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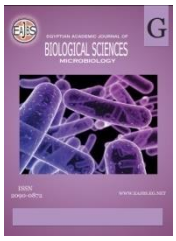

Screening and Production of Fungal L-Asparaginase Enzymes as Anticancer Agents from High-Contrast Soil Environments in Egypt

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Fungal; L-asparaginase; production; anticancer agent.

ABSTRACT

Background: Acute lymphoblastic leukemia represents a great crusader of death for children globally. *Aspergillus niger* is the source of the L-asparaginase enzyme from various soil environments. Similar to the human L-asparaginase enzyme. L-asparaginase enzyme in bacteria, commonly produced by *Erwinia chrysanthemum* or *Escherichia coli*, is currently used for acute lymphoblastic leukemia management and to reduce carcinogenic acrylamide creation during food preparation. **Study Objectives**: Manufacture of fungal L-asparaginase enzyme as an antineoplastic factor. **Methodology:** In the current study, fungal L-asparaginase was produced on mineral asparagine agar (MAM) selective medium (only fungi that utilize asparagine as a sole source for carbon and nitrogen can grow on it) at PH 6.5, temperature 25^0C, and incubation for 3 days. Malt agar medium was used for sub-culturing fungal L-asparaginase-producing strains. The biological activity and cytotoxicity activity were obstinate through nessleriazation and MTT[(dimethylthiazol-2-yl) diphenyl tetrazonium] assays respectively. **Results:** L-asparaginase in fungi showed high potency and bioavailability as an anticancer agent. According to morphological and biochemical tests and DNA blot hybridization, the major fungal isolate producing this enzyme was *Aspergillus niger*. The molecular mass was 65 KDa as discovered aside from a mass spectrometer. Vmax of 163.8 UML^{-1} min^{-1} and a Km value of 3.41×10^{-3} M were displayed via L-asparaginase. **Conclusion:** This was a promising approach study and fungal L-asparaginase was generated without hypersensitivity reactions and glutaminase-like effects with superior bioactivity to bacteria.

INTRODUCTION

Acute lymphoblastic leukemia was a starring drive of death among children worldwide.Growth and structure of mycotic: Fungi(yeasts and molds) are eukaryotic organisms(Parveen Kumar, 2020). There are styles of fungi: yeasts and molds (Caroline S and Michael G, 2018). Molds develop as lengthy filaments (hyphae)and shape a mat(mycelium)(Trevor, Katzung and Hall Marieke, 2021). Some hyphae shape transverse walls (septate hyphae); while others do now no longer consist of those transverse walls (nonseptate hyphae) (Bardal Stan, Waechter Jason and Martin Douglas, 2020).

The increase of hyphae does now no longer arise via way of means of the molecular department however takes place via way of means of the extension of the end of the hyphae (Olson, 2020). The nonseptate hyphae are multicellular (coenocytic) (Levinson, 2021). Most fungi are obligate aerobes, a few are facultative aerobes, however, none are obligate anaerobes (Swanson, County, Muntnick and Shargel, 2019). Several medically important fungi are thermally dimorphic (Fisher, Champe and Harvey, 2021). They exist as yeasts in human tissues at frame temperature and as molds withinside the surroundings at ambient temperature (Dnipro, Schwinghammer, Dnipro, Barbara, 2021).

Characterization of microbial L-asparaginase as an anticancer element: Bacterial L-asparaginase enzyme generally constituted of *Erwinia chrysanthemum* or *Escherichia coli* turned into applied withinside the remedy of acute lymphoblastic leukemia and lowering acrylamide manufacturing that's carcinogenic all through meals preparation (Golderg, 2020). L-asparaginase converts L-asparagine amino acid (that's important for the increase of most cancers cells which cannot synthesize it and achieve it from outside environments consisting of blood, lymph, and extracellular fluids) into aspartic acid and ammonia (Wilson, 2019). Both *Escherichia coli* and *Erwinia chrysanthemum* are prokaryotes(Souza *et al.,* 2017). Bacterial L-asparaginase reasons many aspects consequences consisting of hypersensitive reactions (anaphylactic reactions and drug neutralization) (Metting Patricia J, 2019) and glutaminase-like activity(Larissa *et al.,* 2016). The fungal enzyme is much like human L-asparaginase due to the fact each is from eukaryotic cells(S. A. Alrumman *et al.,* 2019). Our look may want to triumph over those drawbacks via way of means of the manufacturing of this enzyme from new fungal assets consisting of *Aspergillus niger*. This new enzyme turned devoid of those aspects' consequences and had higher efficacy and better yield than bacterial enzymes. In this work, we searched for new managements for auxotrophic cancers for L-asparagine consisting of fungal L-asparaginase enzyme. The enzyme originated from fungi because of better productivity, yield, and purity of the mycotic enzymes than different assets consisting of bugs and mammals. Our look at geared toward amassing grassland soil samples from extraordinary environments in Egypt; moreover, isolation of L-asparaginase surrendering fungi on selective media postdated via way of means of uncovering physiologic and organic technology factors influencing the increase of a few targeted fungal isolates secreting the enzyme of clinical hobby and characterization of L-asparaginase exhibition and activeness via way of means of a few decided on fungal isolates.

MATERIALS AND METHODS

Ethical Statement:

In the attendant study, we preceded All applicable national, international, and/or institutional guidelines for the attention and utilization of humans and animals. All processes carried out in the study including humans and animals were authorized by the local authorities, the Ethical committee for human and animal handling at Cairo university (ECAHCU), at the Faculty of Pharmacy, Cairo University, Egypt in agreement with the recommendations of the Weatherall report with approval number P-18-4-2020. All efforts were performed to ablate the number of humans and animals utilized and their suffering during the study.

Type of Study: Screening experimental study.

Place and date of the study: This study was done by pharmacy faculty at, University of Cairo, Egypt between November 2021 and August 2022.

Source of animal models: They were obtained and legalized from the department of pharmacology and toxicology, pharmacy faculty, University of Cairo, Egypt.

The inclusion criteria for animal models were: Adult Female animal models; can be

induced by acute lymphoblastic leukemia; blood ammonia levels can be easily estimated; obese animals**.**

The exclusion criteria were: young animals; male animal models; Animal blood ammonia levels cannot be easily estimated; Non-obese animals.

Table 1. It represents the list of instruments.

Instrument	Model and manufacturer
Autoclaves	Tomy, Japan
Aerobic incubator	Sanyo, Japan
Digital balance	Mettler Toledo, Switzerland
Oven	Binder, Germany
Deep freezer -80	Artiko
Refrigerator 5	whirlpool
PH meter electrode	Mettler-toledo, UK
Deep freezer -20	whirlpool
Gyratory shaker	Corning gyratory shaker, Japan
190-1100nm Ultraviolet-visible spectrophotometer	UV1600PC, China
Light(optical) microscope	Amscope 120X-1200X,China

Collection of the Samples:50 Soil samples were collected from different environments in Egypt.

Methods:

Screening of Positive Fungal L-Asparaginase-Producing Isolates:

Mineral Asparagine Medium (MAM):

The selective medium was used for screening fungal L-asparaginase-producing isolates. It was composed of the following components: KH2PO4(1.2), MgSO4(0.6), FeSO4(0.005), KCL (0.4), D-glucose (7), Agar (13), L-asparagine (15), Thiamphenicol antibiotic (0.15). Thiamphenicol was added to prevent bacterial growth. The PH of the medium was 6.5 and for 3 days incubation was performed. Only colonies which were capable of employing L-asparagine as an origin of carbon and nitrogen were grown in this selective medium. The positive isolates were stored at 4^0C for later studies.

Malt Extract Agar Medium:

It is a general-purpose growth acidic medium for the cultivation and isolation of fungi. PH was adjusted at 6.5 using NaOH. Subcultures of the positive isolates (grown on MAM) were grown and isolated on malt extract agar medium at temperature 20c, PH 6.5 after 3 days incubation period.

Microscopic examination of positive fungal isolates after dissolving in 10%KOH. Molecular detection of positive fungal isolates using DNA probe hybridization(Rachel A. Egler, Sanjay P. Ahuja, and Yousif Matloub, 2016).

L-asparaginase Manufacture Aside Submerged Fermentation:

Exhibition of L-asparaginase extracellularly from *Aspergillus niger* was performed victimizing the environment incorporating (g/L): L-asparagine11, Dextrose 3, KNO3 2, starch 19, MgSO4.7H2O 0.6, NaCl 0.2, K2HPO4 2, chloramphenicol 2, pH 7.4. sixty mL of the medium of broth was distributed in a 250 mL Erlenmeyer cone-shaped flask. The inoculated flasks with an inoculum size of 5% v/v were incubated on a rotary shaker incubator at 300 rpm and 25 °C. Later on a week of the time of incubation, with a cooling centrifuge at 5000 rpm for 45 minutes at 5 °C, the broth was centrifuged and the distinct superannuate sufficed as refined protein.

Determination of Fungal L-Asparaginase Production and Activity:

Direct nesslerization test:

It was used to characterize the enzyme production and activity from positive fungal isolates grown in MAM. The hydrolysis of L-asparagine amino acid to the acid of L-aspartic and ammonium was catalyzed by L-asparaginase. The released ammonium was identified and assayed spectrophotometrically at a wavelength of 425 nm. The strength of the light was straightaway relative to ammonium concentration. The amount of released ammonium was directly proportional to the enzyme activity.

Salicylate Method:

This method is a variation of the well-known phenate method. It is free from mercury salts and phenol. it is useful for low-range ammonium nitrogen determination.

L-asparaginase catalyzes the hydrolysis of L-asparagine amino acid to L-aspartic acid and ammonium. The released ammonium was identified and assayed spectrophotometrically at UV wavelength 425 nm. The intensity of

the light was directly proportional to ammonium concentration. The amount of released ammonium was directly proportional to the enzyme activity.

In Vitro Cell Viability Assay:

CCL-120 cancer cell line was used for assessment of the physiologic, pharmacologic, and toxicological effects of the enzyme on the cancer cells.

The Vero cell line was used for the assessment of the physiologic, pharmacologic, and toxicological effects of the L-asparaginase enzyme on the mammalian cells.

MTT**(**(dimethylthiazol-2-yl) diphenyl tetrazonium**)** method was used for in vitro cell viability assay of fungal L-asparaginase.

Antioxidant Testing Assays:

DPPH Radical Scavenging Activity:

Using DPPH free radical as a reagent, the free radical scavenging activity of different fractions was determined. Briefly, 600 µl of sample solution (different concentrations) was mixed with 2 ml of 5% (w/v) DPPH stock solution in ethyl alcohol. For 30 min in the dark at room temperature, the mixture was incubated. The trapping capacity was interpreted with a UV spectrophotometer by observing the decrease in optical density at 517 NM. The higher free radical scavenging activity was indicated by the lower absorbance of the reaction mixture. Ascorbic acid has been used as a standardized antioxidant. According to the formula:

DPPH scavenging activity (%) = [(ODblank - ODsample)/ODblank] × 100, DPPH scavenging activity was measured. ODblank is the optical density of the control reaction carrying all reagents. ODsample is the optical density of the test compound. The concentration of the extract yielding a fifty percent inhibition (IC50) was measured from a plot plotting the concentration of the extract versus the percentage of inhibition. Attempts were done in triplicate.

β-Carotene Bleaching Assay:

The antioxidant effect was determined based on the β-carotene bleaching test. We prepared a stock solution of a linoleic acid/β-carotene mixture as follows: 0.6 mg of β-carotene was liquefied in 1.1 ml of chloroform and 190 mg of Tween-20 with 26 µl of linoleic acid. Evaporation of the chloroform was completely achieved by means of a rotary vacuum evaporator. And so we saturated 100 ml of distilled water with oxygen and the resulting solution was intelligently stirred. 5 ml of this reaction mixture was dispensed in test tubes and 199 µl of each sample, was treated at different concentrations. The emulsion system was incubated for 1 h at 45°C. We repeated the same procedure with blank as the negative control and butylated hydroxytoluene (BHT) as the positive control. After this annealing time, the optical density of each mixture was calculated at 480 nm. The activity of the antioxidant in the bleaching form of β-carotene as a percentage (A%) was measured using the following equation:

A % = 1 − (A0 – At/A' 0−A' t) × 100, where A0 and A'0 are the optical densities of the blank and sample, respectively, deliberated at time 0 and A t and A't are the optical density of the sample and the blank measured after 1 h, respectively. Each attempt was achieved in triplicate.

Assessment of Antiproliferative Actions of L-Asparaginase via BrdU Incorporation Assay:

Cell proliferation rates and genomic DNA synthesis rates were evaluated using the 50-Bromo-20-deoxyuridine (BrdU) colorimetric ELISA kit (Roche, Germany). The various cell lines were plated at a density of 5000 cells per well in a 96-well culture dish with the right concentrations of L-asparaginase induced for 48 h and then incubated with BrdU marker solution at 37° C for 8 h (Liboskaet al.2012). Then, the cells were fixed and the DNA was denatured with FixDenat solution. Fixed cells were incubated with peroxidase-conjugated anti-BrdU antibody and then exposed to a tetramethylbenzidine substrate. Finally, the plates were read at 370 nm using a microplate reader. DNA synthesis per cell was calculated by dividing total DNA synthesis by the percentage of viable cells.

Caspase-3 Activity Assay for Evaluation of Programmed Auxotrophic Cancer Cell Death Activity Of L-Asparaginase:

A caspase-3 assay kit was utilized to detect caspase activity that plays a fundamental role in programmed cell death. The caspase-3 action colorimetric assay was used according to the manufacturer's protocols (Sigma-Aldrich Chemie Gmbh). Cell lines were lysed with L-asparaginase, 10 micrograms of supernatant incubated with 85 microliters of assay buffer and 10 microliters of caspase-3, acetyl-Asp-Glu-Val-Asp substrate -nitroanilide (Ac-DVD-pNA), in 96 wells for 4 h at 37°C. The successive changes in caspase-3 activity were assessed by measuring p-ni-troanilide (p-NA) levels. This compound was discharged by caspase-3 enzyme action by computing the values of absorbance of p-NA at 405 nm.

The Design of A New Primer For The Expression Of Fungal L-Asparaginase:

The design of the novel primer of expression was carried out by bio-informatics utilizing NCBI website and NEB cutter V2.0 Microsoft program. Table 2 represents Primer for expression of L-asparaginase.

Table 2. Primer for expression of L-asparaginase.

Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self-complementarity	Self-3' complementarity
Forward primer	GCTGGCGACATCCCATTTTC	Plus	20	829	848	59.90	55.00	4.00
Reverse primer	ATTGGCATAAGTCCCTGCCC	Minus	20	1809	1790	60.11	55.00	4.00

Production of L.Asparaginase By Recombinant DNA Technology:

Synthesis of L-asparaginase was finished by recombinant DNA technology using *Saccharomyces cerevisiae BJ1824* as the expression host. The C-terminus was histidine 6x, the promoter was AUG1, the inducer was methanol, and PYES2-DEST52was the expression system vector. The L-asparaginase genes were transcribed by PCR and then cloned to PYES2-DEST52 using the restriction endonucleases Hind III and EcoR II for plasmid digestion, followed by ligase enzyme ligation. The design and propagation of the recombinant plasmid were performed first in *Escherichia coli Top 10* and then transformed into *Saccharomyces cerevisiae BJ1824*. For the production of L-asparaginase using galactose as an inducer, a selective medium of YNBG(0.67% yeast nitrogen base without amino acids supplemented with suited nutrients and 2% galactose) was used. For the growth of modified yeast at 30°C, followed by maintenance in YPG-rich medium (bacteriopeptone, 1% yeast extract and 2% galactose).

Formulation of Intramuscular Injectable L-Asparaginase Preparation:

In our study, different pharmaceutical dosage forms and routes of administration were attempted to determine the optimal dosage form and route of administration of fungal L-asparaginase enzyme as an anticancer agent against auxotrophic cancers for L-asparagine:

Injectable products were prepared in presence of isotonic aqueous solutions which had PH adjacent to that of blood and body tissues (PH 7.4). 1% w/v Thiamphenicol antimicrobial agent was added to injections prepared in containers. Aqueous solutions were given through intramuscular injection, and the release of 10% w/v L-asparaginase was controlled by increasing vehicle viscosity by using 3%w/v carboxymethylcellulose (CMC). 2% w/v Ethylene glycol was added afterward for prolonging the duration of action to once-daily dose administration instead of multiple-dose injections.

L-glutaminase Assay:

According to Imada et al method 2018; the action of L-glutaminase was dictated by victimizing L-glutamine as a substrate and the discharged ammonia was deliberated via exploiting Nessler's reagent.

Assessment of Secretion Of Antibodies To L-Asparaginase:

The quantity of IgG anti-L-asparaginase antibodies in mouse serum was sealed with efficient ELISA operator.

The Kinetic Parameters Km and Vmax Determination:

The kinetic parameters, maximum velocity (Vmax) and Michaelis–Menten constant (Km) of purified L-asparaginase were ascertained with appropriate concentrations of L-asparaginase (2–11 mM) as the substrate and throughput. information was attached to the nonlinear exponential phase association regression curve. using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). The effect of L-asparaginase was estimated by measuring the hydrolysis rate of L-asparaginase under standard laboratory conditions using the Michaelis-Menten equation.

The Deactivation Rate Constant (KD) and Half-Life Time ($t_{1/2}$) Estimation:

The thermal inactivation constant (KD) and thermal inactivation half-life (t1/2) of the purified L-asparaginase enzyme secreted by Aspergillus niger was determined by running GraphPad Prism 5 software (GraphPad Software Inc.) ., San Diego, CA).

Molecular Weight Determination:

The mass and purity of the sublimated L-asparaginase enzyme were observed by Western blot consisting of 0.2 %SDS according to Laemmli perception with a 10 percent detaching acrylamide gel (pH 8.8) and a 5% stacking gel. pH was 6.8. Gel staining was performed with R-250 blue brilliant coomassie recorded by a staining step with a mixture of methanol-acetic acid and water in a ratio of 5:2:6. Molecular Weight of L-asparaginase determined using a standard molecular weight protein marker in the range of 10-177 kDa. Therefore, the molecular weight of L-asparaginase was confirmed by mass spectrometry.

Characterization of Physicochemical Factors Of The Sublimate Enzyme:

The optimum pH of the purified enzyme was considered in the range of pH 5-12 with the L-asparagin substrate liquefied in different buffers of Tris-HCl (pH 8), glycine-NaOH (pH 9-) 10) and 0.06M: citric acid-Na2HPO4 (pH 5–8). In addition to incubating the test mixture at a temperature range of 25-60°C in 0.06 M Tris-HCl buffer under the test conditions; The effect of temperature on L-asparaginase activity was evaluated. Besides incubation of the purified enzyme in the presence of different substrate concentrations (1–10 mM); High substrate concentrations were observed for enzymatic activity. The chemical reaction mixture was incubated for different times (0, 10, 20, 30, 40, 50, 60 and 80 min) to evaluate the effect of incubation time on L-asparaginase activity. The activity is determined by the properties of the enzyme.

L-Asparaginase Thermal Stability:

The temperature stability of L-asparaginase was determined by pre-incubating the reaction mixture (substrate-free) containing the buffered enzyme for different times (0.0 to 90 min) at different temperatures. different (40, 50, 60, 70 and 80 °C). After the end of the incubation time, the enzyme cooled and residual activities were tested.

A Consequence of Ph on Unchangingness Of L-Asparaginase:

To investigate the optimal pH for L-asparaginase stability, this enzyme in the absence of substrate was pre-incubated at room temperature for 0, 6, 12, 18 and 24 h in valuable buffer solutions. Different pH (pH 4–11) were attempted. The remaining operation has been tested under standard test conditions.

Experimental Animals:

Female Swiss albino mice weighing 20-30 g were obtained from the Department of Urology and Nephrology, Cairo University, Egypt and cultured with purified L-asparaginase from *Aspergillus niger* and *E. coli* (Sigma-Aldrich, no. product: B4517,

CAS). Pets were allowed free access to laboratory standard food and water in a conditioned environment with a temperature of 24 ± 1°C and a relative humidity of 55 ± 5% with a typical 12 am/12 cycle evening hours. raised in the state All experiments were performed according to regulatory and ethical guidelines for laboratory animals authorized by the Ethics Committee of the Faculty of Pharmacy, Cairo University, Egypt.

Tumor Transplantation:

Ehrlich ascites carcinoma (EAC) cells were supplied by Dutch Cancer Institute. Cells were maintained in vivo in female Swiss albino mice by serial intraperitoneal transplantation in the laboratory of the Faculty of Pharmacy, Cairo University, Egypt. In vivo experiments and evaluation of anti-tumor activity: 7-10 days old EAC cells were used for the experiment. Ascites fluid from tumor-bearing mice was removed from the peritoneal cavity by needle aspiration extraction under sterile conditions, washed three times with saline, and then centrifuged at 67 × g. Tumor viability was observed using the trypan blue exclusion test as the victim and cells were counted with a sphygmomanometer. Saline solution was used to obtain ascites fluid at a concentration of 5 x 10^5 viable EAC cells/0.1 ml of tumor cell suspension and injected into the right thigh of the rat shin to obtain ascites tumor. The maximum tumor diameter and its perpendicularity were measured with a digital caliper and used to determine tumor growth. When the primary tumors reached a size of 50 × 100 mm^3, Swiss albino mice were divided into 3 groups of 6 each. Group (1) received saline infusion(control containing EAC, 5 ml/kg). Group (2) received commercially available L-asparaginase. Group (3) received L-asparaginase produced by *Aspergillus niger*. The treatment with L-asparaginase was given 5 days after vaccination and was done twice a week for 2 weeks. 24 h after the last dosing, after 18 h of fasting, the animals of each group were sacrificed by cervical dislocation and the anti-tumor activity of L-asparaginase tested was determined. Antu's complement activity was calculated by determining T(change in tumor size for treatment groups) and C(change in tumor size to control). The degree of tumor growth inhibition was obtained from 100 X ΔT/ΔC. Tumor size (mm^3) = 0.5 Xa Xb2

Assessment of Immunogenicity:

Two groups of 6 mice, each receiving a commercial intraperitoneal injection of L-asparaginase (250 U/kg) or L-asparaginase from *Aspergillus niger*(250 U/kg) twice a week for 4 weeks. Specific antibody (IgG immunoglobulin) levels against L-asparaginase or commercial products were measured in serum by ELISA and a microplate reader was used at 450 nm absorbance. A direct enzyme immunoassay (ELISA) was performed to evaluate the presence of asparaginase-specific IgG antibodies in serum samples using horseradish peroxidase-conjugated anti-rat IgG (purchased from Southern Biotech). All ELISA steps were performed at room temperature and 2.5 ucm (w/v) was used as a binding and blocking buffer. The test steps and conditions were as follows: 2 h blocking asparaginase-coated plates, then rinsed thoroughly with PBS-Tween, incubated with diluted serum samples for 2 h, rinsed, incubated with the detectable antibody for 1 h, rinsed, cultured with 3 substrates, 3',5,5'-tetramethylbenzidine for 30 min and cooled with 9.8% (v/v) H2SO4 in water. The IgG titer was measured.

Determination of Selectivity Index of L-Asparaginase:

The selectivity index of an antineoplastic agent was determined as follows: SI=IC_{50} of the test enzyme in the natural cell line/IC_{50} of the test enzyme in the cancer cell line. IC_{50} represents the test enzyme concentration required to kill 50% of cells. It was deliberated using Graph Pad Prism version 5 software.

Fig. 1. It shows molds of *Aspergillus niger spp* with septate hyphae producing L-asparagine degrading enzymes as anticancer agents.

Fig. 2. It represents *Aspergillus niger* growing on MAM.

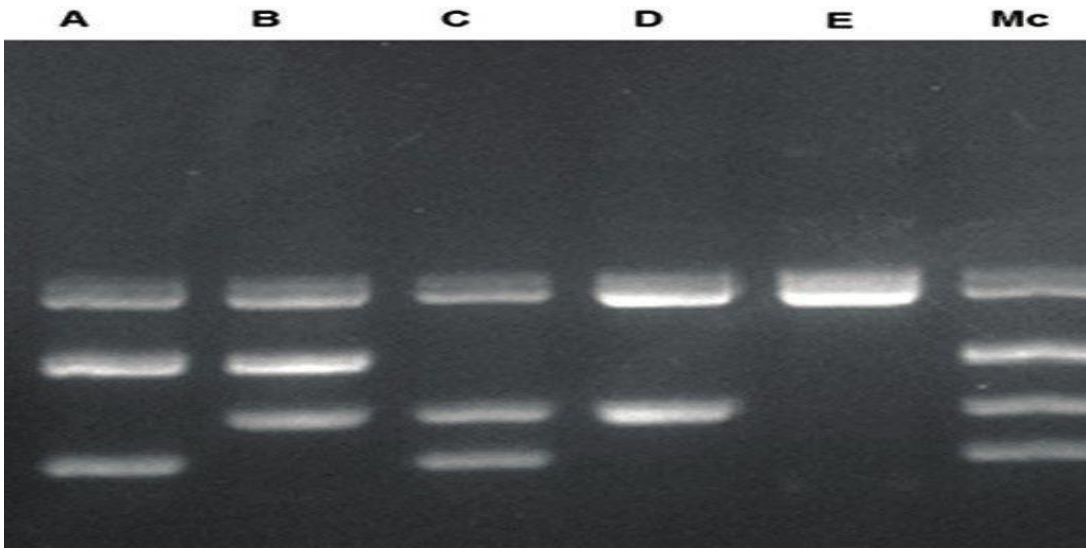

Fig. 3. It shows recombinant proteins of L-asparagine degrading enzymes measured via the Northern blot technique. The maximum yield of recombinant proteins was 65 mg/l. The purity of recombinant Asparagine degrading enzymes was approximately 86%.

Statistical Analysis:

All cultures were conducted in triplets. Their presentation was by means and standard deviation. One-way analysis of variance (p value≤.05) was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software.

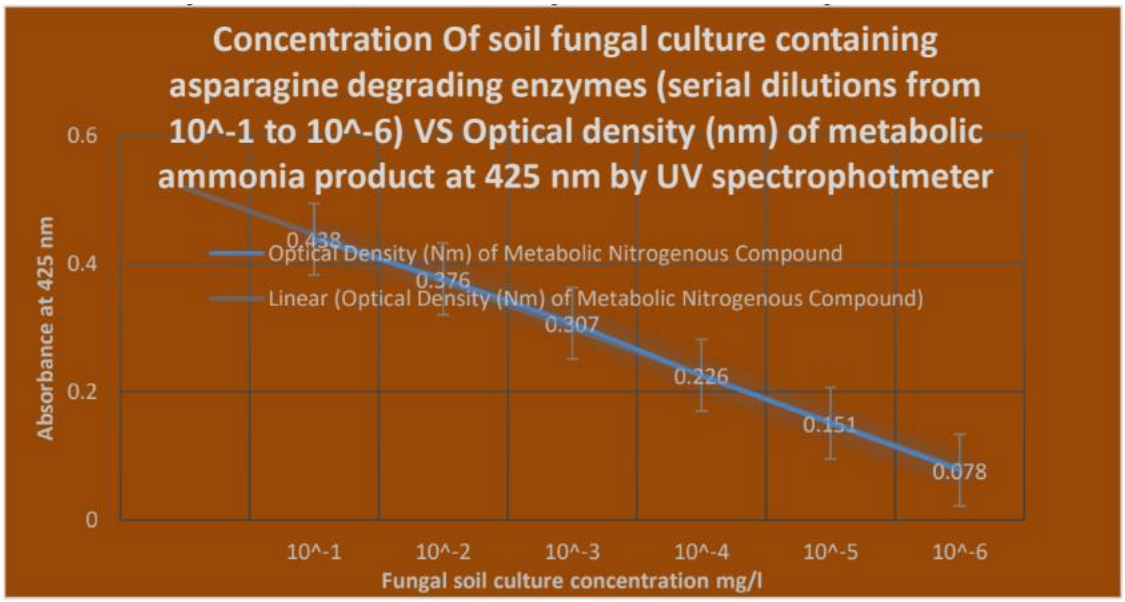

Fig. 4. It represents a Nesslerization screening assay of different concentrations of soil fungal L-asparaginase.

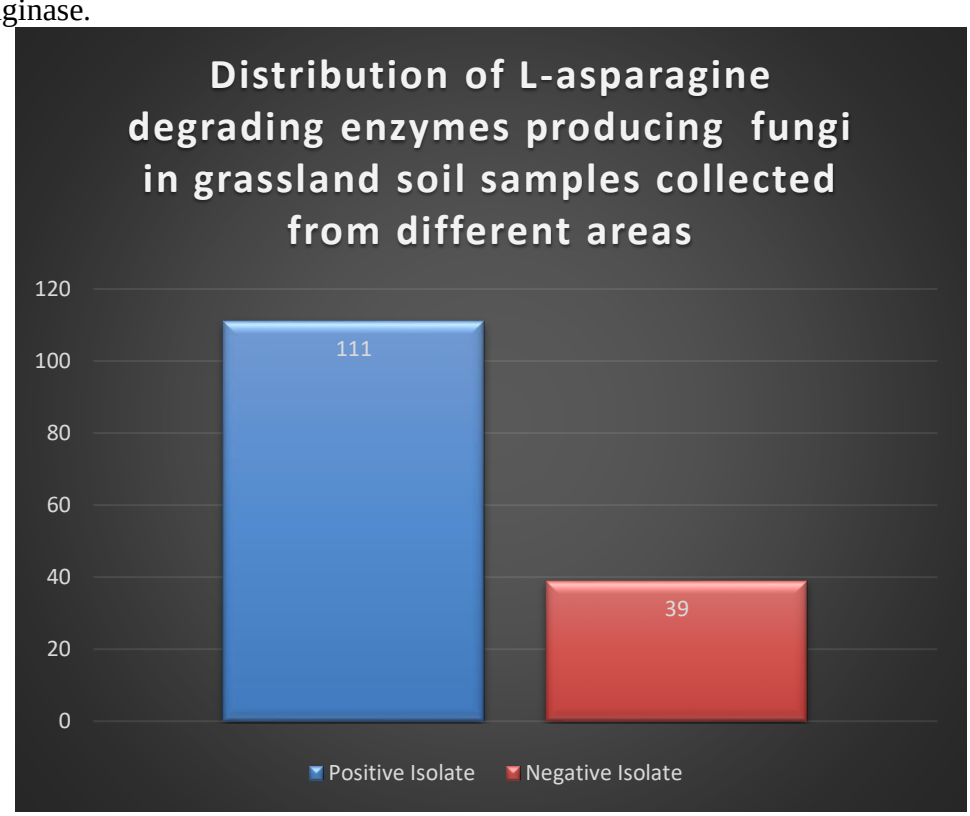

Fig. 5. It represents a distribution of L-asparagine degrading enzymes producing fungi.

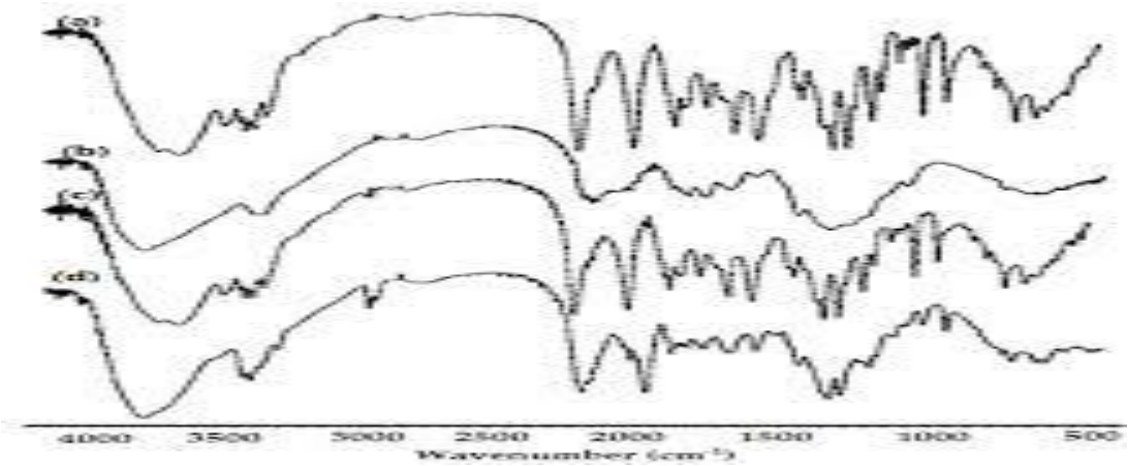

Fig. 6. FTIR spectroscopy shows no drug-drug interaction or incompatibility between L-asparaginase and excipients**.**

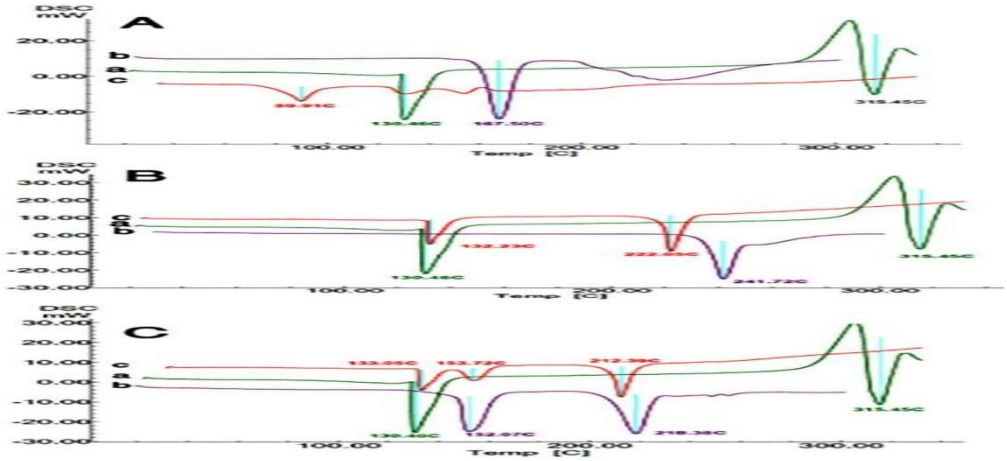

Fig. 7. DSC study shows no drug-drug interaction or incompatibility between L-asparaginase and excipients.

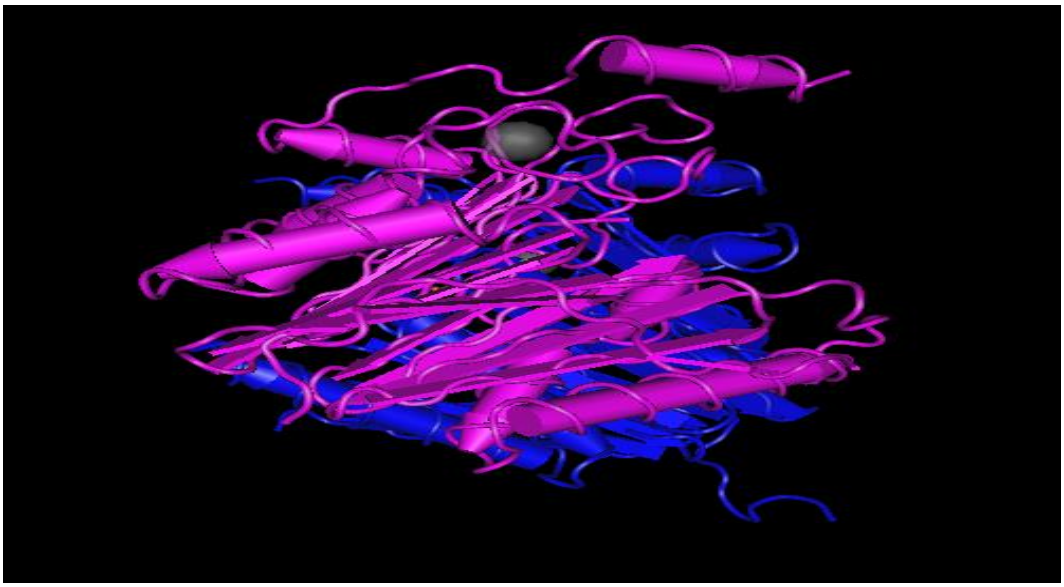

Fig. 8. It shows a 3D structure of L-asparaginase. It comprises 295 amino acids.

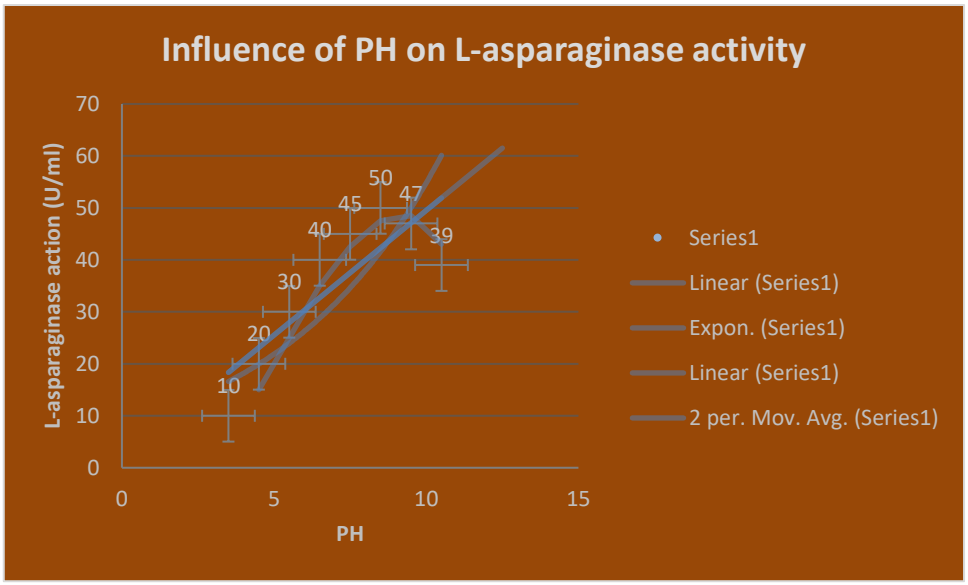

Fig. 9. It shows the action of fungal L-asparaginase as a function of the pH of the reaction**.**

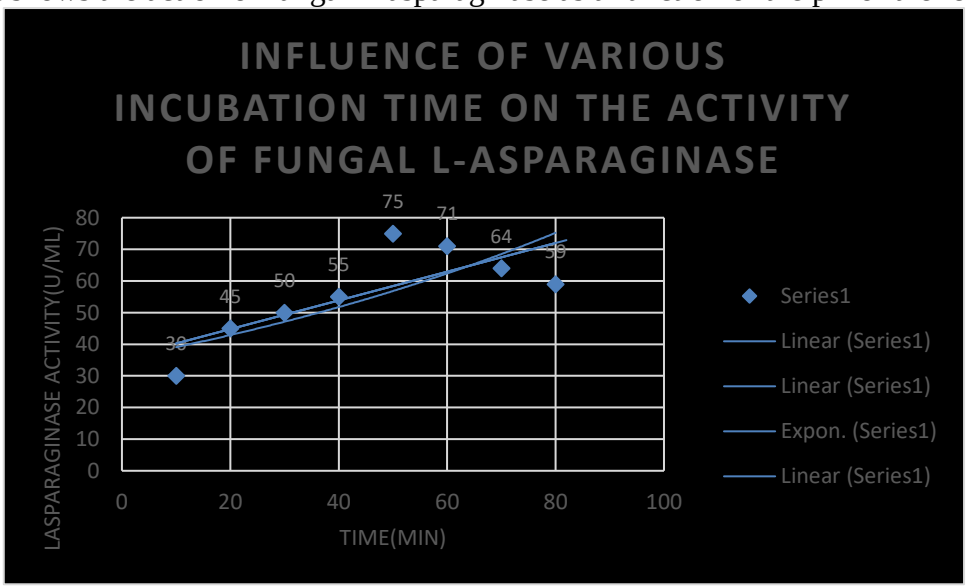

Fig. 10. It shows the influence of various incubation times on L-asparaginase activity**.**

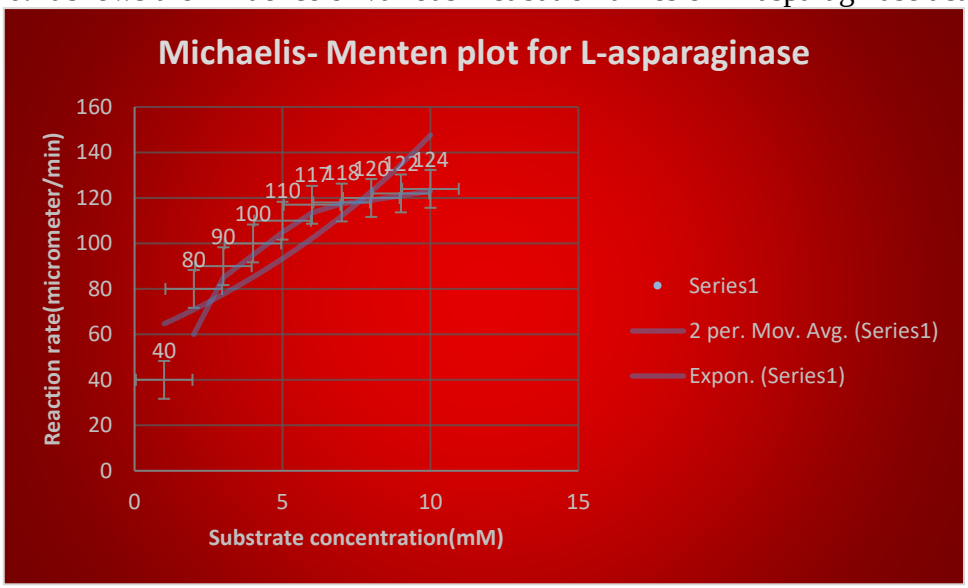

Fig. 11. It displays the Michaelis-Menten plot for L-asparaginase.

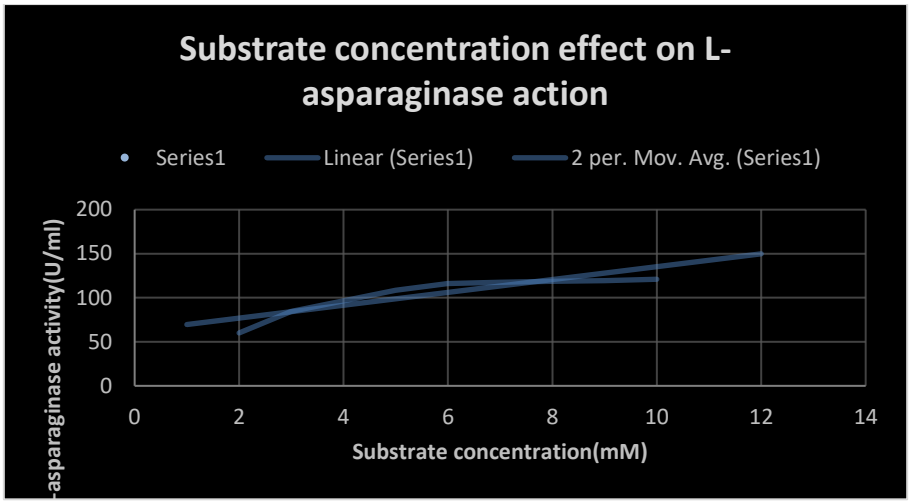

Fig. 12. It shows the Influence of substrate concentration on the enzyme activity of L-asparaginase.

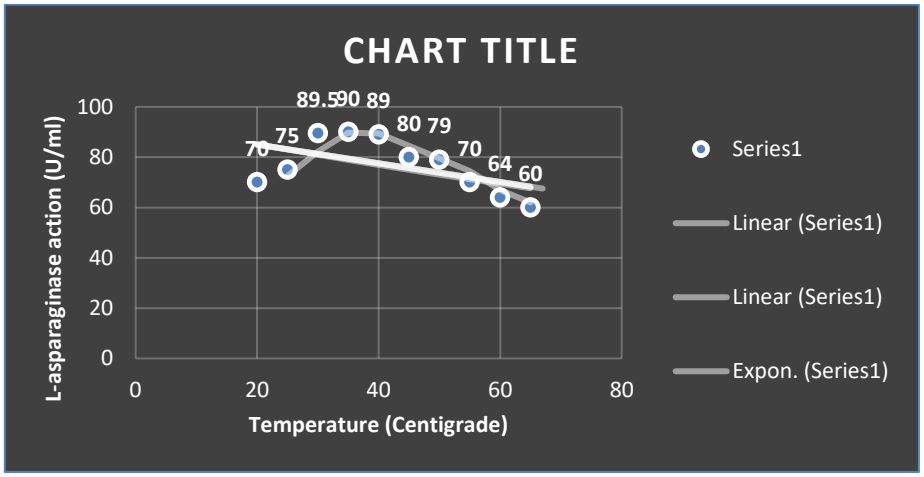

Fig. 13. It represents the effects of temperature on L-asparaginase activity**.**

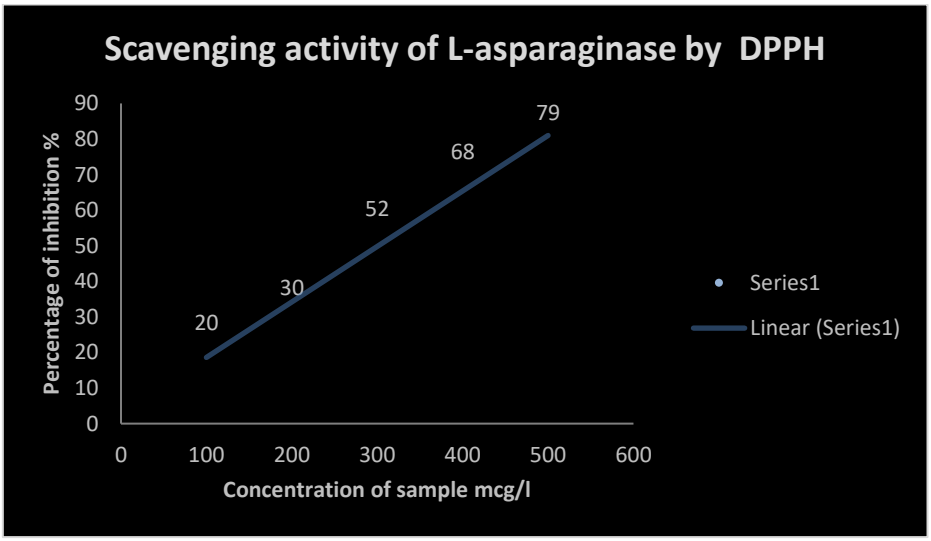

Fig. 14. It represents the scavenging activity(antioxidant activity) of L-asparaginase**.**

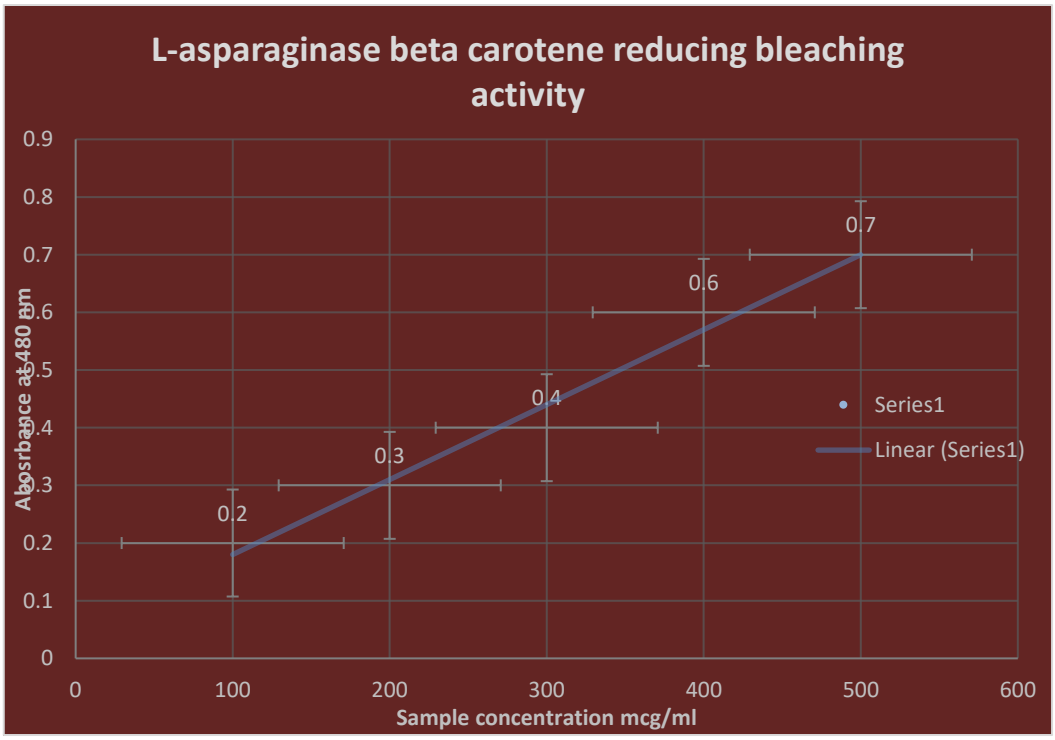

Fig. 15. It represents the beta-carotene bleaching-reducing action(antioxidant power) of L-asparaginase.

RESULTS

Production of L-asparaginase by *Aspergillus niger* was detected by plate assay. Enzyme production was demonstrated by the growth of a positive fungal isolate secreting L-asparaginase. The release of L-asparaginase and mycelium growth during shaking deposited as large spherical pellets indicating an improved production of L-asparaginase over development as free filaments. When L-asparaginase-glutaminase action was examined, L-asparaginase was not affected by glutaminase. Reaction pH exhibited a substantial character in the activity of most L-asparaginases. The purified L-asparaginase was active over a wide pH range from 5 to 11 with a value of 50.672 U/ml at pH 9. The enzymatic effect decreased at higher pH. Even at pH 11, L-asparaginase retained 69.18% and at pH 6 retained 62.426%. The effect of L-asparaginase from *Aspergillus niger* was gradually enhanced with explosive incubation times up to 49 minutes (L-asparaginase effect 68.129 U/ml). Only a slight decrease in L-asparaginase action was subsequently observed. The molecular mass was 65 KDa as ascertained by a mass spectrometer. Vmax of 163.8 UML^{-1} min^{-1} and a Km value of 3.41×10^{-3} M were shown by L-asparaginase. The heat inactivation half-life time ($t_{1/2}$) was70.13 min at 65°C(Kd 0.040 min^{-1}); while it was 72.05 min at 55°C(Kd 0.053 min^{-1}). 83% tumor growth prohibition was observed in mice treated with test L-asparaginase; while it was 71% in mice challenged with commercial L-asparaginase. The pure L-asparaginase presented a 5.246 − purification fold and a total action of 801.032 with a particularized activeness of 68.129 U/mg protein. No detectable IgG antibodies to L-asparaginase were noticed utilizing the ELISA technique. L-asparaginase displayed peak action at pH 9, 37 °C temperature, time of incubation about 49 min, and optimal concentration of substrate 7 mM.

Table 3. It represents a distribution of positive and negative fungal isolates secreting L-asparaginase:

Positive samples	Negative samples
37	13

Table 4. It shows an estimation of L-asparaginase activity by the direct salicylate technique:

Concentration Of soil fungal culture containing L-asparaginase enzyme(serial dilutions from 10*-1 to 10*-6 mg/l)	The optical density of liberated metabolic nitrogenous compound (liberated urea) at 425 nm by UV spectrophotometer
0.00	0.00
1/10	0.438
1/100	0.376
1/1000	0.307
1/10000	0.226
1/100000	0.151
1/1000000	0.078

Table 5. It shows a computation of fungal L-asparaginase action via the direct Nesslerization technique.

Concentration Of soil mycotic culture containing L-arginase enzyme(serial dilutions from 10^{-1} to 10^{-6} mg/l)	The absorbance of liberated ammonia at 425 nm by UV spectrophotometer
0.00	0.00
1/10	0.410
1/100	0.382
1/1000	0.341
1/10000	0.283
1/100000	0.177
1/1000000	0.076

Table 6. It shows the action of fungal L-asparaginase as a function of the reaction PH:

PH	L-asparaginase action
3.5	10
4.5	20
5.5	30
6.5	40
7.5	45
8.5	50
9.5	47
10.5	39

Table 7. It shows the influence of various incubation times on L-asparaginase activity**:**

Incubation time(min)	L-asparaginase action(U/ml)
10	30
20	45
30	50
40	55
50	75
60	71
70	64
80	59

Table 8. It shows the Michaelis-Menten plot for L-asparaginase:

Substrate concentration (mM)	Rate of reaction (µM/min)
1	40
2	80
3	90
4	100
5	110
6	117
7	118
8	120
9	122
10	124

Table 9. It shows the Influence of substrate concentration on the enzyme activity of L-asparaginase:

Substrate concentration (mM)	L-asparaginase activity(U/ml)
1	42
2	78
3	91
4	102
5	115
6	117
7	118
8	119
9	120
10	122

Table 10. It shows the effect of the temperature on L-asparaginase activity.

Temperature ^{0}C	L-asparaginase activity U/ml
20	70
25	75
30	89.5
35	90
40	89
45	80
50	79
55	70
60	64
65	60

Table 11. It demonstrates the scavenging activity of L-asparaginase via DPPH assay: sample concentration(mcg/l) versus percentage of inhibition (%).

Concentration of sample(mcg/l)	Percentage of inhibition(%)
100	20
200	30
300	52
400	68
500	79

Table 12. It represents beta carotene bleaching reducing the activeness(antioxidant power) of L-asparaginase:

Concentration mcg/ml	Absorbance at 480 nm wavelength
100	0.2
200	0.3
300	0.4
400	0.6
500	0.7

No L-glutaminase action was detected via the Nesslerization test.

Purified L-asparaginase worked over a wide temperature range from 20 to 55 °C. The optimal action of L-asparaginase was 77.45 U/ml at 37°C, with lower L-asparaginase action observed at higher temperatures. The selectivity index of fungal L-asparaginase against cancer cell lines was 3.97 against the CCL-120 cancer cell line. To investigate whether L-asparaginase treatment has an inhibitory effect on DNA synthesis in auxotrophic cancer cell lines, we applied a colorimetric BrdU proliferation assay. A dose-dependent decrease in the proliferation of auxotrophic cancer cells was observed after treatment with L-asparaginase for 48 hours. 84% a decrease in cell proliferation of CCL-120 cancer cell line, by L-asparaginase, was observed at 0.9 IU/mL.

The caspase-3 apoptotic index of the L-asparaginase-treated CCL-120 cancer cell line increased in a dose-dependent manner. The results supported the fact that L-asparaginase produced induced apoptotic cell death by increasing the enzymatic activity of caspase-3. L-asparaginase at a concentration of 0.5 IU/ml induced up to 18% caspase-3 action in the CCL-120 cancer cell line. Even at a concentration of 0.9 IU/ml, this induction was increased by up to 24% in CCL-120 cancer cell line.

Results showed the effect of different substrate concentrations ranging from 1 to 10 mM on the action of L-asparaginase and confirmed the optimal substrate concentration required for maximum yield of L-asparaginase action. The results obtained showed a gradual increase in enzyme action as the substrate concentration increased up to 7 mM, indicating an optimal substrate concentration for enzyme action. However, increasing substrate concentrations (8–10 mM) decreased enzymatic action. Activation of L-asparagine hydrolysis was studied in terms of changes in rate constant values(Km and Vmax). Km and Vmax values were deliberated by Michaelis-Menten plots plotting the relationship between different substrate concentrations [S] and enzyme activity (V). A Michaelis-Menten plot showed the Km and Vmax values for the L-asparaginase enzyme. Plots showed the hydrolysis of L-asparagine by *Aspergillus niger* L-asparaginase with a Km value of 3.415 × 10-3 M, a Vmax of 163.8 UML^{-1} min^{-1}; but a Km value of 1.82×10^{-4} M and a Vmax of 119.6 UML^{-1} min^{-1} following Hydrolysis of L-asparagine by a commercially available *E. coli* L-asparaginase. Purified L-asparaginase was more stable at alkaline pH than at acid pH. It retained a potency of 92.81 at pH 9 after 5 hours of incubation and a potency of 70.59

at 24 hours. Furthermore, the enzyme retained approximately 85.15% of its activity after 6 hours at pH 9.5. At pH 4.5, the enzyme retained an activity of 70.31 at 6 hours and a residual activity of 19.84 at 24 hours. Half-life in hours based on pH studies of L-asparaginase yielded from *Aspergillus niger*. Nevertheless, cessation of enzyme action at pH 4.5 was recorded with a short half-life (14.68 hours). The effect of temperature on L-asparaginase stability was investigated and maximum L-asparaginase stability was recorded at 40 °C, with 96.98% of the initial enzymatic activity retained after a 20 min incubation period. However, when the enzyme was exposed to high temperatures and long incubation times of 90 min at 50 °C and 80 °C, a rapid decline in L-asparaginase activity was observed, with residual activities of 29.71% and 8.25%, respectively. Meanwhile, a linear regression of the results obtained was performed using Graph-Pad Prism software to determine the heat-inactivation half-life (T1/2) and heat-inactivation constant (KD). Via fitting the data showing a linear equation, L-asparaginase stability was expressed as a percentage of residual activity compared to the initial (100%) activity of the untreated enzyme. The enzymatic half-life (T1/2) of L-asparaginase was 64.87 min at 50°C and 61.48 min at 60°C. However, termination of enzymatic action was recorded at 80°C with a short half-life (50.06 min).

Table 3 represents a distribution of positive and negative fungal isolates secreting L-asparaginase. Table 4 shows an estimation of L-asparaginase activity by the direct salicylate technique. Table 5 shows a computation of fungal L-asparaginase action via the direct Nesslerization technique. Table 6 shows the action of fungal L-asparaginase as a function of the reaction PH. Table 7 It shows the influence of various incubation times on L-asparaginase activity. Table 8 shows the Michaelis-Menten plot for L-asparaginase. Table 9 shows the Influence of substrate concentration on the enzyme activity of L-asparaginase. Table 10 shows the effect of the temperature on L-asparaginase activity. Table 11 demonstrates the scavenging activity of L-asparaginase via DPPH assay: sample concentration(mcg/l) versus percentage of inhibition. Table 12 It represents beta carotene bleaching reducing the activeness (antioxidant power) of L-asparaginase. Figure 1 shows molds of *Aspergillus niger spp* with septate hyphae producing L-asparagine degrading enzymes as anticancer agents. Figure 2 represents *Aspergillus niger* growing on MAM. Figure 3 shows recombinant proteins of L-asparagine degrading enzymes measured via the Northern blot technique. The maximum yield of recombinant proteins was 65 mg/l. The purity of recombinant Asparagine degrading enzymes was approximately 86%. Figure 4 represents a Nesslerization screening assay of different concentrations of soil fungal L-asparaginase. Figure 5 represents a distribution of L-asparagine degrading enzymes producing fungi. Figures 6 and 7 FTIR spectroscopy and DSC study show no drug-drug interaction or incompatibility between L-asparaginase and excipients. Figure 8 shows a 3D structure of L-asparaginase. It comprises 295 amino acids. Figure 9 shows the action of fungal L-asparaginase as a function of the pH of the reaction. Figure 10 shows the influence of various incubation times on L-asparaginase activity. Table 11 demonstrates the scavenging activity of L-asparaginase via DPPH assay: sample concentration(mcg/l) versus percentage of inhibition. Figure 12 shows the Influence of substrate concentration on the enzyme activity of L-asparaginase. Figure 13 represents the effects of temperature on L-asparaginase activity. Figure 14 represents the scavenging activity (antioxidant activity) of L-asparaginase. Figure 15 displays the beta-carotene bleaching-reducing action (antioxidant power) of L-asparaginase.

DISCUSSION

Thirty-seven soil samples from various settings in Egypt showed that the fungus *Aspergillus niger* was the major L-asparaginase-producing isolate grown on MAM selective medium and malt extract

agar. It was characterized by a mold with septal hyphae visible by light microscopy. A mold with green spores and conidia in the radiant tube was grown on MAM selective medium and malt extract agar. This was confirmed by molecular engineering hybridization with DNA probes. L-asparaginase catalyzes the hydrolysis of L-aspartic acid amino acids to L-aspartic acid and ammonia. Ammonia concentrations were determined by direct Nesslerization measurement and a salicylic acid test. The amount of ammonium released was directly proportional to the enzymatic activity. Optimal conditions for enzyme production were 6.5 and temperature 25°C. Activators of enzyme production were KH2PO4 (1.2 g), MgSO4 (0.6 g), FeSO4 (0.005 g), and KCL(0.4 g). Compared to the bacterial enzyme, this fungal enzyme showed higher potency as an antileukemic and anticancer agent than a bacterial L-asparaginase produced from *Escherichia coli or Erwinia chrysanthemum*. In addition, this fungal enzyme can overcome the shortcomings of L-asparaginase in bacteria. It has lower glutaminase activity and fewer hypersensitivity reactions (such as drug neutralization and anaphylactic reactions) than bacterial L-asparaginase. Cell lines used to evaluate cytotoxicity and anticancer activities of L-asparaginase enclosed: I-CCL-120 cancer cell line demonstrated by in vitro cell viability MTT assay test. II-Vero cell line was utilized to assess the biological, physiological, pharmacological, and toxicological effects of the L-asparaginase enzyme on mammalian cells. L-asparaginase displayed fantabulous DPPH scavenging power at an extract concentration that provided 50% abstinence (IC50) of 19.073 micrograms/ml and powerful beta-carotene bleaching after 94 min incubation with an IC50 of 32.89 micrograms/ml showed inhibition. The molecular weight of L-asparaginase was 65 kDa as estimated by mass spectrometry. It exhibited its peak activity at pH 8.5 with an optimum temperature of 37 °C. A Michaelis-Menten constant study yielded a Vmax of 163.8 UML^{-1} min^{-1} and a Km value of 3.41×10^{-3} M with L-asparagine as substrate. The half-life (t1/2) was 72.05 minutes at 55°C and 70.13 minutes at 65°C. Mice challenged with test L-asparaginase produced greater cytotoxic events (83% tumor growth inhibition) compared to the commercial L-asparaginase extract (71% tumor growth inhibition).

Determining the Optimal Dosage Form and Route of Administration of Fungal L-Asparaginase: In our study, the optimal dose and route of administration for the fungal L-asparaginase enzyme was intramuscular or subcutaneous injection. It had a short half-life (t1/2 = 3 hours) and required dosing several times a day. In our study, we were able to solve this problem by prolonging the action time by adding polyethylene glycol. It is recommended to be injected intramuscularly or subcutaneously once daily. Oral drug delivery systems for L-asparaginase have been unsuitable due to the chemical instability of the enzyme in the acidic environment of the stomach and degradation by proteolytic enzymes in the gastrointestinal tract such as pepsin and trypsin protease enzymes.

Selectivity Index of Fungal L-Asparaginase Against Cancer Cell Lines:

In previous studies and a comparison with standard doxorubicin, our fungal L-asparaginase showed activity against auxotrophic cancers for L-asparagine such as acute lymphoblastic leukemia and was observed to have higher selectivity against auxotrophic cancer types for L-asparagine. L-asparaginase mechanism of action: In our study, fungal L-asparaginase exerts anticancer activity against cancers auxotrophic to L-asparagine through its cytostatic effect on cancer. and was found to induce apoptosis in auxotrophic cancer cells. Deprivation of L-asparagine, a major metabolic source of auxotrophic cancer cells causing their death is another mechanism of its anticancer activity.

The occurrent study detected L-asparaginase secreted by *Aspergillus niger*. The enzyme produced was partially purified by ammonium sulfate precipitation and ion exchange chromatography was used to obtain

pure enzyme. Recently, the L-asparaginase enzyme from *Aspergillus niger* was successfully run with 83.43 total purifications in the final purification.

Purification of L-asparaginase from *Streptomyces albedo flavus* and *Pseudomonas aeroginosa 50.071* was finished by CM Sephadex C-50 column chromatography. SDS-PAGE separation showed only one band characteristic of the enzyme preparation and no detectable contamination. Therefore, L-asparaginase was a homogeneous protein. The molecular weight of each band was determined at a molecular weight of 65 kDa (Sumit Gupta *et al.,* 2020). The molecular weight of L-asparaginase has been found to vary with the enzyme source of purified *Bacillus* species. *Streptomyces gulbargensis, Streptomyces albidoflavus, Streptomyces PDK2 and Streptomyces noursei* showed molecular weights of 45, 85, 112, 140 kDa and 102 kDa, respectively. Amidase enzymes such as L-asparaginase are generally stable and active at neutral and alkaline pH. Previous reporters showed optimal amidase activity in the pH range of 5.0-9.0. The optimum pH for L-asparaginase purified from *Aspergillus niger* was 9 and PDK7. Streptomyces albidoflavus reportedly exhibited maximal L-asparaginase at pH 7.5. *Aspergillus niger* L-asparaginase activity increased gradually with increasing incubation time up to 49 min. Nevertheless, longer incubation times with substrate decreased the L-asparaginase effect. This may be due to product abstinence. Purified L-asparaginase from *Streptomyces noursei* showed maximal activity after an incubation time of 35 minutes. Furthermore, L-asparaginase purified from *Pseudomonas aeruginosa 50,071* reached maximal effect after 30 min, L-asparaginase was active over a wide temperature range of 25–60 °C, and optimal L-asparaginase action was established at 88.16 U/ml at PH9 and Seen at 37 °C. Lower L-asparaginase activity was observed at higher temperatures. Siddalingeshwara and Lingappa recorded an optimal temperature of 37 °C for maximal enzymatic activity in *Aspergillus terreus KLS2*. This property of the enzyme makes it ideal for the complete removal of L-asparagine from the body when treating tumor patients with L-asparaginase.Comparable results have been reported for the maximum activity of purified L-asparaginase at 40°C. The Michaelis-Menten plot showed a Km value of 3.415×10^{-3} M with L-asparagine as the substrate, and the Vmax for the hydrolysis of L-asparagine by *Aspergillus niger* L-asparaginase was 163.8 UML^{-1} min^{-1}. Values for the hydrolysis of L-asparagine by commercially available *E. coli* L-asparaginase was 1.82×10^{-4} M with a Vmax of 119.6 UML $^{-1}$ min, which was half the maximal rate of the enzymatic reaction as a function of substrate concentration. Nevertheless, the enzymatic rate constants for Km and Vmax were dependent on the type of enzyme, the different forms of the enzyme (crude, modified, or purified), the source of the enzyme and the conditions used. The rate of enzyme-catalyzed reactions increased when the substrate concentration exceeded a certain level called the maximum velocity (Vmax). Since there was no enzyme to react with the substrate at Vmax, increasing the substrate concentration did not accelerate the reaction. The maximum velocity Vmax indicated the number of substrate molecules converted to product by an enzyme per unit of time when the catalytic center of the enzyme was completely saturated with its substrate. According to the data reported in this study, the maximum thermostability behavior of L-asparaginase was 40 °C. A previous study by Senthil Kumar and Selvam showed that pre-incubation of purified L-asparaginase from *Streptomyces radiopugnans MS1* at 40 °C for 60 min did not result in a significant loss of enzymatic activity. Similar results have been recorded for many other microorganisms, including *Streptomyces noursei, Pseudomonas stutzeri MB 405, and E. carotovora*. “Enzyme stability and heat inactivation were considered to be major obstacles to the rapid development of biotechnological processes. They were also, regarded as a very important enzyme selection tool for industrial use. Purified L-

asparaginase was more stable at alkaline pH, similar results were reported for L-asparaginase from *Pseudomonas stazzeri MB-405*, which was maximally stable over the pH range. Among the various metal ions tested, Mn^{2+} increased L-asparaginase activity by 18%, as reported by previous researchers on *Fusarium culmorum ASP-87 and Mucor hiemalis*. Hussein et al. reported that monovalent cations such as Na^{+} and K^{+} enhanced the activity of *Enterobacter cloacae* L-asparaginase. K^{+} also functioned as an enhancer of Pectobacterium carotovorum asparaginase. Radha et al. reported that the activity of L-asparaginase of *Vibrio cholerae* increased to 130% in the presence of Ca^{2+}. Additionally, Meghavarnam and Janakiraman reported that the nonionic surfactant Tween 80 was found to increase enzymatic activity by 16%. Senthil Kumar and Selvam reported that EDTA acts as an inducer of *Streptomyces radiopugnans MS1*. EDTA as a metal chelator had no effect on *P. carotovorum* asparaginase. However, as reported by Borkotaky and Bezbaruah, L-asparaginase was completely inhibited by EDTA. In contrast, EDTA inhibited the activity of fungal L-asparaginase from *Trichoderma viride* by 88%. *Fusarium culmorum ASP-87* L-asparaginase activity was also inhibited by EDTA, SDS, and Cu^{2+}. Hussein et al. reported that the divalent and trivalent cations Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{3+} inhibit enzymatic activity. Radha et al. reported that the L-asparaginase activity of Vibrio cholerae was inhibited by the divalent cations, Fe^{2+} Mg^{2+}, Ni^{2+}, Mn^{2+}, and Zn^{2+}, and a complete loss of activity was observed in the presence of Cu^{2+}. Archana and Raja reported that Cu^{2+} inhibited the activity of L-asparaginase produced by *Aspergillus nidulans* by 84%. Kumar et al. reported that Hg^{2+} inhibited L-asparaginase activity produced by *Pectobacterium carotovorum* by 80%. However, L-asparaginase was completely inhibited by Fe^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+}. Husayn et al. Reported that no L-asparaginase activity was noticed when the enzyme was incubated with the metal ions Ni^{2+},Cd^{2+}, and Hg^{2+}. Other metal ions such as Ca^{2+} and Mg^{2+} possessed no significant effect on the enzymatic activity, and β-mercaptoethanol had no effect on the fungal L-asparaginase activity of Trichoderma viride. $Co2^{+}$, Mn^{2+}, and Mg^{2+} ions increased enzymatic activity, suggesting that these metal ions can act as cofactors to aid in triggering enzymatic reactions. Metal ions showed a crucial role in keeping the progressive configuration of enzymes at raised temperatures by protecting them from thermal denaturation (Luke *et al.,* 2022). The divalent cationsMg^{2+} and Mn^{2+} have been rumored to increase the thermostability of *Bacillus* alkaline protease. On the other hand, for monovalent cations, Na^{+} was found to enhance L-asparaginase activity, indicating that the enzyme may contain Na^{+} ions (El-Naggar *et al.*, 2020).

Conclusion

This was a promising approach study due to the novel production of L-asparaginase with high anticancer activity and few adverse effects against acute lymphoblastic leukemia. Fungal L-asparaginase was produced from *Aspergillus niger* grown on MAM selective medium. This enzyme exhibited anti-leukemic and anti-cancer activities superior to bacterial L-asparaginase produced by *E. coli* and *Erwinia chrysanthemum*. It also produced less glutaminase than bacterial L-asparaginase and produced fewer hypersensitivity reactions.

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