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A HIGHLY PURIFIED L-GLUTAMINASE FROM IMMOBILIZED *PSEUDOMONAS* SP. RAS123 CULTURES WITH ANTITUMOR AND ANTIBACTERIAL ACTIVITIES

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ABSTRACT

L-glutaminase (E.C.3.5.2.1) is an antineoplastic enzyme and in the present study, an extracellular L-glutaminase was produced from a marine local strain identified as *Pseudomonas* sp. RAS123. The enzyme was produced from free cultures and from cultures immobilized on and in different supports. *Pseudomonas* sp. RAS123 L-glutaminase produced from immobilized cultures was purified to homogeneity. The specific activity of the enzyme reached 698.655 U/mg protein, with Km and Vmax value of 3.2 mg/ml and 2000 U/ml, respectively. A single band with a molecular weight of about 32.0 kDa was produced by the purified enzyme on SDS-PAGE. Further findings indicated that the pure enzyme's maximum activity occurred at 50°C and pH 9. The enzyme was stable at 60°C for 60 min and in the pH range of 8.0 to 10.0, The effect of chemicals showed that Mn^{2+}, Mg^{2+}, Ni^{2+} and Fe^{2+}activated the enzyme, while SDS (10% w/v) strongly inhibited the activity of the enzyme. The purified enzyme showed cytotoxic activity against HCT-116, HepG2, MCF-7, HeLa, and CCL-86 cell lines tested with IC_{50} values of 122, 175, 195, 306, and > 500 µg/ml, respectively. Also, the antibacterial effect of the enzyme showed activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus mutants*, *Enterobacter cloacae* and *Escherichia coli*. These findings demonstrate that L-glutaminase might be used in numerous biotechnological applications, particularly food and pharmaceutical processing.

Keywords: L-glutaminase, *Pseudomonas* sp, Enzyme purification, Enzyme properties, Anticancer activity, Antibacterial activity

INTRODUCTION

L-Glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) is a hydrolytic enzyme. It catalyzes the deamination process that transforms L-glutamine into L-glutamic acid and ammonia. The enzyme has recently received a lot of attention because of its prospective uses in the food and pharmaceutical industries **(Dutta *et al*., 2015).** Both prokaryotes and eukaryotes use the enzyme to regulate cellular nitrogen metabolism **(Bülbül and Karakuş, 2013).** L-glutamine catabolism and formation are primarily reliant on two enzymes, L-glutaminase and glutamine synthetase. L-glutaminase is responsible for the breakdown of L-glutamine into glutamate and ammonia in the mitochondria. Glutamate is also deaminated, resulting in keto-glutarate, which then enters the citric acid cycle. As a consequence, the hydrolysis of L-glutamine by L-glutaminase is increased but no considerable amounts of particular metabolites are produced **(Cruzat *et al*., 2018).**
Food industries have been using glutaminase widely in order to produce food products rich in glutamic acid, the enzyme is then added to final foods to enhance the savory or "umami" flavor of meals **(Cruzat *et al*., 2018; Vo *et al*., 2020)**. The enzyme enhances the food flavor indirectly by increasing the L-glutamate in the intermediates food ingredients, resulting in a flavor close to that produced by monosodium glutamate (MSG) and so decreasing the use of MSG in foods.
L-medical glutaminase's significance refers to its potential application in the therapy of several disorders. It possesses antioxidant and anti-tumor properties, as well as a role in HIV treatment **(Zhao *et al*., 2004; Kiruthika and Swathi, 2019).** Amino acids depleting enzymes, plays a promising role in cancer treatment as treating acute lymphoblastic leukemia and lymphosarcoma **(Saxena *et al*., 2015).** The role of these enzymes rely on that some cancer cells depend heavily on glutamine for their growth, which can be provided exogenously **(Cluntun *et al*., 2017).** Glutamine is a nitrogen donor for nucleotide biosynthesis and a carbon source for the tricarboxylic acid cycle, which generates glutathione, a crucial precursor for the production of nucleotides and lipids by reductive carboxylation. L-glutaminase works by lowering glutamine levels in the tumor environment **(Cluntun *et al*., 2017; Orabi *et al*., 2019).**
Since the discovery of L-anticancer glutaminase's capabilities, several microbial sources have been crucial for the enzyme's presence. L-glutaminase was also produced by animal, plant, and microorganisms, including bacteria, fungi, and actinomycetes. However, microbiological sources are the most commonly employed for industrial applications because of their low production costs, consistency, modulation capability, and optimization methods **(Binod *et al*., 2017).**
Previous research has shown that marine habitats are a suitable source for the creation of a variety of enzymes with unique qualities such as stability in severe conditions and salt tolerance **(Pandian *et al*., 2014).**
Immobilized cell systems have been increasingly popular for a variety of biotechnological applications, including bioremediation (**Farag *et al*., 2022**), and the production of metabolites such as organic acids, alcohols, antibiotics, and enzymes **(El-Borai *et al*., 2022)**. Immobilization of whole cell methods has sparked a lot of interest because of its potential to improve bioprocesses and increase product output. Several microorganisms, such as bacteria, fungus, and yeast, were immobilized in or on a variety of support materials. Furthermore, entire cell immobilization for fermentation has a number of advantages, one of which is that it can be utilized repeatedly in multiple cycles **(Kumar and Chandrasekaran 2003).**
This study's emphasis was on marine bacterial strain producing L-glutaminase. Moreover, immobilization of *Pseudomonas* sp. RAS123 cultures was applied by entrapment and adsorption techniques. Then purification of the enzyme produced from immobilized cultures was achieved, followed by characterization of the enzyme, and determination of its antimicrobial, and antitumor activities on cell lines.

MATERIAL AND METHODS

Bacterium and Culture Medium

Various marine water samples were obtained from Burullus lake, Kafr El-Sheikh, Egypt. Using sterile saline solution, one millilitre of each water sample was diluted prior placing over a ZoBell agar medium containing L-glutamine with the following components (g/l): peptone, 5; yeast extract, 1; agar-agar, 15; $FeSO_4.7H_2O$ traces; distilled water 200 ml; filtered sea water 800 ml, and the pH was set at 7.5 **(ZoBell, 1941)**. Rapid plate assay was used to screen for the production of L-glutaminase. Colonies isolated in the previous step were spread onto modified M-9 medium containing the subsequent ingredients (g/l): $Na_2HPO_4.2H_2O$, 6.0; KH_2PO_4, 3.0; NaCl, 20.5; L-glutamine, 5.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.15 and Glucose, 2.0; bacteriological agar, 18.0 in 1000 ml of distilled water. As a pH indicator, a stock solution of 2.5 ml of a 3% (w/v) of phenol red in ethanol at pH 7 was added. Plates were incubated for 72 h at 35°C. Pink zones developed after incubation around colonies was an indication for L-

glutaminase production **(Gulati *et al*., 1997)**. These colonies were purified and chosen for more screening in liquid medium.

To produce L-glutaminase, 50 ml aliquots of mineral medium was seeded with one ml of an overnight culture in ZoBell medium, and then shaken at 35°C for 24 h. For optimum L-glutaminase synthesis, statistical methods were employed to optimize the production medium. The optimal medium used to produce the enzyme was made up of (g/l): L-glutamine, 3.75; Na_2HPO_4, 6.0; NaCl, 20.5; KH_2PO_4, 3.0; $MgSO_4$, 0.125; $CaCl_2$, 0.1125 and glucose, 2.0 **(Roberts, 1976).** 0.1 N HCl or 0.1 N NaOH, were used to adjust the medium pH to 7.0±0.2 before sterilizing at 121°C for 20 min Thereafter, the flasks were incubated at 35°C under shaking conditions for 48 h.

GACT Company-Germany used the primers 16S rRNA Forward primer and 16S rRNA Reverse primer for the PCR process to identify the most potent bacterium utilising 16S rRNA gene sequencing analysis. Primer 27f (20Pmol/µl) **(**5'-AGA GTT TGG ATC M TGG CTC AG-3') and 1492r (20 pmol/µl) (5'-CGG TTA CCT TGT TAC GAC TT -3') were used. The PCR amplification was done using IU of Taq DNA polymerase. The PCR amplification programme utilised the following cycling parameters **(Altschul *et al*., 1990)**: Denaturation at 95°C for 5 min, followed by 20 cycles of denaturing for 30 s at 95°C, annealing for 30 s at 56°C, and elongating for 90 s at 72°C, with the final extension taking place at 72°C for seven min. The BLAST database was used to analyse the nucleotide sequences **(Lipman, 1997)**.

For tree construction, the Maximum Likelihood method **(Tamura and Nei, 1993)** and bootstrapping (1000 replicates) were applied through MEGA7 **(Kumar *et al*., 2016)**. The evolutionary distances were computed using the p-distance method **(Nei and Kumar, 2000)** and are in the units of the number of base differences per site. The neighbor-joining approach (with the BioEdit sequence alignment editor) of obtained sequences of the 16S rRNA gene was estimated.

Crude Enzyme Preparation, Enzyme Assay and Protein Determination

The crude enzyme source was considered as the clear supernatant. 0.5 ml of 0.04 M L-glutamine solution, 0.5 ml of distilled water, 0.5 ml of 0.1 M phosphate buffer at pH 8, and 0.5 ml of supernatant were combined and then incubated for 30 min at 37°C to determine L-glutaminase activity. In order to stop the reaction, 0.5 ml of 1.5 M trichloroacetic acid was added. Centrifugation (10000 rpm for 20 min) was used to extract the precipitated proteins. After that, in order to prepare control tubes, the enzyme preparation was added and the nesslerization procedure was carried out with the supernatant **(Imada *et al*., 1973; Gulati *et al*., 1997)**. The standard graph of ammonium chloride was used to figure out the quantity of ammonia released per millilitre of enzyme preparation per minute, and L-glutaminase (U/ml) was determined by the amount of ammonia produced per millilitre of enzyme solution per minute. Following the **Lowry *et al*. (1951)** method, the protein content preparation was also evaluated using bovine serum albumin as a standard reference.

Immobilization of Pseudomonas sp. RAS123 cells

Entrapment in sodium alginate

Bettmann and Rehm (1984) method of cell trapping in alginate beads was used in this study. Alginate (4% w/v) was dissolved in distilled water and then autoclaved at 121°C for 15 min. 2 ml of bacterial cell suspension, diluted with 8 ml sterile distilled water, was combined with 15 ml sterilized alginate solution and stirred using a magnetic stirrer. 10 ml of the prepared alginate/cell mixture was drawn drop by drop into a cool, sterile $CaCl_2$ solution (2% w/v) using a thin needle syringe, while stirring. Gel beads with a diameter of around 2 mm were obtained. The beads were gently stirred for 2 h while being resuspended in a fresh $CaCl_2$ solution to harden them. Beads were then rinsed several times using distilled water and were added to a 250 ml Erlenmeyer flask containing 50 ml of sterile optimum medium and left for incubation in the shaker for 48 h at 35°C and 120 rpm.

Entrapment in agar-sodium alginate

Cells were entrapped in agar-sodium alginate as explained previously by **Bettmann and Rehm (1984),** instead**,** alginate (2% w/v) and agar (2% w/v) replaced alginate (4% w/v).

Entrapment in agar

As described by **Chapatwala *et al*. (1993)**, bacterial cells were entrapped in 2% (w/v) agar gel cubes. At 100°C, 2 g of agar were dissolved in 90 ml of distilled water, and the mixture was sterilized at 121°C for 20 min, then cooled to 40°C. To achieve a final concentration of 2% (w/v), the sterile agar was mixed with 10 ml of bacterial cell suspension obtained from overnight culture. After mixing, 10 ml of the obtained mixture were poured aseptically into a sterile petri-dish. The agar was then sliced into small cubes using a sterile cutter after it had been solidified. The small gel cubes were placed in Erlenmeyer flask containing the sterile optimum medium previously described and incubated following the previous conditions.

Immobilization of bacterial cells by adsorption

Fifty ml culture media were added into 250 ml Erlenmeyer flasks containing small bits of polyurethane foams, wood chips, luffa pulp, or wire fragments, and the flasks were sterilized for 15 min at 121°C. The flasks were then seeded with one ml of bacterial culture (OD_{600}, 1.0) for each flask, and shaked for 48 h at 35°C and 120 rpm **(El-Borai *et al*., 2022)**.

Scanning electron microscope (SEM)

Entrapped and adsorbed *Pseudomonas* sp. RAS123 cells were scanned using a Jeol electron microscope (Electron Microscope Unit in Faculty of Science, Alexandria University). Bacterial growth immobilized in various porous supporting materials were harvested, rinsed with phosphate buffer, and fixed with glutaraldehyde (2% w/v) before being treated with osmium tetraoxide (1% w/v). The samples were then dehydrated in ethanol after being rinsed in a buffer solution. The samples were completely dried in a critical point dryer before being gold-coated. Samples were examined using a scanning electron microscope with a 45° beam angle and a 20 kV voltage **(El-Borai *et al*., 2022)**.

Purification of L-Glutaminase

Crude culture supernatant collected from *Pseudomonas* sp. RAS123 cultures grown under optimal conditions was purified by two-step process including precipitation and gel filtration. In a sequential manner, the crude culture supernatant was successively precipitated at various ethanol concentrations.

Ethanol was added to achieve 25, then 50% (v/v) saturation, and the mixture was maintained at 4°C overnight. To collect the precipitated protein, centrifugation was done at 6000 rpm for 20 min, then ethanol was added to the supernatant to saturate it to 75% (v/v). The generated precipitates were then separated, evaporated, and concentrated using centrifugation. Samples were assayed for the protein and enzyme activity and kept at − 20°C for the next step of purification **(El-Borai *et al*., 2013)**. About 55.64 mg of the partially purified L-glutaminase was diluted in 10 ml of 0.1M phosphate buffer at pH 8.0 and purified using Sephadex G-100 gel column (1.6 cm × 50 cm). The elute was obtained at a rate of 0.5 ml/min adjusted with a peristaltic pump and collected in 5 ml **(Beltagy *et al*., 2016; Farag *et al*., 2021)**. Protein content and L-glutaminase activity were assessed as previously mentioned. Following **Laemmli (1970)** method, the molecular weight of the enzyme was detected using electrophoresis into Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Gel electrophoresis was run in Delta Scientific Services Company (Alexandria, Egypt).

Characterization of L-Glutaminase

Different substrate levels ranging from 1 till 10 mg/ml were tested at 37°C for 30 min to find the optimal concentration. The Km and Vmax values for L-glutaminase were ascertained by linear regression analysis of **Lineweaver-Burke (1934)**, double reciprocal plots of initial velocity data was recorded under the pre-described conditions. The optimal enzyme temperature was evaluated by testing the enzyme activity at incubation temperature ranging from 30 to 60°C. To assess the enzyme's thermal stability, identical quantities of each preparation were preheated individually for 15, 30, and 60 min at varied temperatures (50, 60, 70, and 80°C) in the absence of the substrate. In each case, an inactivating enzyme solution was used as a control. The enzyme activity was determined by adding the substrate and performing the enzyme assay under optimal conditions. Enzymatic reaction was conducted in the pH range of 4.0 to 10.5 to identify the optimum pH. **(**0.1M acetate buffer, 0.1M phosphate buffer and 0.1 M Tris-HCl). pH stability of pure L-glutaminase was also tested by incubating identical enzyme solutions separately in different buffers in a pH range of 4–10.5 for 2 h at room temperature in the absence of the substrate. After adding the substrate and running the enzyme assay under optimal conditions, the activity was then determined. To test the effects of metal ions, sodium dodecyl sulphate (SDS 10 % w/v), and EDTA on the enzyme's activity, 0.1M of each chemical was mixed with the enzyme and incubated for 2 h, afterthat the activity was determined. As a control, the reaction mixture was made without any chemicals (**Farag *et al*., 2021**).

Anticancer Efficiency

Pseudomonas sp. RAS123 L-glutaminase anticancer potency was assessed against human lymphoma cell line (CCL-86); human breast adenocarcinoma cell line (MCF-7); human hepatocarcinoma cell line (HepG2); human colorectal carcinoma cells (HCT-116); and human cervical carcinoma cell line (HeLa)**,** obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were allowed to grow in RPMI-1640 media containing 10% (v/v) fetal calf serum that had been inactivated and 50 µg/ml gentamycin. The cells were kept at 37°C in a humid environment with 5% CO_2 and subcultured every 2 to 3 weeks. Different tumor cell lines were maintained in medium at a $5x10^4$ cell/well concentration, in Corning® 96-well tissue culture plates, and left for incubatation for 24 h to assess antitumor efficiency. After that, L-glutaminase was applied to 96-well plates (three duplicates) to achieve varied concentrations ranging from 3.9 to 500 µg protein/ml.

For each 96 well plate, six vehicle controls with medium or 0.5% (v/v) dimethyl sulfoxide (DMSO) were run as a control. The number of viable cells was evaluated using the Micro culture tetrazolium (MTT) test, as described by **Hansen *et al.* (1989)**, after incubation for 48 h. Briefly, after removing the media from the 96-well plates, 100 µl of new culture RPMI-1640 medium without phenol red was added. Each well, then received 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 ml of phosphate buffer solution), and so the untreated controls. Following that, the 96-well plates had a 4 h incubation period at 37°C and 5% CO_2. A portion of the media (about 85 µl) was withdrawn from each well, and then 50 µl of DMSO was introduced, mixed thoroughly with the pipette, and incubated for 10 min at 37°C. The number of viable cells was then calculated using the optical density recorded by a microplate reader at 590 nm (SunRise, TECAN, Inc., USA), to calculate the viability percentage as [(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relationship between surviving cells and L-glutaminase concentration is used to depict the survival curve of each tumor cell line following L-glutaminase treatment. Using the Graphpad Prism programme, the dose response curve graphic plots for each concentration were used to calculate the 50% inhibitory concentration (IC_{50}), or the dosage necessary to produce detrimental consequences in 50% of intact cells (San Diego, CA. USA) **(Gomha *et al.*, 2015).**

Antimicrobial Assay for L-Glutaminase

A variety of pathogenic microorganisms were tested using the diffusion agar technique with well diameter of 6.0 mm (100 µl). *Staphylococcus aureus* (RCMB010010), *Bacillus subtilis* RCMB 015 (1) NRRL B-543, *Streptococcus mutants* RCMB 017 (1) ATCC 25175, *Enterobacter cloacae* RCMB 001 (1) ATCC 23355, *Escherichia coli* (RCMB 010052)ATCC 25955 and *Pseudomonas aeruginosa* ATCC 27853 were used as bacterial tested strains. In addition, *Candida albicans* RCMB 005003 (1) ATCC 10231 and *Aspergillus fumigatus* (RCMB 002008) were used as antifungal tested strains **(Awad *et al.*, 2019)**.

Statistical Analysis

Results were expressed as mean ± standard deviation; with probability values $P < 0.05$ being considered as statistically significant. The ANOVA test was performed to compare the different examined groups, and Duncan's method was utilized to determine the significant differences between them: The same small letters show that there was no significant difference between the two groups, whereas the difference letters show that there was a significant difference between these two groups.

RESULTS

Screening and Identification of L-Glutaminase Producing Bacterial Isolate

Preliminary screening of sea water samples for bacteria that produce L-glutaminase had led to the isolation of eight different bacterial isolates, which were identified as L-glutaminase producers. As an indication for L-glutaminase production, the isolates when cultured on modified agar medium with phenol red, they caused the medium's colour to change from yellow to pink after 72 h. However, one isolate was selected for further studies **(Supplementary file 1)**.
Identification of the target strain was made and NCBI's blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed the sequence alignment between the obtained sequence and the most closely related bacterial species (*Pseudomonas)* with obtained similarity about 98.61%. The nucleotide sequence (802 base pairs) was deposited to GenBank sequence database and given the accession number MN900616, and the isolate has been identified as *Pseudomonas* sp. RAS123. Additionally, the strain's phylogenetic tree demonstrated the neighborhood relationships to other strains (**Fig. 1)**.

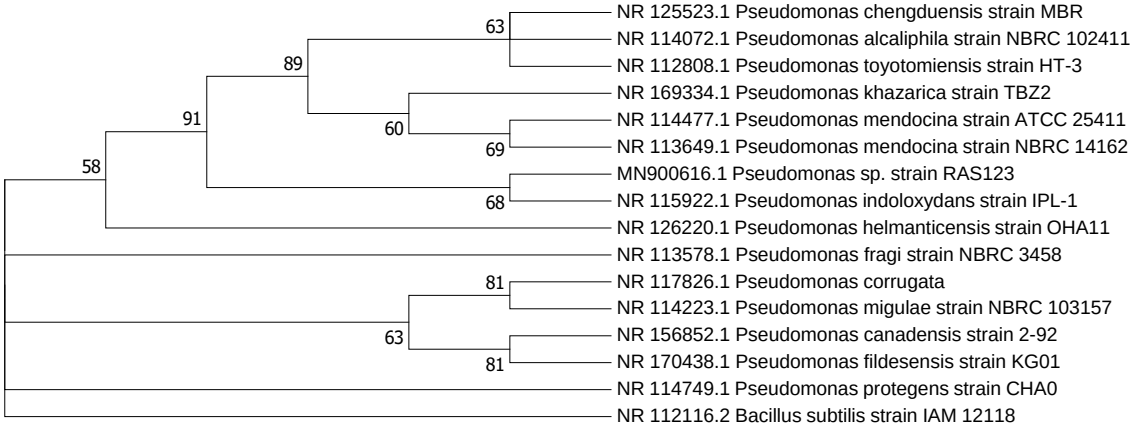

Figure 1 The evolutionary history was inferred based on the partial 16S rRNA gene sequence of *Pseudomonas* sp. strain RAS123 (accession number: MN900616). Maximum Likelihood method and bootstrapping (1000 replicates) were applied through MEGA7. *Bacillus subtilis* strain IAM 12118 16S rRNA gene sequence was used as an outgroup. Branches supported by less than 50% bootstrap value which are hidden.

Production of Extracellular Pseudomonas sp. RAS123 L-glutaminase by Immobilized Cultures

Different gel materials namely; sodium alginate, agar and agar-sodium alginate were used for entrapment of *Pseudomonas* sp. RAS123, and free cells were used as a control. Different support materials namely; polyurethane foams, luffa pulp, wood cubes and wire pieces were used for adsorption of *Pseudomonas* sp. RAS123 cells. The highest L-glutaminase activity was obtained from cells entrapped within sodium alginate (180.42±12.89 U/ml) which was higher than that obtained by free cultures (124.25±11.30 U/ml), followed by cells adsorbed on polyurethane foams (142.26±10.94 U/ml). On the other hand, the agar-sodium alginate beads recorded the lowest L-glutaminase activity (76.47±5.88 U/ml) **(Fig. 2).**
Scanning electron micrographs of *Pseudomonas* sp. RAS123 illustrated morphological characters of the bacterial cells. Also, entrapped *Pseudomonas* sp. RAS123 in sodium alginate or adsorbed on polyurethane foams revealed good growth of bacterial cells either inside the beads of alginate or on polyurethane foams **(Supplementary file 2)**.

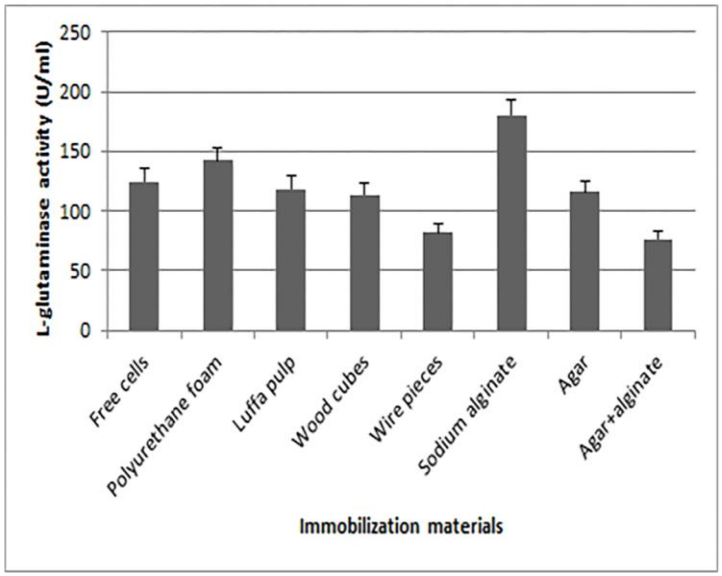

Figure 2 Production of *Pseudomonas* sp. RAS123 L-glutaminase by immobilized cells in different gel materials and on solid porous materials

Purification of L-Glutaminase Produced from Immobilized Pseudomonas sp. RAS123 Cultures

Pseudomonas sp. RAS123 L-glutaminase produced from entrapped culture within alginate beads was succesfully purified using purification procedure which involved different steps and the purification results were summarized in **Table (1)**. L-glutaminase was partially purified using ethanol precipitation; the enzyme was purified about 1.99 times with the yield rate up to 21.99 %. Gel filtration with Sephadex G-100 was used to further purify L-glutaminase, two protein peaks were obtained; L-glutaminase was located in one peak **(Table 1, Fig. 3A)**. The enzyme was purified with gel filtration till 2.47 times giving a a yield rate which reached 15.56%. Following each step, the purity of the enzyme was assessed with 12% (w/v) SDS-PAGE. After purification processes, the purified enzyme's molecular weight was measured and was found to be 32 kDa. It was clearly evident the purification improvement which was noticed by the reduction of the protein bands that were present to one single band **(Fig. 3B)**.

Table 1 Summary of purification steps of *Pseudomonas* sp. RAS123 L-glutaminase

Purification step	Total protein (mg)	Total Activity (U)	L-glutaminase Specific activity (U/mg protein)	Purification (Fold)	Recovery (%)
Culture filtrate	440	142550	283.07	1.00	100
Ethanol fraction (75%)	55.64	31352.16	563.48	1.99	21.99
Sephadex-G100	31.75	22182.30	698.655	2.47	15.56

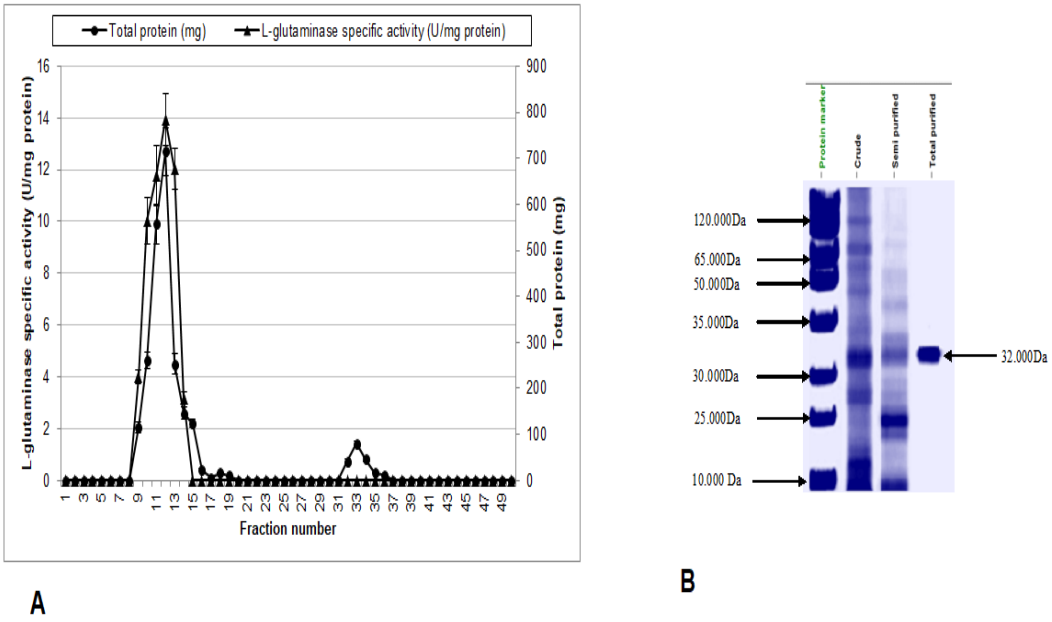

Figure (3) (A) A typical elution curve for the chromatography of *Pseudomonas* sp. RAS123 L-glutaminase by gel filtration in Sephadex G-100. **(B):** Protein fingerprinting patterns of protein marker; *Pseudomonas* sp. RAS123 crude L-glutaminase; semi purified L-glutaminase and purified L-glutaminase sample

Characterization of Pseudomonas sp. RAS123 L-Glutaminase

Values of Km and Vmax of purified *Pseudomonas* sp. RAS123 L-glutaminase were studied and were determined to be 3.2 mg/ml and 2000 U/ml, respectively as recorded in **Fig. (4)**. Temperature had also an impact on activity and stability of L-glutaminase, with an increase in temperature from 30 to 50°C, the enzyme activity increased with the highest activity at 50°C (1344.85±96.06 U/mg protein) (**Fig. 5A**), however, at 60°C the activity was negatively impacted, with about 26.2% of the overall activity lost. Thermal stability of L-glutaminase was shown in **Fig. (5B)**, after 60 min of exposure at 50°C and 60°C, the enzyme retained all of its activity. Additionally, the enzyme maintained 84.11% of its activity after 60 min of treatment at 70°C. When heated at a higher temperature for 60 min at 80°C, the enzyme maintained 66.27% of its initial activity. These findings confirmed the enzyme's thermostability. According to studies on pH effect on the stability and activity of L-glutaminase, the enzyme was most active at pH 9.0 (1440.73±130.98 U/ mg protein). Above or below this value, the activity of the tested purified enzyme decreased. The lowest activity was obtained at the acidic range, and at pH 4.0, the enzyme lost nearly 75.09% of its activity. However, at a pH range of 8 to 9.5, the enzyme displayed a relatively high level of activity, while at the pH range of 8.0 to 10.0, it displayed good stability. However, the enzyme at pH 4.0 and 10.5 exhibited a decrease about 72.23% and 33.75 %, respectively, in activity with respect to the activity of optimal pH (**Fig. 5C, D**). Additionally, results examining the impact of enzyme activators and inhibitors on enzyme activity, showed that Mn^{2+}, Mg^{2+}, Ni^{2+} and Fe^{2+}activated purified L-glutaminase by 2.06, 1.36, 1.35 and 1.28- fold, respectively. The enzyme was partially inhibited by K^{2+}, Na^{+}, and Pb^{2+}. SDS (10% w/v), on the other hand, significantly reduced the enzyme's activity and inwhich it retained only 43.50% of its original activity when compared to an untreated enzyme, but EDTA has no effect on enzyme activity **(Table 2)**.

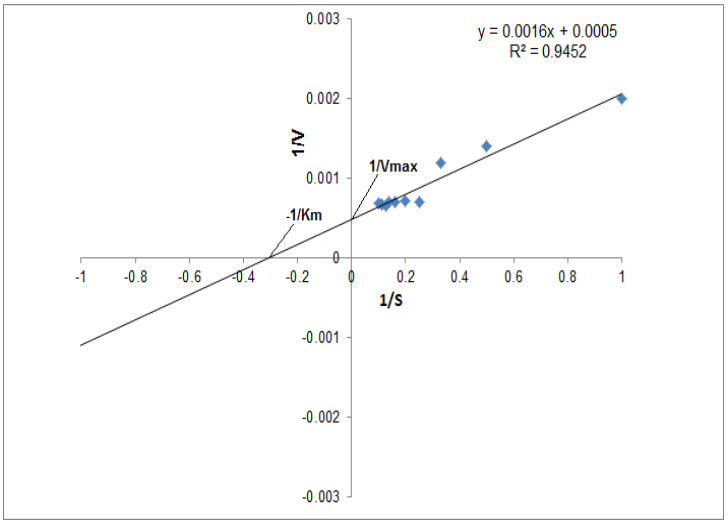

Figure 4 Lineweaver-Burk plot for evaluation of kinetic constants (Km and Vmax) of purified *Pseudomonas* sp. RAS123 L-glutaminase.

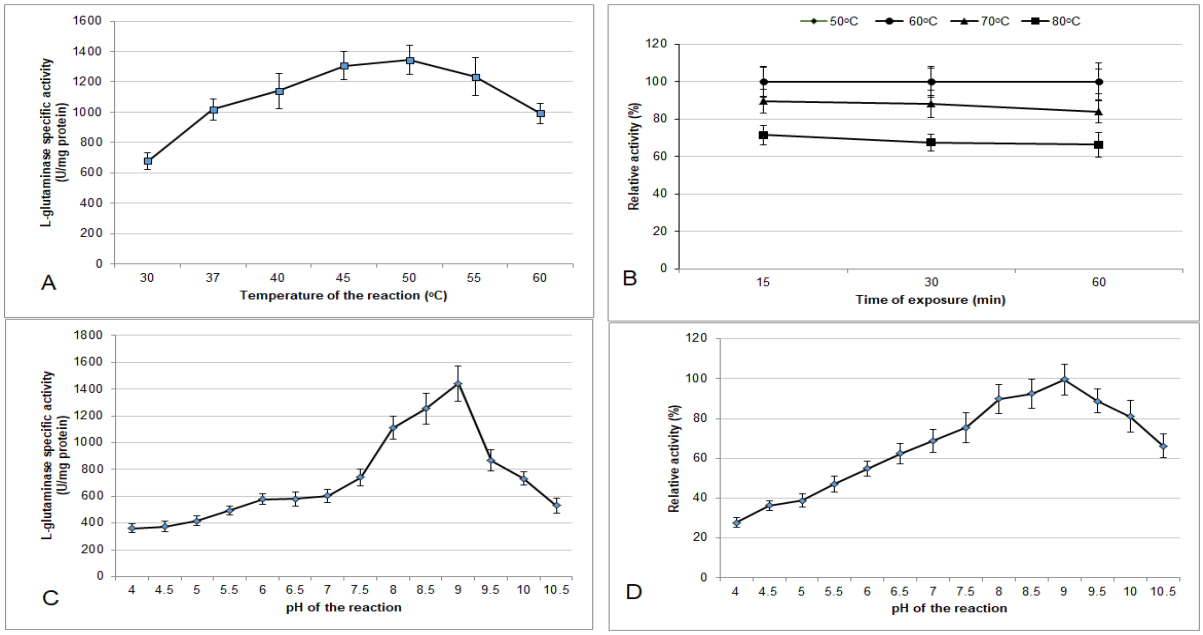

Figure 5(A): Effect of temperature of the reaction on purified *Pseudomonas* sp. RAS123 L-glutaminase activity. **(B):** Thermal stability of the purified *Pseudomonas* sp. RAS123 L-glutaminase. **(C):** Effect of pH of the reaction on purified *Pseudomonas* sp. RAS123 L-glutaminase activity. **(D):** pH stability of purified *Pseudomonas* sp. RAS123 L-glutaminase, probabilities for all outcomes statistics were regarded as significant when $P < 0.05$.

Table 2 Effect of some chemicals on the activity of the purified *Pseudomonas* sp. RAS123 L-glutaminase.

Metal ions (0.1 M)	Relative activity (%)
Control	100±9.09^{c}
ETDA	99.6±6.64 ^{c}
$MnCl_2$	205.53±20.55^{a}
$MgSO_4.7H_2O$	135.87±9.06^{b}
$NiCl_2$	134.98±12.27 ^{b}
FeSO4	127.8±11.62 ^{b}
CaSO4	99.4±8.28 ^{c}
CuSO4	107.17±8.24 ^{c}
ZnSO4	102.99±8.58 ^{c}
$HgCl_2$	90.43±7.54 ^{c}
KCl	67.41±6.13^{d}
NaCl	67.41±6.74 ^{d}
Pb acetate	77.58±5.54 ^{d}
SDS (10%)	43.5±3.95^{e}
ANOVA	21.5
P value	0.001*

Values followed by different letters (^{a,b,c,d,e}) belong to different studied groups. ANOVA test was applied for comparing between different groups.
P value : 0.001* , probabilities for all outcomes statistics were regarded as significant when $P < 0.05$.

Anticancer Activity of Purified Pseudomonas sp. RAS123 L-Glutaminase on Cancer Cell Lines

The viability (%) of various cancer cell lines as MCF-7, CCL-86, HepG2, HCT-116, and HeLa were examined after being treated with various levels of *Pseudomonas* sp. RAS123 L-glutaminase for 48 h. Since the enzyme's toxicity against CCL-86, MCF-7, HepG2, HCT-116, and HeLa cells was dependent on dose level, increasing the enzyme's dose gradually led to a steady decrease in cell growth **(Table 3)**. At the concentrations tested, purified *Pseudomonas* sp. RAS123 L-glutaminase was found to preferentially suppresses cancer cell multiplication. The toxic activity of *Pseudomonas* sp. RAS123 L-glutaminase against HCT-116 cells was significant with 83.51% inhibitory effect (IC_{50} value of 122 µg/ml), which was greater than the impact on HepG-2, MCF-7 (IC_{50} values of 175, 195, µg/ml, respectively), and a moderate inhibitory effect against HeLa (IC_{50} = 306 µg/ml), and a weak effect against CCL-86 (IC_{50} > 500 µg/ml) cells was noticed. ANOVA test was applied, and the results showed that the probability values $P < 0.05$, which were considered as statistically significant.

Table 3 Cytotoxic activity of purified *Pseudomonas* sp. RAS123 L-glutaminase on different cell lines

L-glutaminase concentration (µg/ml)	HCT-116 Viability %	HepG-2 Viability %	MCF-7 Viability %	HeLa Viability %	CCL-86 Viability %
500	16.49±1.27	23.14±1.93	31.97±3.20	37.08±2.65	69.24±6.29
250	30.62±2.55	39.75±2.65	48.21±4.38	63.85±4.56	88.06±8.01
125	49.31±4.48	68.93±4.92	82.64±7.51	89.42±6.39	97.82±8.89
62.5	76.78±5.48	85.02±7.09	93.29±8.48	98.13±7.01	100±8.33
31.25	91.36±6.53	97.48±7.50	99.63±8.30	100±10.00	100±6.67
15.6	98.65±8.97	100±10.00	100±7.14	100±8.33	100±6.67
7.8	100±8.33	100±6.67	100±6.67	100±6.67	100±7.14
3.9	100±7.69	100±9.09	100±10.00	100±9.09	100±8.33
0	100±7.14	100±6.67	100±10.00	100±6.67	100±7.69
ANOVA	51.24	42.5	32.5	33.6	18.25
P value	0.001*	0.001*	0.001*	0.001*	0.013*

Antimicrobial Activity of Purified Pseudomonas sp. RAS123 L-Glutaminase

The results recorded in **Table (4)** showed the antimicrobial activity of purified *Pseudomonas* sp. RAS123 L-glutaminase against some pathogenic microorganisms. The enzyme showed no effect on *Aspergillus fumigatus* (RCMB 002008) and *Candida albicans* RCMB 005003 (1) ATCC 10231.The highest antimicrobial activity of *Pseudomonas* sp. RAS123 L-glutaminase was achieved against *Bacillus subtilis* RCMB 015 (1) NRRL B-543 followed by *Streptococcus mutants* RCMB 017 (1) ATCC 25175 with mean inhibition zone diameter 35.6 ± 1.8 mm and 31.8 ± 1.4 mm, respectivly. Also, the antibacterial activity against *Pseudomonas aeruginosa* ATCC 27853 had the lowest mean inhibition zone diameter (13.7±0.9 mm).

Table 4 Mean inhibition zone of purified *Pseudomonas* sp. RAS123 L-glutaminase on various pathogenic strains

Tested microorganisms	Inhibition zone diameter (mm)	Control
FUNGI		*Ketoconazole*
Aspergillus fumigatus (RCMB 002008)	NA	19.40± 1.20
Candida albicans RCMB 005003 (1) ATCC 10231	NA	20.30 ± 0.80
Gram Positive Bacteria:		*Gentamycin*
Staphylococcus aureus (RCMB010010)	26.40 ± 1.20	24.80 ± 1.60
Bacillus subtilis RCMB 015 (1) NRRL B-543	35.60 ± 1.80	26.20 ± 1.90
Streptococcus mutants RCMB 017 (1) ATCC 25175	31.80 ± 1.40	25.30 ± 1.50
Gram Negatvie Bacteria:		*Gentamycin*
Enterobacter cloacae RCMB 001 (1) ATCC 23355	28.90 ± 2.30	27.50 ± 1.70
Escherichia coli (RCMB 010052) ATCC 25955	26.30± 1.50	30.10 ± 2.30
Pseudomonas aeruginosa ATCC 27853	13.70 ± 0.90	25.90± 2.40

- **NA***: No Activity

DISCUSSION

A marine bacterial isolate capable of producing antineoplastic enzyme was obtained from marine habitat in the current study, suggesting the possibility of identifying new bioactive chemicals from marine sources **(Wang *et al*., 2009; Abdelfattah *et al*., 2016)**. Marine microorganisms were discovered to be a significant source of anticancer enzymes using submerged fermentation **(Krishnakumar *et al*., 2011; Orabi *et al*., 2019; Mostafa *et al*., 2021)**. Chemical composition of seawater may provide a source of microorganisms that can synthesize enzymes with fewer side effects when used for therapeutic purposes **(Kiruthika and Swathi, 2019)**. The possibility of distinct immunological characteristics is due to the changing surface structure of anticancer holoenzymes caused by the high salinity **(Zolfaghar *et al*., 2019)**. The synthesis of L-glutaminase by marine microorganisms has recently received attention of many researches **(Mostafa *et al*., 2021)**. Hence, the development of new microbial resources for L-glutaminase with distinctive features is still ongoing for productivity, medicinal potential, and industrial challenges **(Soren *et al*., 2020).**

Immobilized cells are usually shielded from shear pressures and have a specific level of resistance to environmental stresses (such pH and temperature), which impede substrates and products. Additionally, immobilized cells can be kept active, viable, and productive for a long time, which allows continuous cultivation operations easier and improves the stability of operations (**Zhu, 2007**). So, in the current expermint, freely suspended and immobilized cell approaches were done to assess L-glutaminase synthesis. Entrapped bacterial cultures in sodium alginate beads showed a greater enzyme activity compared to what was observed by free cultures by 1.5-fold, followed by adsorbed cultures on polyurethane foams by 1.15-fold. This could be due to sodium alginate beads' porosity nature, which allows diverse metabolic products to easily permeate from the matrix. Under ambient settings, a variety of porous polymers were used to entrap microorganisms from suspended solution **(Martins *et al*., 2013).** Also, polyurethane foams showed an importance as microbial supports as they are inexpensive, and due to their strong mechanical qualities, large porosity, wide adsorption surface, simplicity of handling, regenerability, and resistance to microbial attack and organic solvents **(Patil *et al*., 2006)**. Agar-alginate beads, on the other hand, are mechanically fragile and very vulnerable to shear force, making them unsuitable for large-scale L-glutaminase synthesis. Several studies showed that entrapping bacterial cells in calcium alginate gel enhanced L-glutaminase production as in that found by *Pseudomonas* sp. BTMS-51 **(Kumar and Chandrasekaran, 2003)**, and *Pseudomonas nitroreducens* sp.001 cultures **(Shen *et al*., 2020)**.

Purified L-glutaminase from immobilized *Pseudomonas* sp. RAS123 cultures gave high specific activity (698.655 U/mg protein), which was higher than that detected by some *Pseudomonas* spp **(Katsumata *et al*., 1972; Roberts, 1976)**. In accordance to our work, **Awad *et al.* (2019)** purified L-glutaminase from culture of *Streptomyces rochei* SAH2_CWMSG by gel filtration using Sephadex G-100 column, chromatography. Other investigators used ion-exchange chromatography for purification of L-glutaminase as that produced from *Alcaligenes faecalis* KLU102 **(Pandian *et al*., 2014)**, and *Bacillus subtilis* OHEM11 **(Orabi *et al*., 2019),** while **Reda (2015)** combined DEAE-Cellulose and Sephadex G100 column, chromatography for purification of L-glutaminase from *Streptomyces canarius* FR. In addition, SDS-PAGE has been utilized by many researchers to detect the purity of the L-glutaminase enzyme. Most L-glutaminases enzymes have molecular weights ranging from 30 till 60 kDa. **(Nandakumar *et al*., 2003; Wakayama *et al*., 2005 Pandian *et al*., 2014; Mostafa *et al*., 2021; Saleem and Ahmed, 2021).** In addition, **Hiremath (2011)** produced L-glutaminase from *Pseudomonas* VJ6 of about 31 kDa.

Km and Vmax of *Pseudomonas* sp. RAS123 L-glutaminase were considered as low Km and high Vmax values, revealing the high affinity of the enzyme. **Desai *et al*. (2016)** reported that Km and Vmax were 2.8 mM, and 7.57 U/ml, respectively, for L-glutaminase produced from *Streptomyces* sp. Also, the kinetic studies of the purified L-glutaminase from *Achromobacter xylosoxidans* strain RSHG1 exhibited Km and Vmax of 0.236 mM and 443.8 U/mg, respectively **(Saleem and Ahmed, 2021).**

In many investigations, the ideal temperature for maximal glutaminases activity was around 40-50°C **(Nandakumar *et al.*, 2003; Ito *et al*., 2013; Saleem and Ahmed, 2021)**. The purified enzyme in our investigation was considered to be heat stable in absence of substrate. Its thermostability was higher than *Stenotrophomonas maltophilia* NYW-81 L-glutaminase which still had about 50% of its original activity after being heated for 10 min at 70°C **(Wakayama *et al*., 2005)** and *Streptomyces rochei* SAH2_CWMSG L-glutaminase which retained only 40% of its activity after incubating for 15 min at 80°C, and completely loses its activity after 30 min of heat treatment **(Awad *et al*., 2019).**

The purified preparation of L-glutaminase had an optimal pH of 9.0. The enzyme, on the other hand, demonstrated a reasonably high activity in the pH range of 8-10. Similarly, other studies for the optimal pH of the L-glutaminase enzyme was found to be in the alkaline range (8-9) **(Dura *et al*., 2002; Ito *et al*., 2013; Abu-Tahon and Isaac, 2019; Mostafa *et al*., 2021)**. On the contrary, **Desai *et al*. (2016)** and **Iyer and Singhal (2008)** found that the optimum pH value of the purified L-glutaminase enzyme was 7.0. **Singh and Banik (2013)** stated that glutaminases which have optimum pH above neutral are appropriate for therapeutic use. The purified *Pseudomonas* sp. RAS123 L-glutaminase was also found to have good pH stability throughout a pH range of 8-10. These findings are comparable to those of other L-glutaminases with a pH stability range of 7.5 to 9.0 **(Dura *et al*., 2002)**.

The purified *Pseudomonas* sp. RAS123 L-glutaminase was activated by Mn^{2+}, Mg^{2+}, $Ni2^{+}$, and Fe^{2+}, and these results were consistent with those of many other authors, as **Awad *et al.* (2019)**, who claimed that Mn^{+2} activated the L-glutaminase enzyme. The enzyme also was highly inhibited by SDS (10% w/v), which could be owing to the presence of disulphide linkages, which are important for maintaining the enzyme's appropriate conformation for catalytic activity. However, *Penicillium brevicompactum* NRC 829 L-glutaminase was suppressed by Hg^{+2} and Cu^{+2} **(Elshafei *et al*., 2014).** No effect on the activity of *Pseudomonas* sp. RAS123 L-glutaminase was observed by EDTA and this is in a good agreement with some investigators **(Dura *et al*., 2002; Elshafei *et al*., 2014).**

Vellard (2003) has stated that enzymes can bind and work on their targets with remarkable affinity on their targets, then convert and catalyze large number of target molecules into the desired products. Because of these two aspects, they are extremely particular and effective medications that can perform a specific therapeutic role in the body that other molecules cannot. Furthermore, **Tanaka *et al*. (1988)** mentioned that amidases deprive tumor cells from L-glutamine, resulting in the selective death of tumor cells that depend on L-glutamine.

The antitumor effect of *Pseudomonas* sp. RAS123 purified L-glutaminase in the present study exceeds earlier findings for *Streptomyces rochei* SAH2_CWMSG L-glutaminase which suppressed HepG2, MCF-7, and HCT-116 growth with IC_{50} of 279.7, 405.1, and 354.2 µg/ml, respectively **(Awad *et al*., 2019).** However, in other studies as that reported by L-glutaminase produced by actinomycetes, two human cell lines (HepG-2 and HeLa) were inhibited by the enzyme, with IC_{50} values of 102 µg/ml and 116.1 µg/ml, respectively **(Mesta and Onkarappa, 2017).** L-

glutaminase from *Bacillus cereus* MTCC gradually reduced the proliferation of hepatocellular carcinoma (Hep-G2) cell lines in the presence of various doses of L-glutaminase (10-100 µg/l) with an IC_{50} value of 82.27 µg/ml **(Singh and Banik, 2013)**. Furthermore, according to **Abu-Tahon and Isaac's (2019),** *Aspergillus flavus* L-glutaminase displayed significant cytotoxicity against the two cell lines Hela and Hep G2, and the IC_{50} values for them were about 18 and 12 µg/ml, respectively, while the IC_{50} values for HCT-116 and MCF7 cells were 44 and 58 µg/ml, respectively, indicating a moderate impact. The variations in cytotoxic effect among different L-glutaminases produced from different strains is related to the variation in glutamine levels reduction in plasma throughout therapy. The level of glutamine reduction in can cer cells, is resulting in their death with varying efficacy, and this depends on the biological half-life in the animal, the kinetic properties of the enzymes, and the rate at which the amino acid enters circulation **(Wriston and Yellin, 1973).**

Antibacterial activity of *Pseudomonas* sp. RAS123 L-glutaminase was discovered against some of the bacteria examined, with inhibitory diameter zones ranging from 14 to 36 mm. Few reports have been observed for the antimicrobial activity of L-glutaminase**. Emelda (2016)** examined the antibacterial activity of L-asparaginase and L-glutaminase producing isolates with potent activity and the maximum antibacterial activity was observed against *Staphylococcus aureus, Pseudomonas aeroginosa, Shigella flexneri, Salmonella Typhi, Vibrio cholerae and Klebsiella pneumoniae*. The lowest activity was observed against *Bacillus subtilis* and *E. coli* with inhibatory zones ranging from 10-26 mm.

CONCLUSION

In this study a new L-glutaminase was produced from free and immobilized cultures of local marine bacterial isolate, which was identified as *Pseudomonas* sp. RAS123. The enzyme was then purified, characterized and its molecular weight was found to be 32 kDa. Because the enzyme was highly specific to L-glutamine as a substrate, it could be utilized in low amounts while yet having a significant impact, also, the enzyme showed broad pH and thermostability. Moreover, purified L-glutaminase had shown substantial anticancer and antibacterial action. Consequently, it might be suggested that L-glutaminase is a promising bioactive compound and could be useful in both therapeutic and food industries.

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REFERENCES

Abdelfattah, M.S., Elmallah, M.I.Y., Hawas, U.W., El-Kassema, L.T.A., Eid, M. A. G. (2016). Isolation and characterization of marine derived actinomycetes with cytotoxic activity from the Red Sea coast. *Asian Pacific Journal of Tropical Biomedicine*, 6, 651–657. http://dx.doi.org/10.1016/j.apjtb.2016.06.004

Abu-Tahon, M.A., Isaac, G.S. (2019). Purification, Characterization and anticancer efficiency of l-glutaminase from *Aspergillus flavus*. *Journal of General Applied Microbiology*, doi:10.2323/jgam.2019.01.002.http://dx.doi.org/10.2323/jgam.2019.01.002

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215 (3), 403-410. http://dx.doi.org/10.1016/S0022-2836(05)80360-2

Awad, H.M., El-Deena, A.M.N., Mostafab, E.E., Hassabob, A.A. (2019). Biochemical studies and biological activities on L-glutaminase from rhizosphere soil *Streptomyces rochei* SAH2_CWMSG. *Egyptian Pharmaceutical Journal*, 18, 27–41. http://dx.doi.org/10.4103/epj.epj_32_18

Beltagy, E.A., El-Borai, A.M., Lewiz, M.M., El-Assar, S.A., 2016. Purification and characterization of alginate lyase from locally isolated marine *Pseudomonas stutzeri* MSEA04. *Acta Biologica Hungarica*, 67 (3), 305-317. http://dx.doi.org/10.1556/018.67.2016.3.8

Bettmann, H., Rehm, H.J. (1984). Degradation of phenol by polymer entrapped microorganisms. J. Applied Microbiology *Biotechnology*, 20, 285-290. http://dx.doi.org/10.1007/BF00270587

Binod, P., Sindhu, R., Madhavan, A. (2017). Recent developments in L-glutaminase production and applications: An overview. *Bioresource Technology*, 245 (Pt B), 1766-1774. http://dx.doi.org/10.1016/j.biortech.2017.05.059

Bülbül, D., Karakuş, E. (2013). Production and optimization of L-glutaminase enzyme from *Hypocrea jecorina* pure culture. *Preparative Biochemistry and Biotechnology*, 43 (4), 385-397. http://dx.doi.org/10.1080/10826068.2012.741641

Chapatwala, K.D., Babu, G.R.V., Wolfram, J.H. (1993). Screening of encapsulated microbial cells for the degradation of inorganic cyanides. *Journal of Indian Microbiology and Biotechnology*, 11 (2), 69-72. http://dx.doi.org/10.1007/BF01583677

Cluntun, A.A., Lukey, M.J., Cerione, R.A., Locasale. J.W. (2017). Glutamine Metabolism in Cancer: Understanding the Heterogeneity. *Trends Cancer*, 3(3), 169-180. http://dx.doi.org/10.1016/j.trecan.2017.01.005

Cruzat, V., Macedo Rogero, M., Noel Keane, K., Curi, R., Newsholme, P. (2018). Glutamine: metabolism and immune function, supplementation and clinical translation. *Nutrients*, 10 (11), 1564. http://dx.doi.org/10.3390/nu10111564

Desai, S.S., Chopra, S.J., Hungund, B.S. (2016). Production, purification and characterization of l-glutaminase from *Streptomyces* sp. isolated from soil. *Journal of Applied Pharmaceutical Science*, 6 (07), 100-105. http://dx.doi.org/10.7324/JAPS.2016.60715

Dura´, M.A., Flores, M., Toldra, F. (2002). Purification and characterization of a glutaminase from *Debaryomyces* spp. *International Journal of food Microbiology*, 76, 117– 126. http://dx.doi.org/10.1016/s0168-1605(02)00024-7

Dutta, S., Ghosh, S., Pramanik, S. (2015). L-asparaginase and L-glutaminase from Aspergillus fumigatus WL002: Production and some physicochemical properties. *Applied Biochemistry and Microbiology*, 51, 425-431. http://dx.doi.org/10.1134/S0003683815040067

El-Borai, A.M., Abedin, R.M., Shall, M.A., El-Assar, S.A. (2013). Purification and immobilization of dextransucrase from immobilized *Lactobacillus acidophilus* ST76480.01. *Journal of Pure and Applied Microbiology*, 7(4), 2915-2923.

El-Borai, A.M., Gadallah E. E., El-Aassar S. A., Beltagy, E.A. (2022). Enhancement of β-1,3-1,4-Glucanase Production by Marine Halomonas meridiana ES021 via Statistical Optimization and Cell Immobilization. *Egyptian Journal of Aquatic Biology and Fisheries*, 26(6), 781-802. http://dx.doi.org/10.21608/EJABF.2022.276391

Elshafei, A.M., Hassan, M.M., Ali, N.H., Abouzeid, M.A.E., Mahmoud, D.A., Elghonemy, D.H., (2014). Purification, kinetic properties and antitumor activity of L-glutaminase from *Penicillium brevicompactum* NRC 829. *British Microbiology Research Journal*, 4 (1), 97–115. http://dx.doi.org/10.9734/BMRJ/2014/5098

Emelda, J. (2016). Isolation and Screening of L-asparaginase and L-glutaminase Producing Bacteria and their Antimicrobial Potential from Environmental Sources. IOSR *Journal of Pharmacy and Biological Sciences*. 11, 47-53.

Farag, A.M., El-Borai, A.M., Morsy, R.A., El-Assar, S.A. (2021). Purification and Characterization of a Thermostable β-Mannanase from Halophilic *Aspergillus terreus* strain ARSA Associated to a Mangrove Plant of Red Sea Coast, Egypt, and its Application in Mannooligosaccharides Production and Juice Clarification. *Egyptian Journal of Aquatic Biology & Fisheries*, 25(6), 1–16. http://dx.doi.org/10.21608/ejabf.2021.209588

Farag, A.M., El-Naggar, M.Y., Ghanem, K.M. (2022). 2,4-Dichlorophenol biotransformation using immobilized marine halophilic *Bacillus subtilis* culture and laccase enzyme: application in wastewater treatment. *Journal of Genetic Engineering and Biotechnology*, 20, 134. http://dx.doi.org/10.1186/s43141-022-00417-1

Gomha, S.M., Riyadh, S.M., Mahmmoud, E.A., Elaasser, M.M. (2015). Synthesis and anticancer activities of thiazoles, 1,3-thiazines, and thiazolidine using chitosan-Grafted-Poly (vinylpyridine) as Basic Catalyst. *Heterocycles*, 91 (6), 1227-1243. http://dx.doi.org/10.3987/COM-15-13210

Gulati, R., Saxena, R.K., Gupta, R. (1997). A rapid plate assay for screening l-asparaginase producing microorganisms. *Letters in Applied. Microbiology*, 2, 23-26. http://dx.doi.org/10.1046/j.1472-765X.1997.00331.x

Hansen, M.B., Nielsen, S.E., Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of Immunological Methods*, 119, 203-210. http://dx.doi.org/10.1016/0022-1759(89)90397-9

Hiremath, J. (2011). Production of L-Glutaminase by Pseudomonas VJ6. *Research journal of biotechnology*. 6, 42-49.

Imada, A., Igarasi, S., Nakahama, K., Isono, M. (1973). Asparaginase and Glutaminase Activities of Microorganisms. *Journal of General Microbiology*, 76, 85-99. http://dx.doi.org/10.1099/00221287-76-1-85

Ito, K., Hanya, Y., Koyama, Y. (2013). Purification and characterization of a glutaminase enzyme accounting for the majority of glutaminase activity in *Aspergillus sojae* under solid-state culture. *Applied Microbiology and Biotechnology*, 97 (19), 8581-8590. http://dx.doi.org/10.1007/s00253-013-4693-4

Iyer, P., Singhal, R.S. (2008). Production of glutaminase (EC 3.2.1.5) from *Zygosaccharomyces rouxii*: statistical optimization using response surface methodology. *Bioresource Technology*, 99, 4300–4307. http://dx.doi.org/10.1016/j.biortech.2007.08.076

Katsumata, H., Katsumata, R., Abe, T., Takenaka, O., Inada, Y. (1972). Purification and physicochemical properties of l-glutaminase from *Pseudomonas*. *Biochimica Biophysica Acta (BBA) – Enzymology*, 289 (2), 405-409. http://dx.doi.org/10.1016/0005-2744(72)90093-9

Kiruthika, J., Swathi, S. (2019). Purification and characterisation of a novel broad spectrum anti-tumor L-glutaminase enzyme from marine *Bacillus subtilis* strain JK-79. *African Journal of Microbiology Research*,13(12), 232-244. http://dx.doi.org/10.5897/AJMR2017.8630

Krishnakumar, S., Alexis, Rajan. R., Ravikumar, S. (2011). Extracellular production of L- Glutaminase by marine alkalophilic *Streptomyces* sp.-SBU1 isolated from Cape Comorin coast. *Indian Journal of Marine Science*, 40, 717-721. http://dx.doi.org/123456789/13084

Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33, 1870-1874. http://dx.doi.org/10.1093/molbev/msw054

Kumar, S.R., Chandrasekaran, M. (2003). Continuous production of L-glutaminase by an immobilized marine *Pseudomonas* sp. BTMS-51 in a packed bed reactor. *Process Biochemistry*, 38 (10), 1431-1436. http://dx.doi.org/10.1016/S0032-9592(03)00035-9

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685. http://dx.doi.org/10.1038/227680a0

Lineweaver, H., Burke, D. (1934). The determination of enzyme dissociation constants, Journal of American Chemistry, 56, 658–666. http://dx.doi.org/10.1021/ja01318a036

Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic *Acids Research*, 25, 3389- 3402.

Lowry, O.H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951). Protein measurement with Folin-phenol reagent. Journal *of Biological Chemistry*, 48, 17-25.

Martins, S.C.S., Martins, C.M., Fiúza, L.M.C.G., Santaella, S.T. (2013). Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. *African Journal of Biotechnology*, 12 (28), 4412-4418. http://dx.doi.org/10.5897/AJB12.2677

Mesta, S. C., Onkarappa, R. (2017). Cytotoxicity of L-glutaminase from Estuarine actinomycete SMRO 38 against HeLa and HePG2 Cell Lines. *International Journal* of *Innovative Science Engineering* and *Technology*, 6, 15875–15884. http://dx.doi.org/10.15680/IJIRSET.2016.0608056

Mostafa, Y.S., Alamri, S.A., Alfaifi, M.Y., Alrumman, S.A., Elbehairi, S.E.I., Taha, T.H., Hashem, M. (2021). L-Glutaminase Synthesis by Marine *Halomonas meridiana* Isolated from the Red Sea and Its Efficiency against Colorectal Cancer Cell Lines. *Molecules*, 26, 1963. http://dx.doi.org/10.3390/molecules26071963

Nandakumar, R., Yoshimune, K., Wakayama, M., Moriguchi, M. (2003). Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *Journal* of *Molecular Catalysis B*: *Enzymatic*, 23, 87-100. http://dx.doi.org/10.1016/S1381-1177(03)00075-4

Nei, M. and Kumar, S. (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Orabi, H., El-Fakharany, E., Abdelkhalek, E., Sidkey, N. (2019). Production, optimization, purification, characterization, and anti-cancer application of extracellular l-glutaminase produced from the marine bacterial isolate. *Preparative Biochemistry and Biotechnology*, http://dx.doi.org/10.1080/10826068.2019.1703193

Pandian, S.R.K., Deepak, V., Sivasubramaniam, S. D., Nellaiah, H., Sundar, K. (2014). Optimization and purification of anticancer enzyme L-glutaminase from *Alcaligenes faecalis* KLU102. *Biologia*, 69 (12), 1644–1651. http://dx.doi.org/10.2478/s11756-014-0486-1

Patil, N.K., Veeranagouda, Y., Vijaykumar, M., Nayak, S., Karegoudar, T. (2006). Enhanced and potential degradation of o-phthalate by *Bacillus* sp. immobilized cells in alginate and polyurethane. *International Journal of Biodeterioration and Biodegradation*, 57, 82-87. https://dx.doi.org/10.1016/j.ibiod.2005.11.007

Reda, F.M. (2015). Kinetic properties of *Streptomyces canaries* l-glutaminase and its anticancer efficiency. *Brazilian Journal of Microbiology*. 46 (4), 957-968. http://dx.doi.org/10.1590/S1517-838246420130847

Roberts, J. (1976). Purification and properties of a highly potent antitumor glutaminase-asparaginase from *Pseudomonas* 7A*. *The Journal of Biological Chemistry*, 251 (7), 2119-2123.

Saleem, R., Ahmed, S. (2021). Characterization of a New L-Glutaminase Produced by *Achromobacter xylosoxidans* RSHG1, Isolated from an Expired Hydrolyzed L-Glutamine Sample. *Catalysts*, 11, 1262. https://dx.doi.org/10.3390/catal11111262

Saxena, A., Upadhyay, R., Kango, N. (2015). Isolation and identification of actinomycetes for production of novel extracellular glutaminase free L-asparaginase. *Indian Journal of Experimental Biology*, 53, 786-793. http://dx.doi.org //pubmed.ncbi.nlm.nih.gov/26742323/

Shen, X., Hua, Y., Luo, Y., Zhang, T., Jiang, B., Shuai, Y. (2020). Permeabilization and immobilization of whole-cell *Pseudomonas nitroreducens* SP.001 to improve its l-glutaminase performance. *Journal of Science Food and Agriculture*, http://dx.doi.org/10.1002/jsfa.10736

Singh, P., Banik, R. M. (2013). Biochemical characterization and anti-tumor study of l-glutaminase from *Bacillus cereus* MTCC 1305. *Applied. Biochemistry and Biotechnology*. 171, 522-531. http://dx.doi.org/10.1007/s12010-013-0371-3

Soren, J.P., Paul, T., Banerjee, A., Mondal, K.C., Das Mohapatra, P.K. (2020). Exploitation of agricultural waste as sole substrate for production of bacterial L-glutaminase under submerged fermentation and the proficient application of fermented hydrolysate as growth promoting agent for probiotic Organisms. *Waste Biomass Valorization*. 11, 4245–4257. http://dx.doi.org/10.1007/s12649-019-00761-3

Tamura, K., Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, 10, 512-526. https://dx.doi.org/10.1093/oxfordjournals.molbev.a040023

Tanaka, S., Robinson, E.A., Appellla, E., Miller, M., Ammon, H.L., Roberts, J., weber, I.T., Wolodawer, A. (1988). Structures of amidohydrolases aminoacid sequence of a glutaminase-asparginase from *Acientobacter glutaminasificans* and preliminary crystallographic data for an asparginase from *Erwinia crysanthemi*. *Journal of Biological Chemistry*, 263 (18), 8583-8591.

Vellard, M. (2003). The enzyme as drug: Application of enzymes as pharmaceuticals. *Current Opinion in Biotechnology*, 14, 444–450. http://dx.doi.org/10.1016/S0958-1669(03)00092-2

Vo, T.D., Sulaiman, C., Tafazoli, S., Lynch, B., Roberts, A., Chikamatsu, G. (2020). Safety assessment of glutaminase from *Aspergillus niger*. *Food Science* & *Nutrition*, 8, 1433–1450. https://dx.doi.org/10.1002/fsn3.1426

Wakayama, M., Yamagata, T., Kamemura, A., Bootim, N., Yano, S. (2005). Characterization of salt-tolerant glutaminase from *Stenotrophomonas maltophilia* NYW-81 and its application in Japanese soy sauce fermentation. *Indian Journal of Microbiology and Biotechnology*, 32, 383-390. http://dx.doi.org/10.1007/s10295-005-0257-7

Wang Z., Liu, J., Su, A., Sun M., Wang, C. (2009). Antiangiogenic activity of low-temperature lysozyme from a marine bacterium in vivo and in vitro. *Chinese Journal of Oceanology and Limnology*, 27(4), 835-844. http://dx.doi.org/10.1007/s00343-009-9181-4

Wriston, J.C., Yellin, T.O. (1973). L-asparaginase: A review. *Advanced Enzymology of Related Areas of Molecular Biology*, 39, 185–248.

Zhao, J., Lopez, A.L., Erichsen, D., Herek, S., Cotter, R.L., Curthoys, N.P., Zheng, J. (2004). Mitochondrial glutaminase enhances extracellular glutamate production in HIV-1-infected macrophages: Linkage to HIV-1 associated dementia. *Journal of Neurochemistry*, 88, 169-180. http://dx.doi.org/10.1046/j.1471-4159.2003.02146.x

Zhu, Y. (2007). Immobilized Cell Fermentation for Production of Chemicals and Fuels. Bioprocessing for Value-Added Products from Renewable Resources, pp, 373-396

ZoBell, C.E. (1941). Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *Journal of Marine Research*, 4, 42-75.

Zolfaghar, M., Amoozegar, M.A., Khajeh, K., Babavalian, H., Tebyanian, H. (2019). Isolation and screening of extracellular anticancer enzymes from halophilic and halotolerant bacteria from different saline environments in Iran. *Molecular Biology Reports*, 46, 3275–3286. http://dx.doi.org/10.1007/s11033-019-04787-7