. Brazilian journal of

microbiology, 47, 77-85.

Batool, T., Makky, E. A., Jalal, M., & Yusoff, M. M. (2016). A Comprehensive Review on L-Asparaginase and Its Applications. Applied biochemistry and biotechnology, 178(5), 900–923. https://doi.org/10.1007/s12010-015-1917-3

Lingnert, H., Grivas, S., Jägerstad, M., Skog, K., Törnqvist, M., & Åman, P. (2002). Acrylamide in food: mechanisms of formation and influencing factors during heating of foods. Scandinavian journal of nutrition, 46(4), 159-172

Kumar, K., Kataria, M., & Verma, N. (2013). Plant asparaginase-based asparagine biosensor for leukemia. Artificial Cells, Nanomedicine, and Biotechnology, 41, 184–188

Muneer, F., Siddique, M. H., Azeem, F., Rasul, I., Muzammil, S., Zubair, M., ... & Nadeem, H. (2020). Microbial L-asparaginase: purification, characterization, and applications. Archives of Microbiology, 202(5), 967-981.

Magaldi S, Mata-Essayag S, Hartung de Capriles C, Perez C, Colella M, Olaizola C and Ontiveros Y. 2004. Well diffusion for antifungal susceptibility testing. Int J infectious diseases., 8(1): 39-45

Badoei-Dalfard A. (2016). L-asparaginase production in the pseudomonas pseudoalcaligenes strain JHS-71 isolated from Jooshan Hot-spring. Molecular biology research communications, 5(1), 1–10.

Imada, A., Igarasi, S., Nakahama, K., & Isono, M. (1973). Asparaginase and glutaminase activities of micro-organisms. Microbiology, 76(1), 85-99.).

Classics Lowry O, Rosebrough N, Farr A, Randall R. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265-75 .

D Hameed, H. A., & Hussein Ali, E. (2021). Extraction and Purification of Extracellular L-Glutamate Oxidase from Streptomyces. Archives of Razi Institute, 76(4), 769-779.

Anand, C. V. (1998). Protein Purification Protocols: Methods in molecular biology. Biochemical Education, 1(26): 98.

El-Naggar, N. E. A., & El-Shweihy, N. M. (2020). Bioprocess development for L-asparaginase production by Streptomyces rochei, purification, and in-vitro efficacy against various human carcinoma cell lines. Scientific reports, 10(1), 1-21.

Elshafei, A. M., Hassan, M. M., Abouzeid, M. A. E., Mahmoud, D. A., & Elghonemy, D. H. (2012). Purification, characterization and antitumor activity of L-asparaginase from Penicillium brevicompactum NRC 829. British Microbiology Research Journal, 2(3), 158.

Brunelle, J. L., and Green, R. (2014). One-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE). Methods in Enzymology, 541, 151–159

Boeckx, J.; Hertog, M.; Geeraerd, A.; and Nicolai, B. (2017). Kinetic modeling: an integrated approach to analyze enzyme activity assays. Plant methods, 13(1), 69

Mahajan, R. V., Kumar, V., Rajendran, V., Saran, S., Ghosh, P. C., & Saxena, R. K. (2014). Purification and characterization of a novel and robust L-asparaginase having low-glutaminase activity from Bacillus licheniformis: in vitro evaluation of anti-cancerous properties. PLoS One, 9(6), e99037.

Mohan Kumar, N. S., & Manonmani, H. K. (2013). Purification, characterization, and kinetic properties of extracellular L-asparaginase produced by Cladosporium sp. World Journal of Microbiology and Biotechnology, 29(4), 577-587.

Gulati, R., Saxena, R. K., & Gupta, R. (1997). A rapid plate assay for screening l-asparaginase-producing micro-organisms. Letters in applied microbiology, 24(1), 23-26

Tortora, G. J.; Funke, B. R. and Case, C. L. (2004). Microbiology. (8th ed.). Pearson Education, Inc. San Francisco. New York.

El–Aziz, A., Amany, B., Hassanein, W. A., Mattar, Z. A., & El-Didamony, R. A. (2021). Production of Chemotherapeutic agent L-asparaginase from Gamma-Irradiated Pseudomonas aeruginosa WCHPA075019. Jordan Journal of Biological Sciences, 14(3).

Mahajan, R. V., Kumar, V., Rajendran, V., Saran, S., Ghosh, P. C., & Saxena, R. K. (2014). Purification and characterization of a novel and robust L-asparaginase having low-glutaminase activity from Bacillus licheniformis: in vitro evaluation of anti-cancerous properties. PLoS One, 9(6), e99037.

Abbas Ahmed. M .M ,Nageh Abo Dahab F, TaherTaha M and Fareed Hassan SM. (2015). Production, Purification, and Characterization of Lasparaginase from Marine Endophytic Aspergillus sp. ALAA-2000 under Submerged and Solid State Fermentation. J MicrobBiochemTechnol, 7:3.

Shakambari, G., Ashokkumar, B., & Varalakshmi, P. (2019). L-asparaginase–A promising biocatalyst for industrial and clinical applications. Biocatalysis and Agricultural Biotechnology, 17, 213-224

Amena S, Vishalakshi N, Prabhakar M and Dayanand A.L.K. (2010). Production, purification, and characterization of l-asparaginase from Streptomyces gulbargensis. Braz J Microbiol, 41: 173-178.

El-Bessoumy A, Sarhan M and Mansour J. 2004. Production, Isolation, and Purification of L-Asparaginase from Pseudomonas aeruginosa 50071 Using Solid-state Fermentation. BMB Reports., 37(4): 387-393.

Akilandeswari, K., Kavitha, K., & Vijayalakshmi, M. (2012). Production of bioactive enzyme L-asparaginase from fungal isolates of water sample through submerged fermentation. Int J Pharm Pharm Sci, 4(suppl 4), 363-366.

Chohan, S. M., & Rashid, N. (2018). Gene cloning and characterization of recombinant L-Asparaginase from Bacillus subtilis strain R5. Biologia, 73(5), 537-543.

Mohammed, N. S., & Ali, E. H. Extraction, Purification, and Characterization of Chloramphenicol Acetyltransferase from Pseudomonas aeruginosa.

Shukla S and Mandal S K. 2013. Production optimization of extracellular L-asparaginase through solid-state fermentation by isolated Bacillus subtilis. Int Appl Biol Pharm Technol., 4 (1): 219-226.