Screening and Exhibition of Fungal L-glutaminase Enzyme as an Anticancer Agent in Different Egypt Soil Environment

Original Article

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ABSTRACT

Background: Acute lymphoblastic leukaemia and hepatic auxotrophic carcinoma for L-glutaminase are two of the leading causes of mortality worldwide. The bacterial L-glutaminase enzyme, which is normally produced under harsh circumstances from *Escherichia coli or Bacillus cereus*, was used to treat acute lymphoblastic leukaemia. Fungal L-glutaminase is preferred over bacterial L-glutaminase due to less hypersensitivity events, such as allergic responses and medicine neutralization; as well as higher efficiency against hepatic carcinoma.

Aim of Study: The objective of the present work was to produce fungal L-glutaminase enzyme from different soil types in Egypt to be used as an oncolytic agent.

Patients and Methods: In the current screening experiment, mineral L-glutamine agar (MGA), a selective medium, was employed for the production of fungal L-glutaminase. Malt agar media was further used to sub-cultivate fungal L-glutaminase producing strains. L-glutaminase-producing strains were molecularly identified by using the Southern blotting method. The fungal L-glutaminase gene was firstly isolated using specific PCR primers then the gene was sub-cloned and inserted into a DNA vector to be produced via recombinant DNA technology. In order to assess anticancer activity, the MTT test was performed. Molecular mass of L-glutaminase was assessed via a mass spectrometer; then confirmed with western blot assay.

Results: *Aspergillus niger Strain ATCC 1015* was identified as the major fungal isolate that produced this enzyme. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay test results showed that the IC50 values for anticancer activity against the cancer cell lines CCL-120 and JHH4 were 38.9 and 40.3 g/ml, respectively. L-glutaminase had a molecular mass of 65 kDa, a specific activity of 15.3 U/mg protein, a yield of 57.6%, and a purity factor of 3.8. Extracellular L-glutaminase, 6.8U/ml, was generated. The conventional vitamin C and L-glutaminase have IC50s for their antioxidant activity of 89 g/ml and 189 g/ml, respectively. The inoculum contained $1*10^8$ spores/ml.

Conclusion: As fungus in diverse Egyptian soil environments evolved into a novel source of the enzyme L-glutaminase, the current investigation was a promising one.

Key Words: Fungal, L-glutaminase, Oncolytic agent, Production.

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INTRODUCTION

Among the major causes of death globally are acute lymphoblastic leukaemia and hepatic auxtrophic carcinoma for L-glutaminase (**Dhankhar *et al.,* 2020**). L-glutamine is hydrolytically deaminated by L-glutaminase, an amidohydrolase, producing L-glutamic acid and ammonia in the process (**Caroline and Zeind 2018**). Molds and yeasts are are eukaryotic creatures. Mold and yeast are the two different categories of fungus (**Trevor *et al.,* 2022**). Molds generate a mat by growing as long filamentous hyphae (**Bardal *et al.,* 2021**). Certain hyphae (septate hyphae) form transverse walls (**Olson, 2020**), but other hyphae do not form these transverse walls (nonseptate hyphae)(**Levinson, 2021**). The expansion of the hyphal tip instead of cell division is the mechanism by which hyphae develop (**Swanson *et al.,* 2019**). The multicellular nonseptate hyphae are coenocytic (**Fisher *et al.,* 2021**). None of the fungi are obligatory anaerobes (**Dipro *et al.,* 2021**), although the majority of them are obligate aerobes (**Golderg, 2020**). Thermally dimorphic fungi include several significant ones for medicine (**Wilson, 2019**). At body temperature, they live as molds in the environment (**Metting, 2019**) and as yeasts in human tissues (**Butheina *et al.,* 2021**).

Acute lymphocytic leukaemia was treated with the bacterial L-glutaminase enzyme, which is typically generated by *Bacillus cereus or Escherichia coli* (**Das *et al.,* 2013**).

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L-glutaminase breaks down L-glutamine amino acid (which is necessary for the proliferation of cancer cells since they cannot synthesis it and must acquire it from extracellular fluids, blood, and lymph) into L-glutamate and ammonia (**Awad *et al.,* 2021**).

Nevertheless, since *Escherichia coli and Bacillus cereus* are both prokaryotes. A number of adverse events, including allergic reactions and medication neutralization, are brought on by bacterial L-glutaminase (**Jambulingam, *et al.,* 2014**). L-glutaminase from fungal eukaryotic cells on the other hand, is comparable to human enzymes (**Hamed and Al-wasify, 2016**).

Excellent scavenging and antioxidant properties were shown by L-glutaminase. For the increased synthesis of fungal L-glutaminase, starch and L-glutamine at 1% w/v have been shown to be the optimum sources of nitrogen and carbon (**Khalil *et al.,* 2020**).

The current study's goal was to produce a fungal L-glutaminase from a novel yeast source in order to overcome the limitations of bacterial L-glutaminase.

PATIENTS AND METHODS:

Ethical statement

All appropriate national, international, and/or institutional standards for the care and use of people and animals were followed in the current study. The ethical committee for animal handling at Cairo University (ECAHCU), at the Faculty of Pharmacy, Cairo University, Egypt, approved all procedures used in the study, including the use of animals, in accordance with the recommendations of the weather-all report with permission number P-18-7-2021. Every attempt was made to reduce the number of animals used in the study and their suffering.

Type of study

A screening experimental investigation was conducted in this one.

Place and date of the study

In Egypt, Cairo University's pharmacy school conducted this study between November 2021 and December 2022.

Collection of the samples

From various locations in Egypt, 100 soil samples with a depth of 10 cm were taken (Menofia, Sharqia, Qalyobia, Giza and Cairo govern-orates).

Material source

The chemical and biological components were bought from the Egyptian Algomhuria Pharmaceutical Chemical Company. From the Accegen biological firm, cell lines were bought.

Screening of positive fungal L-glutaminase producing isolates

Mineral glutamine agar (MGA):

To screen isolates of fungi that produce L-glutaminase, selective medium was employed. Its composition (g/l) included, KH2PO4 (1.2), MgSO4 (0.6), FeSO4 (0.005), KCL (0.4), D-glucose (7), Agar (13), L-glutaminase (15), and antibiotic Thiamphenicol made up the substance (0.15). Thiamphenicol was included to stop the development of bacteria. The incubation period was three days, with a PH of 6.5 for the medium. In this selective media, only the colonies that could use L-glutaminase as a source of nitrogen and carbon could grow. The positive isolates were kept at 4^{o}C for further research.

Malt extract agar medium:

It is an all-purpose acidic growth medium for the isolation and growing of fungi. NaOH was used to alter the pH to 6.5. After three days of incubation, subcultures of the positive isolates (produced on MGA plates) were cultured and isolated on malt extract agar medium at 20^{o}C, PH 6.5.

After being dissolved in 10% KOH, positive fungal isolates could be examined under a standard normal microscope. Using Southern DNA blot hybridization, the molecular identification of positive fungal isolates was completed.

Production of fungal L-glutaminase by recombinant DNA technology:

The following are the primary procedures in designing homologous primers (known sequences): Recovering or obtaining the target gene (nucleotide sequence). The target gene was then subjected to a restriction analysis using the software tool restriction Mapper. The Staden package program's Spin subprogram was then used to determine the reading frames. Also, the nucleotide sequences of the reverse primer on the complementary strand and the forward primer on the parent DNA strand were determined.

The whole L-glutaminase genome from NCBI was cloned using fasta. Moreover, the CDS of the L-glutaminase enzyme from NCBI was copied. The vector *pET-14b* (purchased from Novagene in the United States) plasmid was selected as the expression vector.

According to the webcutter website, suitable restriction enzymes for forward and reverse primers were chosen. Enzyme has to be in the plasmid vector's multiple cloning sites (MCS), accessible, and in line with the orientation of the gene that will be copied and expressed.

Using the PDRAW32 Microsoft tool, the T annealing for forward and reverse primers was calculated after appropriate restriction enzymes, forward and reverse enzymes, and primers were identified. The difference

in annealing temperature between forward and reverse primers shouldn't be greater than 3 degrees Celsius. This provided the best conditions for the protein of interest to be cloned and expressed.

Procedure

The new primer for expression of L-glutaminase as anticancer agent:

Forward primer for expression: Direction of sequence was from 5- to 3-*AGCTGCCCTTGTCATAGTTG*

Annealing temperature= melting temperature-5= 57.88-5=52.88^{o}C.

A-Reverse primer for expression: Direction of sequence is from 5- to 3-*AAGGCAGTTCTCCTTTGCCT*

Annealing temperature= melting temperature-5=59.52-5=54.52^{o}C.

The expression vector was the plasmid *pET-14b* (purchased from Novagene in the United States). According to the webcutter website, the two restriction enzymes utilised and selected were Bam HI and Sphl. The L-glutaminase enzyme's nucleotide sequence (*cDNA*) was successfully isolated from the yeast extract and then put into PCR for amplification. Finally, this sequence was inserted into an appropriate expression vector, such as *pET-14b plasmid*. Also, when the expression vector was transformed into *Escherichia coli BL21(DES) POLYSS* for the activation of the transcription process, inducible IPTG was added to the expression host. Expression was carried out throughout production in *Escherichia coli. T7 lac* served as the promotor, and the tag protein had a 6x histidine tag at the C terminus. L-glutaminase was isolated from yeast and its gene (*cDNA*) was cloned using PCR. The expression vector *pET-14b plasmid* was; then transformed into the *E. coli BL21(DES)POLYS*S, and the recombinant protein was partly purified by ammonium sulphate from the supernatant. Next, using nickel affinity chromatography on immobilised nickel columns, 85% of the L-glutaminase was purified. In order to build and spread plasmids, *E. coli DH5* (obtained from stratagene) was used as the main host. *IPTG* stimulated the gene's expression. For the initial culture, the yield was close to 650mg/L.

For a standard bacterial culture, LB agar and broth were used, and an incubation period of 24 hours at 37^{o}C was completed. In accordance with the manufacturer references' instructions, antibiotics were applied to the medium. The production of fusion proteins containing a 6 histidine tag, an L-glutamine recognition site, and a T7 tag at the N-terminal of the target protein was made possible using the pET-14b plasmid expression vector.

Clarification and purification of L-glutaminase:

Three minutes at a speed of 4,000 rpm were spent centrifuging. By precipitation with ammonium sulphate and partial extraction of L-glutaminase precursor from the culture's supernatant, followed by purification with nickel affinity chromatography, the process was completed. After extraction by precipitation (salting out) of 100 ml of the supernatant with 53ml of a 4.1 M ammonium sulphate saturated solution at 25C, recombinant fused L-glutaminase with polyhistidine-tagged proteins could be quickly purified from the supernatant using nickel columns using immobilised metal affinity chromatography (the metal-ligand was a nickel-metal ion; while the target bio-molecule was polyhistidine tag fusion protein). The preparations were filtered using Whatman-1541-042 filter paper (0.22 micron), which was acquired in the USA, to sterilise them before the final formulation was completed (**Orabi *et al.*, 2019**).

Determination of fungal L-glutaminase production and activity

Direct Nesselerization test:

A positive fungal isolate cultured in MGA was utilised to characterise the enzyme synthesis and activity. The hydrolysis of L-glutamine amino acid into L-glutamic acid and ammonium is catalysed by L-glutaminase. The released ammonium was recognised and measured using a spectrophotometer at 425nm. The relationship between light intensity and ammonium concentration was linear. The enzyme activity was directly inversely correlated with the amount of released ammonium (**El-Sousy *et al.*, 2021**).

Salicylate method:

This approach is a variant of the popular phenate approach. Both phenol and mercury salts are absent. It is helpful for determining ammonium nitrogen in the low range.

The hydrolysis of L-glutamine amino acid into L-glutamic acid and ammonium is catalysed by L-glutamase.

At a UV wavelength of 425nm, the liberated ammonium was detected and measured spectrophotometrically.

The relationship between light intensity and ammonium concentration was linear.

The enzyme activity was directly inversely correlated with the amount of released ammonium (**Binod *et al.*, 2017**).

In vitro cell viability assay:

The physiologic, pharmacologic, and toxicological effects of the enzyme on cancer cells were evaluated using the CCL-120 cancer cell line. The physiologic, pharmacologic, and toxicological effects of the enzyme on hepatic cancer cells were evaluated using the JHH4 hepatic carcinoma cell line.

To evaluate the physiologic, pharmacologic, and toxicological effects of the L-glutaminase enzyme on mammalian cells, the Vero cell line was employed.

Fungal L-glutaminase was tested for in vitro cell viability using the MTT (dimethylthiazol-2-yl) diphenyl tetrazolium) method (**Patel *et al.,* 2013**).

Formulation of fungal L-glutamine degrading enzymes as anticancer agents:

The best dosage form and mode of administration of fungus glutamine-degrading enzymes as an anticancer treatment against auxotrophic tumors for L-glutamine were investigated in the current investigation using several pharmaceutical dosage forms and routes of administration:

The preparation of injectable goods took place in the presence of isotonic aqueous solutions with PH that are similar to that of bodily tissues and blood (PH 7.4). The antibacterial agent thiamphenicol was added to injections that had been prepared in containers. The release of L-glutamine-degrading enzymes from aqueous solutions administered intramuscularly was inhibited by increasing vehicle viscosity with carboxymethylcellulose (CMC). In order to extend the duration of effect to once-daily dosage administration rather than multiple-dose injections, ethylene glycol was added afterwards.

Wet granulation was used to create tablets containing 10 mcg/g of L-glutamine-degrading enzymes. As an excipient, 3% magnesium aluminium silicate was added. It was a glidant, disintegrant, and binder. starch 17% w/w as a diluent A lubricating ingredient, 1% weight/volume magnesium stearate, was applied.

Estimation of antioxidant actions of L-glutaminase:

This was accomplished using an assay for the scavenging of nitric oxide.

In a nutshell, 6ml of the reaction mixture containing sodium nitroprusside (SNP) 6mM in phosphate buffered saline pH 7.4 with L-glutaminase extract was incubated at 25^{o}C for 3 hours in front of a viewable polychromatic light-colored source (tungsten lamp 25 Watt). As a result, the radical of NO produced interacted with oxygen to produce nitrite ion (NO2-), which was measured at intervals of thirty minutes by mixing two millilitres of the incubation mixture with a balanced amount of the Griess reagent (sulphanilamide 2% in phosphoric acid 6% and N-naphthylethylenediamine dihydrochloride 0.2%). The chromophore optical density was measured at 540 nm. The amount of nitrite produced in the presence of L-glutaminase extract was calculated by using a standard curve built on noteworthy sodium nitrite solution values.

Assessment of secretion of antibodies to L-glutaminase:

With the use of an effective ELISA operator, the quantity of IgG anti-L-glutaminase antibodies in mouse serum was sealed.

The kinetic parameters Km and Vmax determination:

Purified L-kinetic glutaminase's parameters, Michaelis-Menten constant (Km), and maximum velocity (Vmax) were determined using the proper substrate concentrations of L-glutaminase (2–11 mM). The nonlinear exponential stage union regression curve was attached to the data. Software called GraphPad Prism 5 was used for this. By measuring the rate at which L-glutamine is hydrolyzed in a conventional laboratory setting, the Michaelis-Menten equation was used to get the L-glutaminase result.

The deactivation rate constant (KD) and half-life time ($t_{1/2}$)Estimation:

The thermal inactivation constant (KD) and thermal inactivation half-life ($t_{1/2}$) of the purified L-glutaminase enzyme were determined by running software of Graph-pad Prism number five.

Molecular weight of L-glutaminase determination:

Using the Western blot technique, which combines a 10% detachable acrylamide gel (pH 8.8) with a 5% stacking gel and 0.2% SDS according to Laemmli perception, it was possible to observe the mass and purity of the purified L-glutaminase enzyme. pH was 6.8. Gel staining was carried out using R-250 blue brilliant Coomassie, followed by a staining stage using a 5:2:6 solution of methanol, acetic acid, and water. Using a protein marker with an observed molecular weight range of 20–130 kDa, the molecular weight of L-glutaminase was determined. As a result, L-molecular glutaminase's weight was determined, and then it was confirmed using a mass spectrometer.

Determination of selectivity index of L-glutaminase:

The following formula was used to calculate an antineoplastic agent's selectivity index: SI is the ratio of the IC50 values for the test enzyme in healthy vs cancerous cell lines. The test enzyme's IC50 value denotes the concentration needed to kill 50% of the cells. Software called Graph Pad Prism version 5 was used for the deliberation. Also, the impact of varying PH, temperature, incubation duration, and substrate concentrations on the enzyme activity was evaluated. By a direct Nesslerizarion test, the location of L-glutaminase within the cell was determined.

Statistical analysis

Every culture was carried out in triplicate. They used standard deviation and means to present their findings.

One-way analysis of variance(p value ≤0.05) and statistical analysis using Excel spreadsheet software were both employed as techniques for doing statistical analysis. For this study, the F statistical test was used.

RESULTS

In present investigation, L-glutaminase was produced majorly via the yeast *Aspergillus niger Strain ATCC 1015*. L-glutaminase had a specific activity of 15.3 U/mg protein, a molecular mass of 65 KDa, a yield of 57.6%, and a 3.8 fold increase. L-glutaminase has an extracellular productivity of 6.8 U/ml. At an acidic pH, this enzyme exhibited no activity. It had more activity at alkaline PH, peaking at PH 9, but not reaching PH 10. L-glutaminase performed equally well in neutral PH 7.3 circumstances with Mg as a cofactor and alkaline conditions. L-glutaminase is administered intramuscularly or subcutaneously via injection. It needs to be administered numerous times daily and has a brief half-life. In the present investigation, we were able to get around this issue by adding polyethylene glycol to make the activity last longer.

With increasing incubation durations up to 50 minutes, the impact of *Aspergillus niger* L-glutaminase was steadily increased (L-glutaminase effect 70.099 U/ml). With the use of a mass spectrometer, the molecular mass was determined to be 65 KDa. L-glutaminase had a Vmax of 159.7 UML in 1 min and a Km value of 4.011 10 3 M. At 66°C (Kd 0.039 min-1), the heat inactivation half-life time ($t_{1/2}$) was 69.24 min, but at 54°C, it was 72.05min (Kd 0.061 min-1). The fungus L-glutaminase has a 3.97 selectivity index against the cancer cell line CCL-120 and a 3.42 selectivity index against the cancer cell line JHH4.

The ELISA test revealed no detectable antibodies against fungus L-glutaminase. The conventional vitamin C and L-glutaminase have IC50s for their antioxidant activity of 89 g/ml and 189 g/ml, respectively. MTT test results showed that the IC50 values for anticancer activity against the cancer cell lines CCL-120 and JHH4 were 38.9 and 40.3 g/ml, respectively.

The purity and yield of the produced test enzyme:

High yield and purity were attributes of the L-glutaminase synthesized in our investigation using the novel expression primer.

Determination of activity of L-glutaminase enzyme by direct Nesslerization and salicylate tests:

According to the results of these tests, the test L-glutaminase catalysed the transformation of L-glutamine amino acid into L-glutamic amino acid and ammonia. A spectrophotometer was used to calculate the concentration of ammonia that was released. The concentration of freed ammonia was directly inversely correlated with the L-glutaminase concentration and activity.

In vitro cell viability assay:

This experiment demonstrated greater anticancer activity of L-glutaminase against auxorophic cancer cell lines, including acute lymphocytic leukaemia cancer cells produced by tissue culture. Acidic PH has no effect on this enzyme's activity. The maximum activity was at PH 9, with increased activity at alkaline PH but not reaching PH 10. L- glutaminase was equally efficient in neutral PH 7.3 circumstances with Mg ions acting as cofactor.

The determination of optimum and suitable dosage form and route of administration of L-glutaminase enzyme:

L-glutaminase was administered intramuscularly or subcutaneously via injection. Given numerous times each day, it has a brief half-life. In our investigation, we were able to get around this issue by adding polyethylene glycol to make the activity last longer. This novel enzyme outperformed bacterial L-glutaminase in terms of performance and yield while being free of its negative side effects.

A calculation of the direct Nesslerization technique's fungal L-glutamine degrading effect is shown in (Table 1). A calculation of the activity of fungal L-glutamine-degrading enzymes using the Salicylate approach is shown in (Table 2). Metallic glutamine Agar's components are listed in (Table 3). Distribution of L-glutaminase cellular locations are displayed in (Table 4).

After adding 10% KOH to the slide specimen, *Aspergillus niger* molds that produce L-glutaminase are visible under a microscope in (Figure 1). Recombinant proteins of glutamine-degrading enzymes determined using the Western blot method are shown in (Figure 2). Around 85% of the recombinant L-glutamine-degrading enzymes were pure. Western blot analysis and a mass spectrometer both revealed the molecular mass to be 65 kDa.

The distribution of fungi that produce L-glutamine-degrading enzymes in grassland soil samples that were taken from various locations was shown in (Figure 3). A Nesslerization screening experiment of various doses of soil-based fungal L-glutamine-degrading enzymes is shown in Figure 4. Figure 2 displays varying concentrations of soil fungal culture harboring L-glutamine-degrading enzymes releasing ammonia at 425nm. The formulation of L-glutaminase is shown in Table 5 as a sterile solution at PH 7. The nitric oxide scavenging experiment in Table 6 illustrates the scavenging (antioxidant) activity of L-glutaminase. Image 3 shows the L-glutaminase enzyme Michaelis-Menten plot. By a nitric oxide scavenging experiment, Figure 5-7 illustrates the scavenging activity (antioxidant activity) of L-glutaminase. The fungal L-activity glutaminase's in relation to the reaction's pH, different incubation periods affect L-glutaminase activity and the Nesslerization assay's cellular location detection of L-glutaminase are demonstrated (figures 8-10).

Table 1: Displays a computation of fungal L-glutamine degrading action via the direct Nesslerization technique:

Concentration of soil bacterial culture containing L-glutamine degrading enzymes (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 2: Displays a computation of fungal L-glutamine degrading enzymes action via the salicylate technique:

Concentration Of soil fungal culture containing L-glutamine degrading enzymes (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	0.421
1/100	0.386
1/1000	0.372
1/10000	0.297
1/100000	0.188
1/1000000	0.082

Table 3: Ingredients of Metallic glutamine Agar:

Ingredient	Unit of measurement
Potassium chloride	0.5 mg
Magnesium sulfate	0.5 mg
$KH2PO_4$	1.0 g
Ferrous sulfate	0.1 g
Zinc sulfate	0.1 g
L-glutamine	1.0 g
Agar	2%

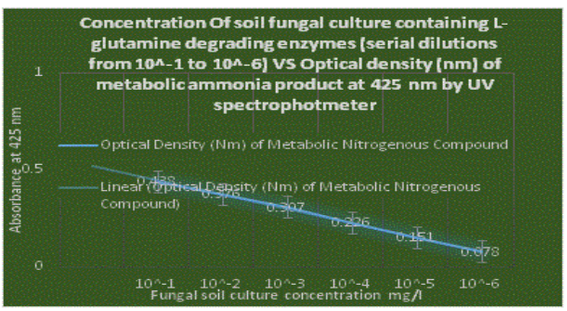

Figure 1: Serves as a Nesslerization screening test for various quantities of L-glutamine-degrading enzymes from soil fungi.

Table 4: Distribution of cellular positions of L-glutaminase:

Proportion	Percent(%)
Extracellular	69
Surface bound	21
Cytosolic	10

Table 5: Shows the formulation of L-glutaminase as a sterile solution at PH 7:

Ingredient	concentration
L-glutaminase	15 mg
PEG20	45 mg
Mono-basic sodium phosphate	USP, 1.5 mg+5%
Di-basic sodium phosphate	USP, 3 mg+5%
Sodium chloride	USP, 7.5 mg+5%
Water for injection	Query size to 1 ml

Table 6: Exhibits the scavenging (antioxidant) activity of L-glutaminase via nitric oxide scavenging assay:

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

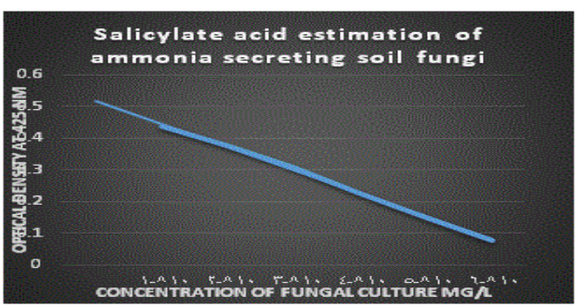

Figure 2: Ammonia was discharged at 425 nm by various concentrations of a soil-based fungus culture that included enzymes that break down L-glutamine.

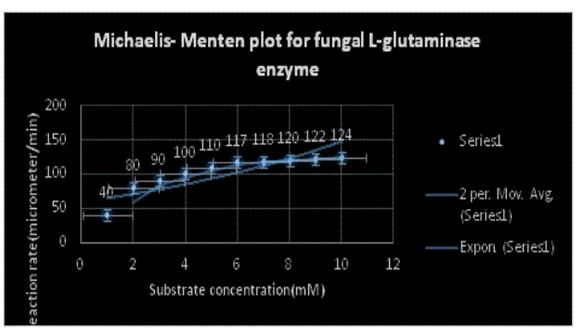

Figure 3: Shows the L-glutaminase enzyme's Michaelis-Menten plot.

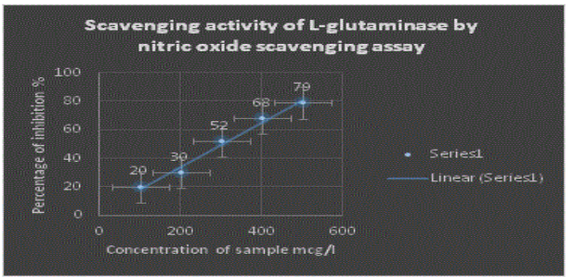

Figure 4: Using nitric oxide scavenging experiment, it illustrates the antioxidant activity (scavenging activity) of L-glutaminase.

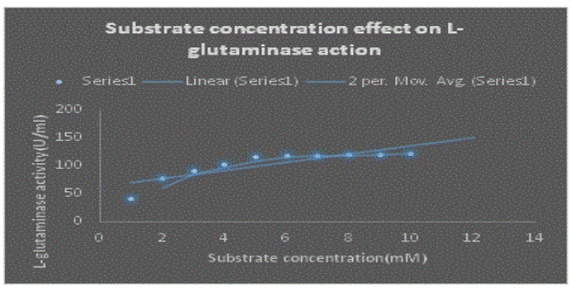

Figure 5: Displays the Influence of substrate concentration on the enzyme activity of L-glutaminase.

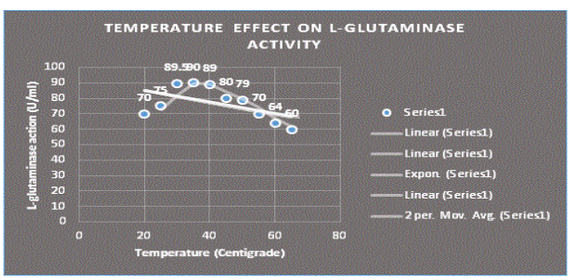

Figure 6: Stands for the effects of temperature on L-glutaminase activity.

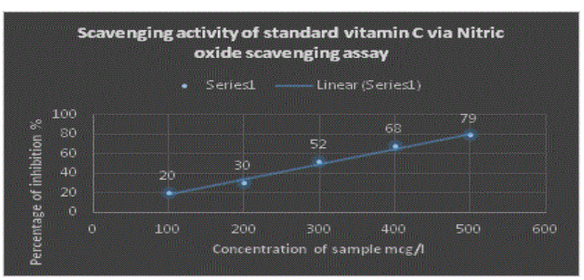

Figure 7: Represents the scavenging activity (antioxidant activity) of standard vitamin C.

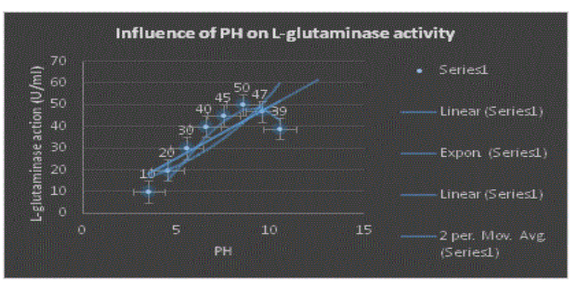

Figure 8: Demonstrates the action of fungal L-glutaminase as a function of the pH of the reaction.

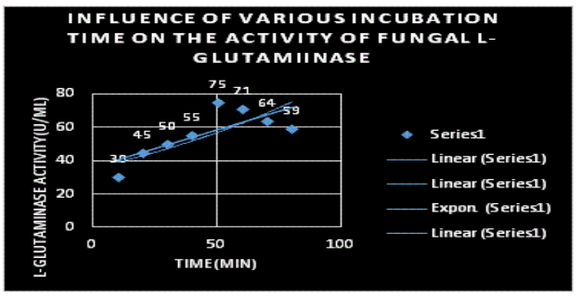

Figure 9: Displays the influence of various incubation times on L-glutaminase activeness.

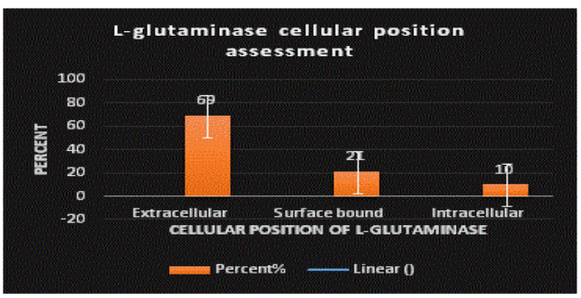

Figure 10: Demonstrates the Nesslerization assay's ability to detect the cellular localization of L-glutaminase.

DISCUSSION

The major isolate that produced L-glutaminase when grown on MGA selective medium and malt extract agar was the fungus *Aspergillus Niger Strain ATCC 1015*, which was discovered through analysis of 74 soil samples from various locales in Egypt. It was distinguished by mould with septate hyphae that could be observed under a light microscope. Using MGA selective medium and malt extract agar, molds with green spores and conidia in radiating channels were developed. By using DNA probes in a molecular method hybridization, this was verified. The hydrolysis of L-glutamine amino acid into L-glutamic acid and ammonia is catalyzed by L-glutaminase. Direct nesslerization and Salicylate assays were used to estimate the ammonia content. The enzyme activity was directly inversely correlated with the amount of released ammonium. pH 6.5 at a temperature of 25°C were the ideal conditions for the synthesis and biological activity of the enzyme. The enzyme synthesis was triggered by KH2PO4 (1.2g), MgSO4 (0.6g), FeSO4 (0.005g), and KCL (0.4g). As compared to bacterial L-glutaminase made from *Escherichia coli or Bacillus cereus*, this fungal enzyme demonstrated greater efficiency as an oncolytic agent against acute lymphocytic leukaemia and hepatic cancer (**Chitra *et al.*, 2022**).

Moreover, this fungal enzyme might compensate for bacterial L-shortcomings. glutaminase's Compared to bacterial L- glutaminase, it displayed less hypersensitive responses (such as medication neutralisation and anaphylactic reactions). This was demonstrated by an in vitro MTT assay test on several cell lines used in this

investigation. According to **Singh and Banik, 2013** study, monovalent cations such Na^{+}, K^{+}, and phosphate ions boosted the enzyme activity more than divalent cations like Pb^{+2}, Hg^{+2}, and Cd^{+2}. As well, this study stated that L-glutaminase enzymes produced mainly from microorganisms were intracellular proteins; however few exhibited extracellular portions (**Singh and Banik, 2013**). Nevertheless, in this study, Mg^{+2}, K^{+} ions enhanced L-glutaminase activity. As well, L-glutaminase in the current study was noticed to be extracellular protein with higher yield than most other origins of L-glutaminase.

According to a study by **Durthi *et al.*, 2020,** purified L-glutaminase from *E. coli or Bacillus cereus* had a maximum activity when incubated at PH 8 at 40°C for 30minutes (**Durthi *et al.*, 2020**); however, in this study, the enzyme's maximum activity was obtained after 50minutes of incubation at PH 10 at 35°C.

According to **Awad *et al.*, 2019**.'s study, L-glutaminase was stable at a broad range of PH between 3.5 and 11, and it demonstrated thermal stability up to 66°C with Tm value 58°C (**Awad *et al.*, 2019**). In this study, L-glutaminase was stable at a broad range of PH between 3.5 and 11, and it demonstrated thermal stability up to 66°C with Tm value 58°C. L-glutaminase had the highest catalytic activity and affinity for L-glutamine (Km 0.129 mM, V max 2.02 U/mg/min), followed by L-asparagine and L-aspartic acid (**Reda *et al.*, 2015**); but in present investigation, it only displayed these characteristics for L-glutamine as a substrate.

CONCLUSION

For the production of fungal L-glutaminase, *Aspergillus Niger Strain ATCC 1015* was grown on MGA selective medium. This enzyme showed more anticancer effect when compared to bacterial L-glutaminase produced from *Escherichia coli or Bacillus cereus*. More so than bacterial L-glutaminase, it exhibited less hypersensitivity reactions.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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REFERENCES

Awad MF., El-Shenawy FS., El-Gendy MMAA., El-Bondkly EAM. (2021) May. Purification, characterization, and anticancer and antioxidant activities of L-glutaminase from Aspergillus versicolor Faesay 4. Int Microbiol. 24(2):169-181. doi: 10.1007/s10123-020-00156-8. Epub 2021 Jan 2. PMID: 33389217.

Awad H., El-Deen A., Mostafa E., Hassabo A. (2019). Biochemical studies and biological activities on L-glutaminase from rhizosphere soil Streptomyces rochei SAH2_CWMSG. Egyptian Pharm J. 18, no. 1p. 27.

Bardal S., Waechter J., Martin D. (2021). Applied pharmacology. 9:376-429.

Binod P., Sindhu R., Madhavan A., Abraham A., Mathew AK., Beevi US. *et al.* (2017). A. Recent developments in l-glutaminase production and applications - An overview. Bioresour Technol. 245(Pt B):1766-1774.

Butheina A., Likaa M., Radwa E. (2021). Evidence of antioxidant activity of novel L-glutaminase purified from L. Gasseri BRLHM. J. applied sci nanotech. 1; 4:298-307.

Caroline S., Zeind MG. (2018). Applied therapeutics, the clinical use of drugs. 16:1102-1131.1.

Chitra J., Rajendren S., Jeyakanthan J., Samy B., Mercy J., Manikandan N., Kumari N. (2022). Chapter 9 - Microbes and their products as novel therapeutics in medical applications, Editor(s): Pradeep Verma, Maulin P. Shah, Bioprospecting of Microbial Diversity, Elsevier, Pages 203-221.

Das, Mousumi, Dayanand, Agsar. (2013). Production and antioxidant attribute of L-Glutaminase from Streptomyces Enissocaesilis DMQ-24. Int J of Latest Research in Sci and Tech. 2:1-9.

Dhankhar R., Gupta V., Kumar S., Kapoor RK., Gulati P. (2020). Microbial enzymes for deprivation of amino acid metabolism in malignant cells: biological strategy for cancer treatment. Appl Microbiol Biotechnol 104, 2857–2869.

Dipro C., Schwinghammer T., Dipro J., Well B. (2021). Pharmacotherapy handbook. 5:101-119.

Durthi CP., Pola M., Rajulapati SB., Kola AK., Kamal MA. (2020). Versatile and valuable utilization of amidohydrolase L-glutaminase in pharma and food industries: A Review. Current Drug Metabolism. 21(1):11-24.

El-Sousy, Sara M.A., Easa, S.M., Hassan, Amira A., Ismail, Abdel-Mohsen S. (2021). Production of a bacterial extracellular L-glutaminase possessing high antioxidant capability. Egy Pharm J. 20(1):p 59-71.

Fisher B., Champe P., Harvey R. (2021). Lippincott illustrated reviews microbiology. 45:1376-1523.

Golderg Stephen. (2020). Clinical physiology is made ridiculously simple. 11:4214-4236.

Hamed SR., Al-wasify RS. (2016). Production and optimization of L-glutaminase from a terrestrial fungal Fusarium oxysporum. Int J. PharmTech Res. 9:233-241.

Jambulingam, Kiruthika and Nachimuthu, Saraswathy (2014). Isolation and characterization of a novel L-glutaminase producing marine Bacillus subtilis strain JK-79. Asian Journal of Microbiology, Biotechnology and Environmental Sciences. 16:601-610.

Khalil, Mary and Moubasher, Hani and El-Zawahry, Mokhtar and Michel, Marina (2020). Evaluation of antitumor activity of fungal L-glutaminase produced by egyptian isolates. 10.33263/LIANBS91.924930.

Levinson Warren (2021). Review of medical microbiology and immunology. 9:111-144.

Metting Patricia (2019). Physiology. 14:637-645.

Olson James (2020). Clinical pharmacology made ridiculously simple. 12:83-104.

Orabi HM., El-Fakharany EM., Abdelkhalek ES., Sidkey NM. (2019). L-asparaginase and L-glutaminase: sources, production, and applications in medicine and industry. Journal of Microbiology, Biotechnology and Food Sciences. 9(2): 179-190.

Patel N., Oudemans PV., Hillman BI., Kobayashi DY. (2013). Use of the tetrazolium salt MTT to measure cell viability effects of the bacterial antagonist Lysobacter enzymogenes on the filamentous fungus Cryphonectria parasitica. Antonie Van Leeuwenhoek. 103(6):1271-80.

Pola M., Babu S., Kola A. (2020). Insights intopotent therapeutical antileukaemic agent L-glutaminase enzyme under solid state fermentation. Journal of current drug metabolism. 3:211-220.

Reda FM. (2015) Kinetic properties of Streptomyces canarius L- Glutaminase and its anticancer efficiency. Braz J. Microbiol. 46(4):957-68.

Swanson L.N., Souney P.F., Muntnick A.H., Shargel L. (2019). Comprehen Pharmacy Rev Napl. 15:878-907.

Singh P., Banik RM. (2013). Biochemical characterization and antitumor study of L-glutaminase from Bacillus cereus MTCC 1305. Appl Biochem Biotechnol. 171(2):522-31.

Trevor A., Katzung B., Kruidering-Hall M. (2022). Katzung Trevor pharmacology examination board review. 21:1142-1257.

Wilson Golder N., (2019). Biochemistry and genetics. 23:698-706.