Screening and Production of Bacterial L-glutaminase as an Anticancer Agent from Different Soil Environments in Egypt

Original Article

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ABSTRACT

Background: Breast cancer and other neoplasms like acute lymphocytic leukaemia pose a serious threat to public health worldwide. Treatment for malignancies including acute lymphocytic leukaemia and breast cancer requires enzymes that break down L-glutamine. An amidohydrolase called L-glutaminase can be an effective chemotherapeutic tool for treating a variety of cancers.

Aim of Study: This is a screening experimental representation was conducted to study and industry of bacterial L-glutaminase as an anticancer substance from several Egyptian soil regions.

Patients and Methods: On mineral L-glutamine agar selective medium (*MGA*), a few bacterial isolates were examined in the current investigation to see if they produced L-glutaminase. The most effective positive bacterial isolates generating L-glutaminase, however, were identified using morphological and biochemical testing. Molecular detection using the direct Southern blotting approach was also used to identify the main positive isolate that produces L-glutaminase. *L-glutaminase* synthesis by bacteria was evaluated for its characteristics. Using an *MTT* test, the anticancer activity was evaluated.

Results: Exclusive bacterial isolates that used L-glutamine as their sole source of metabolic nitrogen exhibited favorable growth on MGA. *PH 7.4* and a temperature of 37^{o}C were the ideal environmental and physiological conditions for developing positive bacterial isolates. From the soil samples taken from various soil conditions in Egypt, the morphological and biochemical analyses showed that *Bacillus cereus 14579* was the main positive bacterial generating L-glutaminase isolate. The produced *L-glutaminase* has shown excellent bioavailability and effectiveness as an anticancer therapy.

The yield [productivity] was *5.2 U/ml* during the original manufacture from MGA, but it increased to 42.96 U/ml by bacterial recombinant DNA manufacturing. L-glutaminase was purified, yielding final enzyme recovery of 55 1.23%, total activity *12,990±19.76 (U)*, specific activity *384.66±8.92 (U/mg of protein)*, and purification fold *2±2.99*. Furthermore, the L-glutaminase activity was raised by *19%, 23%, 15%, and 9%*, respectively, by the enzymatic activators Mn^{2+}, K^{+}, Na^{+}, and $Fe3^{+}$. It demonstrated strong *DPPH* (2, 2-diphenyl-2-picryl-hydroxyl) scavenging activity (*IC50= 61 µg/ml*) and anticancer activity (*IC50= 40.72, 9.7, 7.39, 20.61, 51.28, and 11.55 µg/ml, respectively*) against human liver (*HepG-2*), colon (*HCT-116*), breast (*MCF-7*), lung (*A-549*), lymphocytic (*CCL-120*), and cervical (*Hela*) cancer cell lines. A stronger affinity for its substrate was shown by the kinetic parameters of *Km* and *Vmax*, which were *13.2 10^{-5} M* and *119.86 µmol/ml/min*, respectively.

Conclusion: Acute lymphocytic leukemia and breast cancer are examples of auxotrophic malignancies for which *L-glutamine* serves as the only metabolic source, and L-glutaminase generated by Bacillus cereus was an appropriate enzyme in the therapy of these diseases.

Key Words: Acute lymphocytic leukaemia, Auxotrophic, Breast cancer, L-glutaminase.

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INTRODUCTION

Major causes of death globally include *acute lymphocytic leukemia and breast cancer* (**Stan *et al.*, 2020**). Bacterial sources of L-glutaminase are efficient and cost-effective agents in the treatment of cancer, the food industry, and the production of high-value compounds like threonine (**Zeind and Carlvalho, 2018**). Leukemic and breast cancer cells are auxotrophic cancer cells that are unable to produce the amino acid L-glutamine, which is crucial for their survival and expansion. L-glutaminase is an amide that plays a crucial supporting function in cancer cells' cellular nitrogen metabolism. L-glutaminase is a flavor and fragrance enhancer used in the food industry. The

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use of L-glutaminase in biosensors to monitor L-glutamine levels in mammalian and hybridoma cell cultures without the need for separate measurements of L-glutamic acid is another significant use (**Dipro *et al.,* 2019**).

Normal cells may, however, synthesize it (**Fisher *et al.,* 2021**). It is highly beneficial to deprive these cancer cells of L-glutamine using bacterial L-glutaminase (**Parveen, 2017**). L-glutamine is not needed for normal cells since they can synthesize it (**Saleem and Ahmed, 2020**), but it is necessary for cancer cells as a source of nitrogen for their metabolism (**Trevor *et al.,* 2021**).

L-glutaminase, an enzyme from the hydrolase family, breaks down the amino acid L-glutamine, releasing ammonia and L-glutamate in the process (**Golderg, 2018**). L-glutaminase is an amidohydrolase that can be an effective chemotherapeutic tool for treating a variety of cancers (**Levinson, 2019**). Different microbial sources of *L-glutaminase* have drawn a lot of interest in a variety of biological processes. *Alcaligenes faecalis* L-glutaminase's anti-tumor activity against the *HeLa* cell line (**Meeting, 2019**) and Bacillus cereus *MTCC 1305's* anti-tumor activity against the hepatocellular carcinoma (*Hep-G2*) cell line have both been shown (**Olson, 2020**). *L-glutaminase* from *Pseudomonas 7A* inhibits the translation of *mRNA* and suppresses viral replication, exhibiting antiviral efficacy against *retroviral* illness (**Swanson *et al.,* 2019**). L-glutaminase from *Bacillus amyloliquefaciens* was further utilized in food as a flavor enhancer (**Wilson, 2019**). While the *Bacillus cereus LC13* enzyme demonstrated antioxidant activity when combined with ascorbic acid (**Binod *et al.,* 2017**). The colorectal cancer cells' rapid growth indicates that they have greater nutritional needs. The provision of certain nutrients, including amino acids, that the tumor cells require from normal cells is what drives their growth. Without exogenous L-glutamine, glutamine-dependent tumor cells cannot survive (**Sajitha et al., 2014**). An important step in bio-pharmaceutical research is to purify recombinant proteins attached to short peptide affinity tags. This is achieved by Immobilized-metal affinity chromatography, or *IMAC*. *IMAC* relies on interactions between a large number of electron donors on an affinity tag attached to a transition metal ion (Co^{2+}, Ni^{2+}, Cu^{2+}, *or* Zn^{2+}) chelated to a solid-phase support. Both denaturing and non-denaturing conditions can be used for *IMAC*. Most frequently, 6-His is used as the affinity tag, and the tag's electron donor is the histidine imidazole ring.

The *N*- or *C*-terminus of the target usually has *6–12 polyhistidine* residues added as affinity tags. A recent affinity tag was built using a natural peptide produced from the N-terminus of chicken lactate dehydrogenase.

Six histidine residues may be found throughout the *19*-residue polypeptide (*KDHLI HNVHK EEHAH AHNK*) that makes up the *HAT* tag. Compared to polyhistidine tags, it has a reduced net charge and only binds to Co^{2+}-carboxymethylaspartate. The most popular metal chelators are imidodiacetic acid (*IDA*), aspartic acid carboxymethylated (marketed under the name *TALON*), and nitrilotriacetic acid (*NTA*). The chelator is frequently covalently bonded to polymer beads or ferromagnetic beads to isolate magnetic fields. Proteins with *IMAC* tags are generally adsorbed at neutral to slightly basic *PH* levels to avoid protonation of the histidine imidazole groups. Under moderate elution circumstances, a weak chelator, such as ethylene diamine tetraacetic acid (*EDTA*), as well as proteolytic elution, can be utilized to remove the metal ion. Low pH may occasionally cause the target protein to become denatured, but it will still need elution. For target proteins having a metal core, *IMAC* is not indicated since the metal can be removed by the binding partner chelators.

The *Arg-tag*, a different brief poly-amino acid affinity tag, is employed to raise the fusion protein's isoelectric point and enhance its capacity to bind to a cation-exchange matrix. Mild elution conditions are typically characterized by an alkaline pH gradient of *NaCl*. This tag's ability to cling to flat mica sheets may lead to a variety of brand-new applications (**Vo *et al.,* 2020**). Due to microbial enzymes' lower cost of production, controllable nature, and dependability, attempts have been made to replace classical enzymes like L-glutaminase that were isolated from plant and animal tissues (**Singh and Banik, 2013**). The current work is sought to identify and produce bacterial L-glutaminase from various soil environments in Egypt as an anticancer drug against auxotrophic cancer cells, including leukemia and breast malignancies.

PATIENTS AND METHODS:

This Screening experimental study was carried out in Egypt by Cairo University's pharmacy department between November *2021 and May 2023*.One hundred soil samples were collected from various sites in Egypt (Menofia, Sharqia, Qalyobia, Giza, and Cairo governorates). The samples were grassland soil taken at a *0-10cm* depth, collected from different sites in sterile plastic bags and chosen at random. The samples were deposited in sterile containers and kept at 4^{o}C until processed.

The chemical and biological components were purchased from the Egyptian Algomhuria Pharmaceutical Chemical Company. Cell lines were purchased from the Accegen biological company. A selective medium called mineral glutamine agar (*MGA*) was used to isolate and characterize bacteria with the capacity to utilize L-glutamine as their sole source of nitrogen. The following components (in *g/L*) made up this medium.

After being combined, the materials were dissolved in hot water and repeatedly stirred until entirely dissolved. The medium was then cooled in a water bath to *47–50°C*, with a *PH* of 7.3 at a temperature of *37°C*. The plates were immediately poured into dishes with a 90mm diameter

once the ingredients had been mixed, up to a level of *15-20ml*.

Blood agar medium [BAM]

To identify the L-glutaminase-producing bacterial species together with the gram stain, the biochemical responses, and the colony shape, an enrichment medium was used for the identification of beta-hemolysis of the positive isolates cultivated on *MGA* (**Niederstebruch *et al.*, 2017**).

Isolation of L-glutaminase producing strains under drastic conditions:

In *250ml* Erlenmeyer flasks, one gram of each soil sample was suspended in *99ml* of sterile distilled water and shaken at *4000 rpm* for two minutes in a gyrator shaker. The mineral glutamine agar (*MGA*) medium was used to plate the soil suspensions after they had been serially diluted in sterile distilled water. A 7.3–7.4 PH adjustment was made to the medium at a temperature of *37.0°C*. Forty eight hours were spent incubating the plates at *37°C*. The *MGA* medium could only support the growth of bacteria that could use L-glutamine as their only supply of carbon and nitrogen. Colonies that demonstrated growth underwent two rounds of purification using the streak plate technique before being placed on nutrient agar slants and maintained at *4°C*. Sub-culturing of positive isolated colonies on sheep blood agar was performed to detect whether or not beta-haemolysis had occurred. The selected isolate was routinely sub-cultured every *4 weeks*, and the slant culture was maintained in the refrigerator.

Inoculum preparation

The investigated bacterial strain's inoculum was generated in *250ml* Erlenmeyer flasks containing 50ml of nutrient broth liquid at *PH 7*. After autoclave the medium, it was infected with a loopful of culture from a *24-hour*-old nutritional agar slant. The inoculated flasks were shaken for *24 hours* at *150 rpm* and utilized as the inoculum.

Identification of L-glutaminase producing strains

Bacteria are classified into two categories based on the makeup of their cell walls. Using *Gram stain* the bacterial cells became purple after being treated with a solution of crystal violet and subsequently iodine on a microscope slide. When colored cells were treated with a solvent such as alcohol or acetone, gram-positive organisms kept the stain whereas gram-negative organisms lost the stain and turned colorless. With the addition of the counter-stain safranin, the clear, gram-negative bacteria became pink. Gram staining was used to identify the cell shape, the morphology of the spores and the spore location. The microorganism's capacity to haemolyze the blood was tested on the blood agar media. Motility test under the microscope discriminated between motile and no motile bacteria. A sterile needle was used to penetrate the medium to within 1 cm of the tube's bottom to select a well-isolated colony and test for motility. The needle was certainly retained in the same position as it was inserted and removed from the medium. It took *18 hours* of incubation at *35°C*, or until noticeable growth appeared.

The detailed biochemical reactions and Saccharide fermentation tests are described in the supplementary material.

The determination of optimal environmental and physiological factors affecting growth of some selected bacterial isolates producing L-glutaminase [PH, Temperature, NaCl salt concentration, and incubation time]:

This was accomplished by cultivating several soil samples previously obtained on *MGA* at various *PH*, Temperature, *NaCl* salt concentrations, and incubation durations.

Optimization of L-glutaminase:

There have been investigations into several process variables that increase L-glutaminase yield. Peptone, L-glutamine, and ammonium nitrate at a concentration of *1% W/V* were used as nitrogen sources, and the effects of adding additional carbon sources (such as sucrose, mannitol, and soluble starch) and nitrogen sources (such as ammonium nitrate) were also studied. Additionally, the effects of various sodium chloride concentrations [*2-8.5%*], starting *PH* values [*4-10*], incubation temperatures [*20-50°C*], inoculum concentrations [*4-30%*], and incubation periods [*20-120 hours*] were investigated. When doing this optimization, a specific parameter was used in further experiments when another parameter needed to be optimized. For L-glutaminase activity testing, all extracts derived from the aforementioned parameters were used. The experiments were run in triplicate, and the mean results were presented.

Characterization of L-glutaminase produced by certain selected isolates:

Figuring out the foremost activators such as Co^{+2}, Ni^{+2} and Mn^{+2} to use to get the L-glutaminase enzyme out of the positive bacterial isolates that were cultivated on mineral L-glutamine agar. When bacterial glutamine-degrading enzymes break down L-glutamine in media at *PH 7, 37°C, 0.05 mg/ml* borate buffer, and minute amounts [*5 mcg/ml*] of the metal ions Ni^{+2}, Co^{+2}, or Mn^{+2} as co-factors, the optical density of the resulting compound is estimated using an explicit nesslerization assessment. Following the addition of *10ml* of sterile water to various grassland soil samples received from several Egyptian governments, *1ml* of each diluted sediment sample was extracted. Additionally, a substrate containing *2% L-glutamine* was added to each diluted sediment. The model was then incubated over the following 24 hours at *37°C*. *3ml* of each growth medium was added after 24 hours at *37°C* of incubation. The purpose of this experiment was to determine ammonia concentration as a metric of L-glutaminase activity. When feasible, supernatants were collected, and *1ml* of each

collected supernatant was subjected to *100µl* of Nessler's reagent. To figure out the quantity of ammonia created, the absorbance at *480 nm* was measured using a *UV/VIS* spectrophotometer. The experiment was replicated three times. Under ideal assay conditions, one unit of L-glutaminase was the quantity of enzyme that released *1 µmole* of ammonia per minute per milliliter [*mole/ml/min*]. The enzyme activity was measured in triplicate and represented as a unit per ml [*U/ml*].

Molecular detection:

This was accomplished using the Southern blotting method and the direct hybridization approach. The Southern blotting technique is used to transfer *DNA molecules* to nitrocellulose paper by blotting them there after being separated by electrophoresis, denatured, and hybridized with a *DNA probe. DNA gel* electrophoresis: An electrical field was applied while *DNA samples* were positioned in depressions (also known as wells) at one end of a gel. Depending on the size of the *DNA molecules*, the DNA moved toward the positive electrode at a different rate. Shorter molecules moved more quickly than longer ones because the gel functioned as a sieve. The gel was then taken out of the device. Before using tools to visualize them, the bands were not apparent. Various methods might be used to see the *DNA bands* in the gel. Using a dye stain, such as *ethidium bromide*, made it possible to directly see *DNA* bands under ultraviolet light. The most common method of identifying certain sequences was to use a labelled probe (**Green and Sambrook, 2021**).

Upstream process:

Bacterial recombinant DNA production of L-glutaminase:

The expression vector was *pET-14b* (purchased from Novagene in the United States), the promotor was *T7 Lac*, and the tagged protein was *6x histidine* linked to the C terminus of an L-glutaminase molecule. *Escherichia coli BL21 [DE3] polys S* served as the expression host. All of them were used in the manufacture of L-glutaminase using bacterial recombinant *DNA* technology. *IPTG* was the transcription process's inducer. The principal host for the plasmid synthesis and replication was *Escherichia coli DH5* [obtained from Stratagene corporation, USA].

L-glutaminase genomic *DNA* was isolated from *Bacillus cereus ATCC 14579* discovered in several Egypt soil conditions using restriction endonuclease type II enzymes [*DNA* cutting enzymes] *Xbal* and *ACCI*. These cutting enzymes were bought from the German business Sigma-Aldrich. Furthermore, genomic *DNA* was amplified using the polymerase chain reaction method before being sub-cloned to the prokaryotic expression vector $_{p}$*UC18* using the same restriction endonuclease type II enzymes used to extract genomic *DNA* from *Bacillus cereus ATCC 14579*. This was followed by the transformation of pET-14b into the polys S expression host *Escherichia coli BL21 [DE3]*. The addition of *IPTG* stimulated transcription at the promotor site *T7 Lac*, hence initiating gene expression. Luria agar and broth [*LA, LB*] were used for routine bacterial culture for *24 hours* at *37°C* incubation temperature. *Ampicillin* and/or *Kanamycin* were added to the culture medium according to the manufacturer's guidelines (**Barzkar *et al.*, 2021**).

Downstream process:

Purification was carried out using 500cc of crude enzyme extract.

For recombinant expression yield, a centrifuge tube was spun for 3 minutes at *4000 rpm*. The expressed protein was extracted using 70% ethanol [one volume of *70% ethyl alcohol* was added to each *9 volumes* of supernatant extract]. Furthermore, the expressed protein was purified using immobilized affinity chromatography [*IMAC*] and *Ni-NTA* resin as described before (**Rodriguez *et al.*, 2020**).

Protein content of L-glutaminase estimation:

The protein content of the crude *L-glutaminase* enzyme source was evaluated using Lowery's technique, with bovine serum albumin as the reference, and the data were represented as *mg/ml*. The procedure was as described before (**Waterborg and Matthews, 1994**).

In vitro cell viability assay:

The L-glutaminase enzyme's physiologic, pharmacologic, and toxicological effects on acute lymphocytic leukaemia cancer cells were studied using the *CCL-120* cancer cell line. The *L-glutaminase* enzyme's physiologic, pharmacologic, and toxicological effects on mammalian cells were studied using the Vero cell line. Human cancer cell lines include *HepG-2* (liver), *HCT-116* (colon), *MCF-7* (breast), *A-549* (lung), *CCL-120* (lymphocytic), and *Hela* (cervical). Cell lines were purchased from the Accegen biological firm in the United States. The tumor cells were grown in Dulbecco's modified Eagle's medium [*DMEM* for normal cell lines] and *DMEM-F12* for normal *Vero* cell lines, which were supplemented with 10% heat-inactivated fetal calf serum [*GIBO*], penicillin [*110 mcg/ml*] and streptomycin [*115 mcg/ml*] at *37°C* in a humidified atmosphere containing 5% carbon dioxide. In a 30ml tissue culture flask, cells were planted at a density of 2×10^{6} and cultured at *37°C* till *85-90* confluent sheet. For the in vitro cell viability assessment of bacterial L-glutaminase as an oncolytic agent, the *MTT* {*(dimethylthiazol-2-yl) diphenyl tetrazonium*} technique was utilized. The medium from cell cultures was discarded. For adherent cells, the medium was carefully aspirated. The 96-well plate was spined for suspension cells in a microplate centrifuge at *1,000 x g, 4°C, for 5 minutes* before the media was carefully aspirated. *50ml* of serum-free medium and *50ml* of *MTT* solution were added to each well. The plate was incubated at *37°C* for three hours. During the incubation period, each well got *150µl* of *MTT* solvent. The plate was stirred on an orbital

shaker for 15 minutes while covered in foil. To thoroughly dissolve the *MTT formazan*, the liquid has to be pipetted at times. Within an hour, the absorbance at *OD= 590 nm* was measured (**Jakštys *et al.,* 2015**).

Estimation of antioxidant activity of L-glutaminase:

The *DPPH [2, 2-diphenyl-2-picryl-hydroxyl]* test was used to accomplish this. The procedure involves combining 2ml of *DPPH* solution [purchased from Sigma Aldrich, Germany] with *2ml* of *L-glutaminase* extract solution. The reducing power and scavenging activity of the antioxidants towards *DPPH* could be measured by monitoring the reduction of its absorbance at *225 nm* when the color of *DPPH* changed from purple to yellow of the equivalent *hydrazine* (**Sirivibulkovit *et al.,* 2018**).

Estimation of activity of L-glutaminase via indol phenol assay:

Monochloramine was formed when ammonium ions interacted with hypochlorite in an alkaline solution [*PH 10.8*]. When nitroprusside was employed as a catalyst, the monochloramine generated a blue coloured chemical, indophenol, in the presence of phenol and an excess of hypochlorite. Indophenol was created by combining reagents A and B. To make Reagent A, dissolve *0.2 M* phenol and *0.4 mM sodium nitroprusside dihydrate* in water. To make Reagent B, dissolve *3 mM* sodium dichloroisocyanurate dihydrate and 0.15 in water. Before use, both reagents were kept in the refrigerator. Soil samples were serially diluted with distilled water, and *2 ml* of each soil sample was added to reagent A and combined with reagent B. After shaking vigorously for 1 minute, the reaction was allowed to proceed for *20 minutes*, resulting in the formation of indophenol complex. Its absorbance was measured color-metrically at *650 nm* using a *UV* spectrophotometer (**Prakash *et al.,* 2009**).

Formulation of L-glutaminase:

15 mg L-glutaminase, 45 mg Polyethylene glycol 20 (PEG 20), USP, 1.5 mg+5% Mono-basic sodium phosphate, USP, 3 mg+5% Di-basic sodium phosphate, USP, 7.5 mg+5% Sodium chloride, Query size to 1ml Water for injection.

Detection of immunogenicity of L-glutaminase conjugated with Polyethylene glycol 20:

This was carried out using *ELISA* technique described before (**Tabatabaei and Ahmed, 2022**).

Statistical analysis

All cultures were conducted in triplets. Their presentation was by means and standard deviation. One way analysis of variance (p value ≤0.05) was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software. *F* test was utilized during statistical analysis in the present study.

Table (1) represents the outcomes of biochemical reactions.

Table 1: The outcomes of biochemical reactions:

Test	Result
Gram stain	+
Cell shape	Rod
Spore shape	Ellipsoidal
Spore site	Central
Motility	+
Catalase	+
Oxidase	+
Blood haemolysis	Beta haemolysis
Indol	-
Methyl red	+
Voges-proskauer	+
Citrate utilization	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Growth at 45 ^{0}C	+
TSI	+
Tolerance salinity	
5% NaCl	+
7% NaCl	+
Saccharide fermentation	
Glucose	+
Fructose	+
Maltose	+
Sucrose	+

RESULTS

In the present study, an attempt was made to detect an efficient strain of *Bacillus cereus* from different soil environments in Egypt for the enhanced production of L-glutaminase as anticancer agent. As well as, the antioxidant attribute of L-glutaminase was reported.

Only bacterial isolates which utilized L-glutamine as the sole metabolic source of carbon and nitrogen showed positive growth on selective metallic glutamine agar medium [*MGA*]. The optimal environmental and physiological factors affecting growth of positive bacterial isolates were PH 7.4 at temperature *37^{o}C*. The morphological and the biochemical tests revealed that Bacillus cereus was the major positive bacterial producing L-glutaminase isolate from the soil samples collected from different soil environments in Egypt. The optimal conditions for characterization of L-glutaminase production were with the following activators *0.5 g/l KCL, 0.1 g/l ZnSO4, 1.0 g/l FeSO4, 0.5 g/l MgSO4, KH2PO4 at PH 7.4* and temperature *37^{o}C*. L-glutaminase produced from *Bacillus cereus* showed high efficacy and bio-availability as anticancer agent. Molecular mass of L-glutaminase was

approximately *37 KDa*, The yield [productivity] was *5.2 U/ml* during the initial production from *MGA*; while it raised to *42.96 U/ml* via bacterial recombinant *DNA* production. *L-glutaminase* was purified to yield total activity *12,990±19.76 (U)*, specific activity *384.66±8.92* (U/mg of protein), and purification fold *2±2.99* with final enzyme recovery *55±1.23%*. Furthermore, Mn^{2+}, K^{+}, Na^{+}, and Fe^{3+} were enzymatic activators that increased the L-glutaminase activity by *19%, 23%, 15%*, and *9%*, *respectively*. This bacterial enzyme showed higher efficacy as antileukaemic anticancer agent against acute lymphocytic leukaemia as indicated by the in vitro cell viability test using *MTT* assay. It showed potent *DPPH* scavenging activities with IC50= 203 µg/ml and anticancer activities against human liver (HepG-2), colon (HCT-116), breast (*MCF-7*), lung (*A-549*), lymphocytic (*CCL-120*) and cervical (*Hela*) cancer cell lines with IC_{50} *40.72, 9.7, 7.39, 20.61, 51.28 and 11.55 µg/ml, respectively.*

"These results are represented by graphs from 1-23"

Km and Vmax of L-glutaminase were 13.2×10-5 M and 119.86 µmol/ml/min, respectively, which reflected a higher affinity for its substrate. The inoculate contained $1*10^{7}$ *spores/ml.*

Isolation and the screening of L-glutaminase producing bacteria:

A total of *31* bacterial isolates were isolated from different soil samples and used for various screening studies. The current study involved the screening of isolated bacteria on mineral glutamine agar media with the glutamine as the sole source of nitrogen and carbon. Only the organisms that were able to utilize *L-glutamine* as nitrogen and carbon source could grow.

Identification and screening of L-glutaminase degrading enzyme:

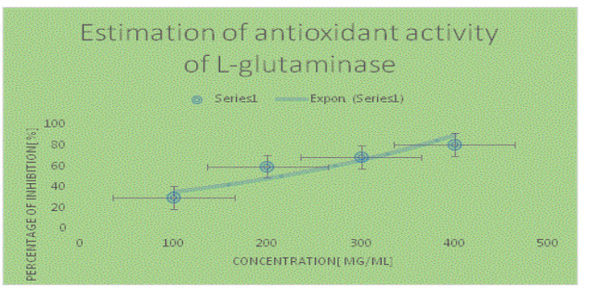
The collected soil samples were further analyzed by nesslerization test. This method has been applied for the determination of the ammonia concentration as a product of enzymatic degradation by glutamine degrading enzyme. The concentration of the liberated ammonia by test samples demonstrated that glutamine degrading enzymes were present in collected samples. Among the collected samples, samples from 1 to 31 which were further identified as *Bacillus cereus species* produced the highest amount of glutamine degrading enzyme.

Identification and the characterization of the most potent bacteria:

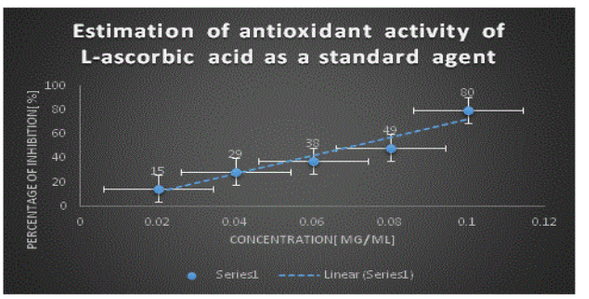
The morphological characteristics of isolates from 1 to 31 which showed high L-glutaminase activity indicated that the organism belonged to the *genus Bacillus*. The biochemical characterization by biochemical tests revealed that it was similar to *Bacillus cereus*. The purified L-glutaminase showed a maximal activity against L-glutamine when, it was incubated at *PH 8.5* at *40.5°C* for 35 minutes. It maintained its stability at a wide range of *PH* from *5-10.5* and was thermostable up to *60°C* with Tm [melting temperature] value 56.81°C. It demonstrated high affinity and catalytic activity for L-glutamine. The crude extract of L-glutaminase contained *83.9 mg* protein content during purification with Nickel immobilized affinity chromatography. The *IC50* values for antioxidant activities for the control [*vitamin C*] and L-glutaminase were discovered to be *99 mcg/ml and 203 mcg/ml* respectively. The optimum of maximum production of L-glutaminase was observed, when it was incubated at *PH 8.5* at *40.5°C* for *35 minutes* in addition to agitation at *195 rev/min*. And inoculum size $1×10^{8}$ *spores/ml*.

Detection of immunogenicity of L-glutaminase conjugated with PEG 20:

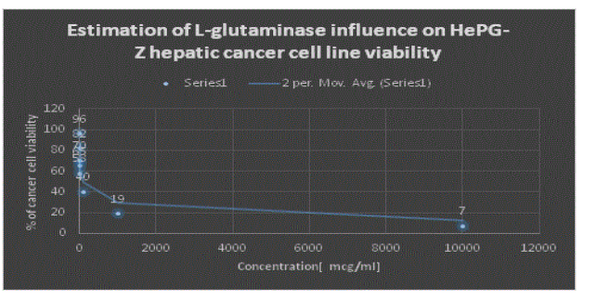
This was accomplished by the use of the *ELISA* method. During the current investigation, few *IgG2* antibodies were detected against *L-glutaminase* produced by *Bacillus cereus*.



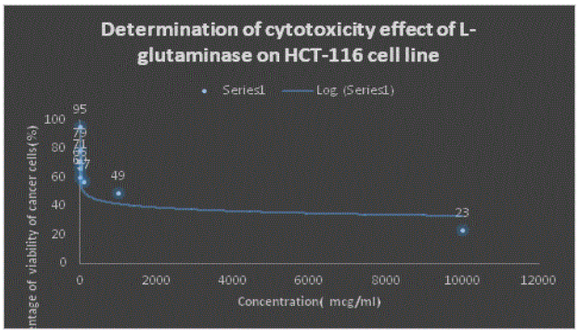
Graph 1: Antioxidant activity of test L-glutaminase via DPPH assay.



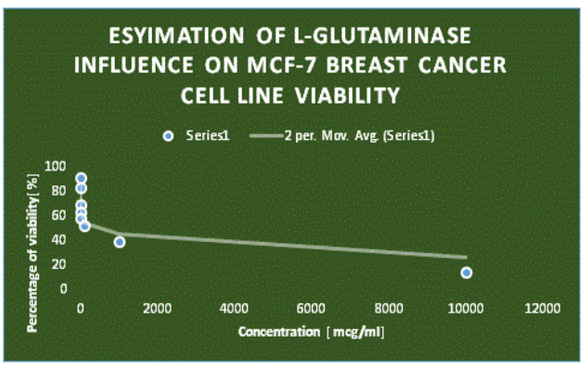
Graph 2: Antioxidant activity of standard L-ascorbic acid via DPPH assay.



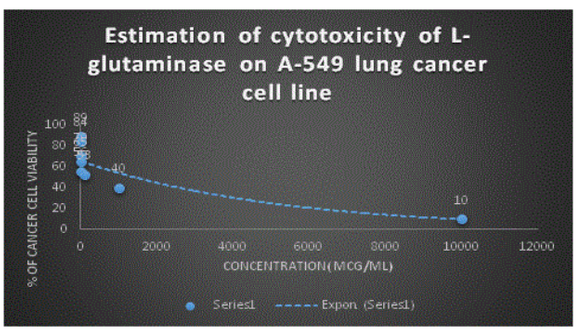
Graph 3: Detection of the cytotoxicity of L-glutaminase on HePG-Z hepatic carcinoma cell line.



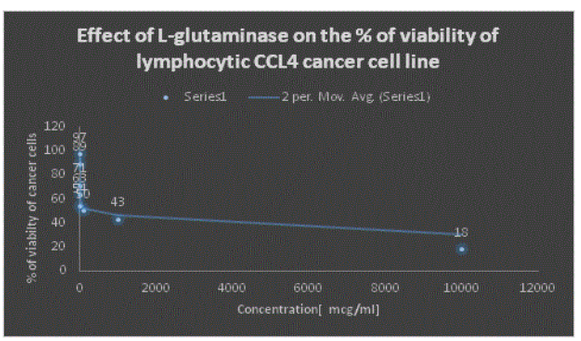
Graph 4: Detection of the cytotoxicity of L-glutaminase on HCT-116 colon cancer cell line.



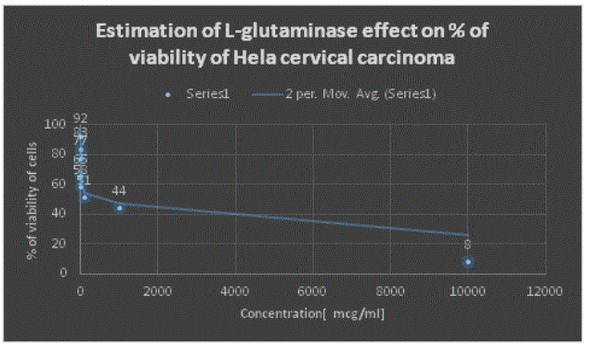
Graph 5: Detection of the cytotoxicity of L-glutaminase on MCF-7 breast cancer cell line.



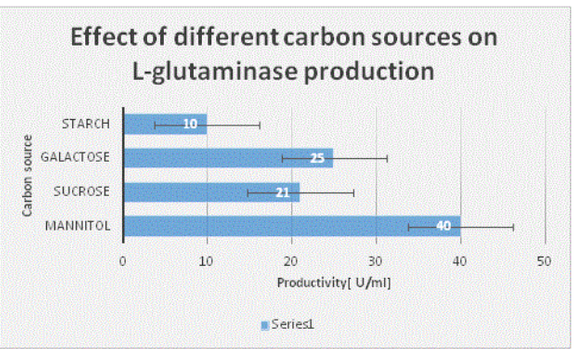
Graph 6: Detection of the cytotoxicity of L-glutaminase on A-549 lung cancer cell line.



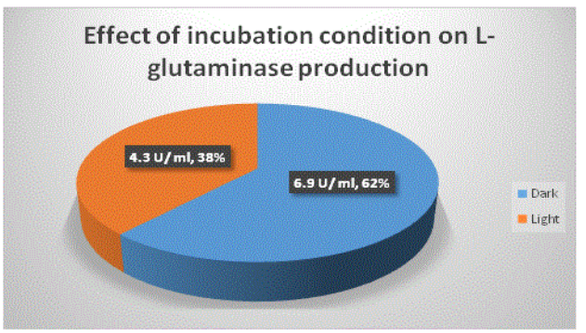
Graph 7: Detection of the cytotoxicity of L-glutaminase on lymphocytic CCL4 cancer cell line.



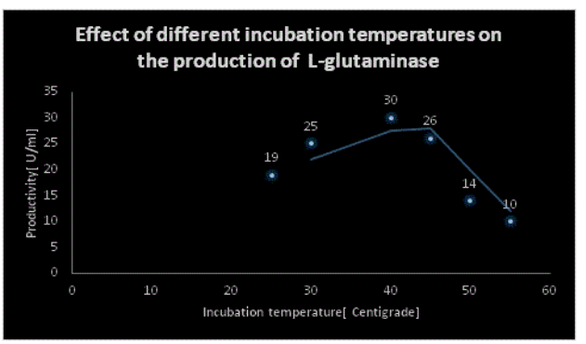
Graph 8: Detection of the cytotoxicity of L-glutaminase on Hela cervical cancer cell line.



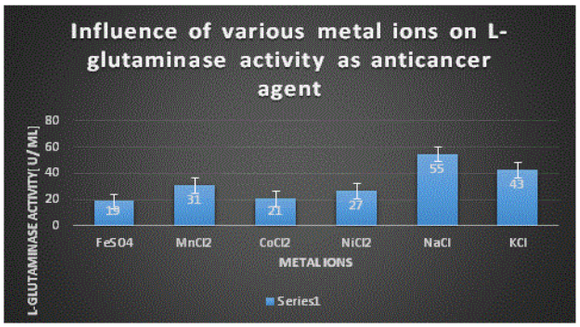
Graph 9: Effect of different carbon sources on L-glutaminase productivity.



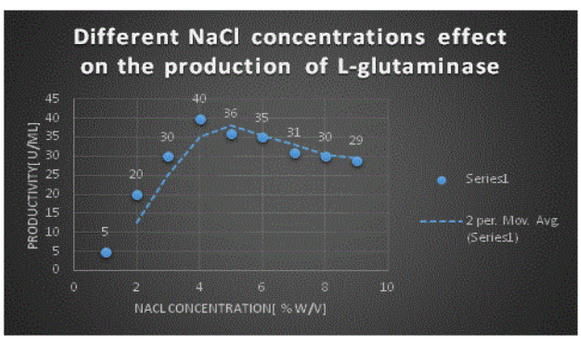
Graph 10: Effect of different incubation conditions[Dark and light] on L-glutaminase output.



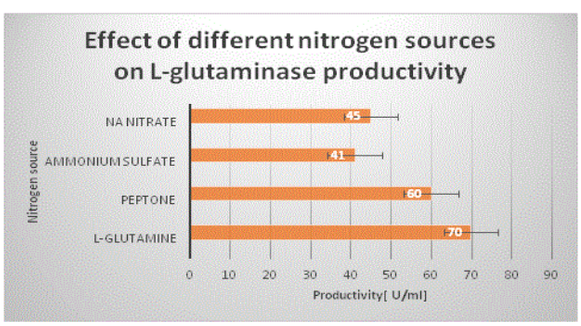
Graph 11: It displays the effect of different incubation temperatures on L-glutaminase production.



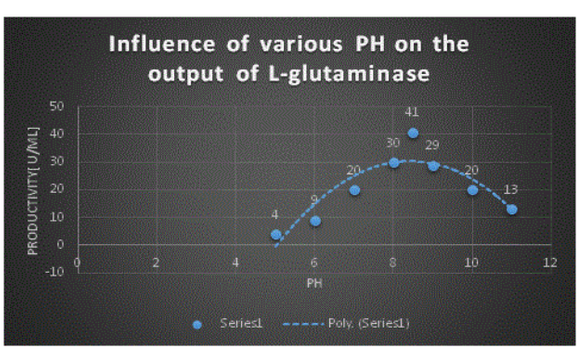
Graph 12: The effect of different metal ions on L-glutaminase cytotoxicity.



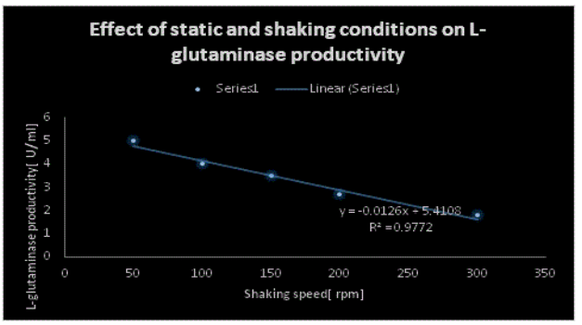
Graph 13: It shows the effects of different NaCl concentrations on L-glutaminase production.



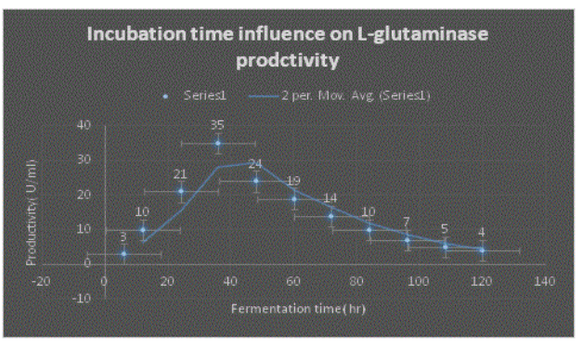
Graph 14: Effect of different Nitrogen sources on L-glutaminase production.



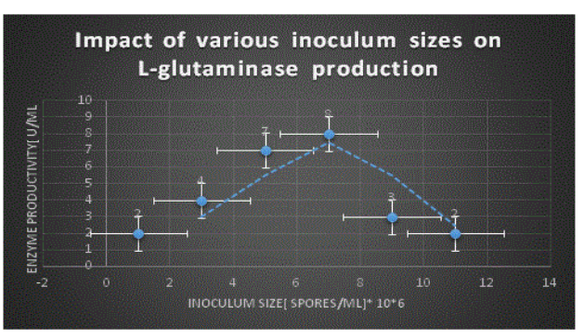
Graph 15: It displays Effect of different PH on the the productivity of L-glutaminase.



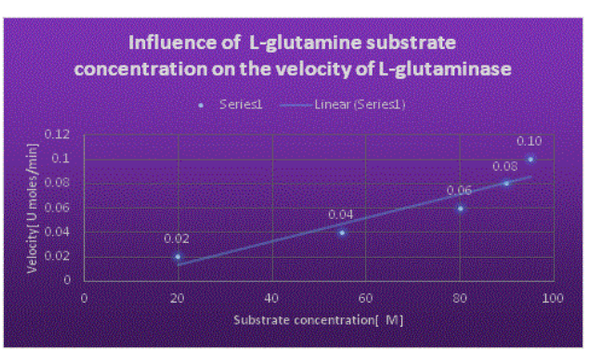
Graph 16: Effect of static and shaking conditions on L-glutaminase productivity.



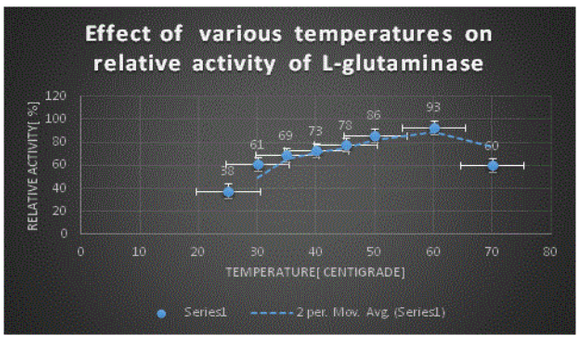
Graph 17: It displays the incubation time for L-glutaminase production.



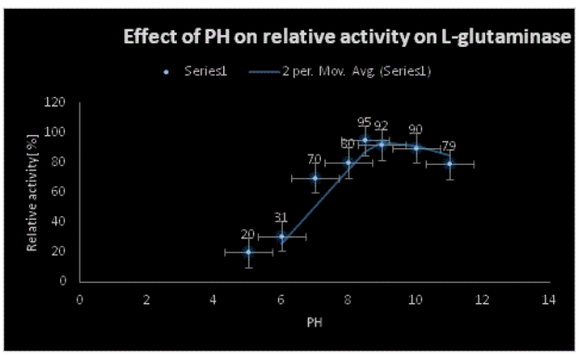
Graph 18: Effect of different inoculum sizes on L-glutaminase productivity.



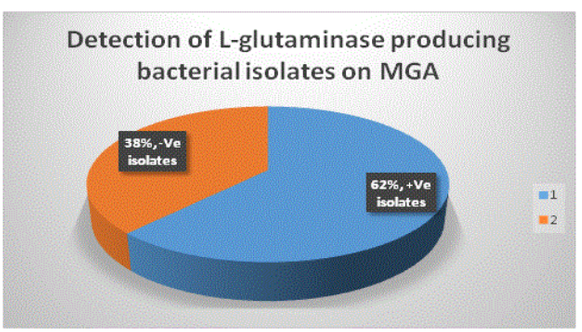
Graph 19: Effect of substrate concentration on the velocity of L-glutaminase.



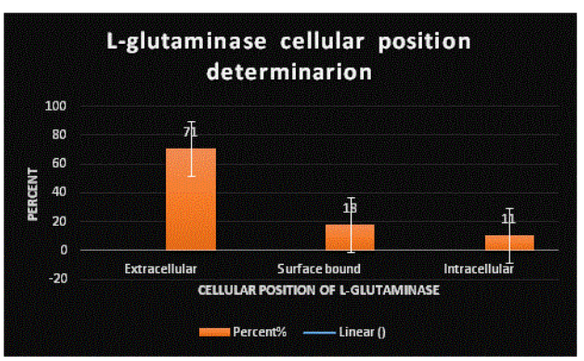
Graph 20: Effect of different temperatures on relative activity of L-glutaminase.



Graph 21: Effect of PH on L-glutaminase activity.



Graph 22: It shows the growth of L-glutaminase secreting bacterial isolates on metallic glutamine agar medium.



Graph 23: It displays the cellular position detection of L-glutaminase through Nesslerization assay.

DISCUSSION

The goal of the current study was to identify and determine the physiological and environmental factors influencing the growth of some L-glutaminase-producing isolates as an anti-cancer agent, as well as to isolate and investigate bacteria that produce the enzyme from various soil environments in Egypt. Additionally, the identification of the characteristics of the enzyme. Mineralized glutamine agar was a particular media used to cultivate bacteria that produced the enzyme L-glutaminase. What grew on mineralized L-glutamine agar was bacterial isolate with the capacity to manufacture the enzyme *L-glutaminase* and the ability to use glutamine as a sole metabolic source of nitrogen and carbon for their growth. By studying the phenotype, molecular detection and conducting biochemical reactions of these isolates, it turned out to be the bacterium *Bacillus cereus 14579*. Comparing results of the present study with previous study conducted in Iran (**Prakash *et al.*, 2020**), it was found that the prevalent producing bacteria were *Bacillus cereus 14579* in the present investigation; while It was *Escherichia coli* species which was predominant in the previous study. The environmental and physiological factors affecting the growth of bacteria was that it was from the soil of the acidic medium and next to flour mills and legumes rich in glutamine and L-metallic glutamine medium *PH* was *7.3* at *37°C* in the presence of oxygen. The catalyst for its production were found to be zinc sulfate, magnesium sulfate, ferric sulfate, potassium chloride and potassium dihydrogen phosphate salts. *Indophenol test* was done to measure the concentration of ammonia resulting from the activity of bacterial *L-glutaminase*, on the other hand direct Nessler,s test was utilized to measure the percentage of ammonia to detect enzyme-producing bacteria and test enzyme activity. For different concentrations of soil samples. it was found that Its activators were the mineral salts: sodium sulfate salts, potassium chloride and manganese sulfate and ferric sulfate at a moderate pH of *7.3* and *37°C* in the presence of oxygen. The test enzyme was tested for the efficacy of the enzyme as an anti-cancer agent, and its efficacy was proven through *MTT assay*. Furthermore, It was produced by bacterial hybrid *DNA* technology (hybrid genetic material) with the ability to produce effective emulsion injections as a anticancer factor. Only bacterial isolates showed positive growth on *MGA* which utilized L-glutamine as the sole metabolic growth source of nitrogen. The optimal environmental and physiological factors affecting growth of positive bacterial isolates were *PH 7.4* at temperature *37°C*. The morphological and the biochemical tests revealed that *Bacillus cereus* was the major positive bacterial producing L-glutaminase isolate from the soil samples collected from different soil environments in Egypt. The optimal conditions for characterization of L-glutaminase production were with the following activators *KCL, ZnSO4, FeSO4, KH2PO4, MgSO4 at PH 7.4* and temperature *37°C*. L-glutaminase

produced from *Bacillus cereus 14579* showed high efficacy and bio-availability as anticancer agent. Molecular mass of L-glutaminase was approximately 37 KDa, The yield [productivity] was *5.2 U/ml* during the initial production from MGA; while it rose to 42.96 U/ml via bacterial recombinant *DNA* production. *L-glutaminase* was purified to yield total activity *12,990±19.76 (U)*, specific activity *384.66±8.92 (U/mg of protein)*, and purification fold *2±2.99* with final enzyme recovery *55±1.23%*. Furthermore, Mn^{2+}, K^{+}, Na^{+}, and Fe^{3+} were enzymatic activators that increased the L-glutaminase activity by *19%, 23%, 15%,* and *9%*, respectively. It showed potent *DPPH* scavenging activities with *IC50= 203 µg/ml* and anticancer activities against human liver (*HepG-2*), colon (HCT-116), breast (*MCF-7*), lung (*A-549*), lymphocytic (*CCL-120*) and cervical (Hela) cancer cell lines with IC_{50} *40.72, 9.7, 7.39, 20.61, 51.28* and *11.55 µg/ml, respectively*. The kinetic parameters of *Km* and *Vmax* were 13.2×10^{-5} M and *119.86 µmol/mL/min*, respectively, which reflected a higher affinity for its substrate. The purified L-glutaminase showed a maximal activity against L-glutamine when, it was incubated at *PH 8.5* at *40.5°C* for *35 minutes* in addition to agitation at *195 rev/min*. And inoculum size 1×10^{8} *spores/ml*. It maintained its stability at a wide range of *PH* from *5-10.5* and was thermostable up to *60°C* with Tm [melting temperature] value *56.81°C*. It demonstrated high affinity and catalytic activity for L-glutamine. Mechanism of action of was found to be because L-glutaminase activates caspase 8 leading to apoptosis of cancer cells which are auxotrophic for L-glutamine; as well as due to deprivation of the cancer cells from obtaining L-glutamine [which is considered a sole metabolic source of carbon and nitrogen], as a consequence of degradation of L-glutamine in the external surrounding media by L-glutaminase. L-glutamine mediated selective death of cancer cells was considered, since the cancerous cells were unable to synthesize *L-glutamine* due to the lack of *L-glutamine* synthase; unlike the normal cells which contains *L-glutamine synthase*.

According to the findings, *L-glutaminase* isolated from *Bacillus cereus* exhibited good antioxidant activities. As the concentration rose, there was a remarkable proportionate increase in scavenging activity. This was approved via *IC50* values for ascorbic acid as the control and *L-glutaminase* which were 99 and 203 mcg/ml respectively. L-glutamine could exhibit antioxidant properties and a proportional increase was observed in scavenging activity along with the concentration of antioxidant molecule. *L-glutaminase* utilized scavenge free radicals generated in vitro by donating their protons (*H*). L-glutaminase resembled other antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and reductase in its ability to convert reactive oxygen species into non reactive oxygen molecule. As a result *L-glutaminase* can be considered protective mechanism in reactive oxygen species [*ROS*] development. According to **Hasson *et al.*, 2021** study, the *IC50* value of antioxidant activity of L-glutaminase reached *165 mcg/ml*; whereas the IC50 of antioxidant activity of L-ascorbic acid reached 65 mcg/ml (**Hasson *et al.*, 2021**). (**Gomaa and Eman, 2022**) study stated that the produced L-glutaminase by the isolated marine bacterial strain *Bacillus sp. DV2-37* showed potential anticancer activity against all the tested cell lines with IC50 values of 3.5, 3.4 and 3.8 mcg/ml for *MCF-7, HepG-2* and *HCT-116*, respectively. As well, it stated that the maximum L-glutaminase productivity (*47.12*) by *Bacillus sp. DV2-37* was obtained at 96 hours of cultivation period. After that the enzyme production decreased, suggesting its association with growth parameters. The present study showed corresponding anticancer activities towards the same these cell lines. (**Nathiya *et al.*, 2011**) study mentioned that L-glutaminase purified from a bacterium was able to stop a breast carcinoma with *IC50* of *256 mcg/ml*. (**Kiruthika *et al.*, 2013**) study confirmed that the maximum L-glutaminase activity by *Vibrio azureus JK-79* isolated from marine environment was at *37°C*. (**Awad *et al.*, 2019**) study stated that the purified L-glutaminase had a molecular mass of *55 kDa, Km* and *Vmax* values of *1.314mmol/l* and *95.24 µ Me/min*, respectively. Of the various physio-chemical parameters tested, *PH 7.5* and temperature *40°C* were optimal for the enzyme activity. (**Sidkey *et al.*, 2019**) study confirmed that the optimized conditions for L-glutaminase production by isolate FIH was found to be 6 days at *35°C* and *PH 7* under static and dark incubation conditions with inoculum size of $4.38\chi\ 10^{6}$ *spores/ml*. The optimization led to the production of *7.66 U/ml* of *L-glutaminase* with great stability at *4%* salt concentration.

Glucose was the best carbon source for L-glutaminase production by *Pseudomonas aurignosa* as indicated by (**Al-Zahrani, 2020**) study.

(**Jambulingam *et al.*, 2014**) study reported that L-glutaminase production by marine *Bacillus subtilis JK-79* was enhanced by using yeast extract.

(**Pandian *et al.*, 2014**) study reported that purified L-glutaminase by *Alcaligenes faecalis KL102* inhibited the growth of Hela cells with an *IC50* value of *12.5 mcg/ml*. In the present study, L-glutaminase exhibited weak toxicity to the normal Vero cell lines indicating that this enzyme possesses high selectivity towards cancer cells. As well the *PH* range *6-9* was reported to be the most favorable range for L-glutaminase production.

(**Krishnakumar *et al.*, 2011**) study mentioned that the highest production of L-glutaminase by the marine alkalophilic *Streptomyces sp*. *SBU1* was at *96 hours* of incubation period. However, maximum *L-glutaminase* production was achieved at 18 hours of incubation time by marine isolated bacterial strain *Bacillus subtilis* as reported by (**Zhang *et al.*, 2021**). The current investigation found less detectable immunogenicity against L-glutaminase

coupled with *PEG* than other bacterial *L-glutaminases* used in prior studies. The duration of the activity, on the other hand, was prolonged due to *PEG* chelation, but it was shorter when L-glutaminase was used without *PEG* conjugation.

CONCLUSION

Bacterial L-glutaminase was an ideal anticancer agent for the treatment of auxotrophic cancers for glutamine such as acute lymphocytic leukaemia.

It was found that *Bacillus cereus group* was the main bacterial isolate producing *L-Glutaminase* enzyme.It is recommended in the future that researchers explore fungal sources of L-glutaminase in the future.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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