International Journal of Technology and Engineering Studies
volume 9 pp. 1-13 doi: https://dx.doi.org/10.20469/ijtes.9.40001

Production, Purification and Characterization of L-glutaminase from Pseudomonas sp. GPB-06

Arti
Department of Biotechnology,
Shimla, Himachal Pradesh, India.

Lalita Kaushal
Department of Biotechnology,
Shimla, Himachal Pradesh, India.

Kumari Alka
Department of Biotechnology,
Shimla, Himachal Pradesh, India.

Ekta Arya
Department of Biotechnology,
Shimla, Himachal Pradesh, India.

Duni Chand*
Department of Biotechnology,
Shimla, Himachal Pradesh, India.

Abstract: Enzymes serve as remarkably efficient biological catalysts synthesized by living organisms, playing a crucial role in various metabolic processes both within and outside the cell. They accelerate the rates of a diverse array of chemical reactions essential for sustaining life. L-glutaminase(EC.3.5.1.2), a member of the amidohydrolase family, is responsible for catalyzing the deamination of L-glutamine which results in the generation of L-glutamic acid and ammonia. Notably, L-glutaminase exhibits wide distribution across the biological spectrum, being found in microorganisms to mammals. The purification of L-glutaminase from *Pseudomonas* sp. GPB-06 was achieved through a two-step process. Initially, cell disruption was carried out using a Bead-Beater in five cycles. Subsequently, the membrane-free enzyme extract underwent an initial precipitation step at 30% ammonium sulphate saturation to eliminate contaminating proteins and a final cut at 80% saturation resulting in the precipitation of the target protein. Dialysed enzyme was further purified using DEAE ion exchange chromatography which resulted in 1.66-fold purification, with a respectable 41.15% yield. The purified protein was identified as a monomer, comprised of subunits with a molecular weight of 41 kDa each. Characterization of the purified L-glutaminase revealed its optimal activity in a 100 mM potassium phosphate buffer at pH 7.0 and a temperature of 30^{o}C. The enzymatic properties of the purified L-glutaminase from *Pseudomonas* sp. GPB-06 included a Vmax of 144.92 μmole/min/mg protein. Furthermore, the Km values for L-glutamine were determined to be 0.6 mg/ml.

Keywords: *L-glutaminase, Pseudomonas, production, purification.*

Received: 02 January 2023; ***Accepted:*** 10 March 2023; ***Published:*** 05 May 2023

I. INTRODUCTION

Enzymes serve as highly efficient biological catalysts produced by living organisms, participating in all metabolic processes both within and outside the cell. They significantly enhance the rates of a diverse range of chemical reactions essential for life [1]. L-glutaminase (EC.3.5.1.2) is a member of the amidohydrolase family, catalyzing the deamination of L-glutamine and resulting in the production of L-glutamic acid and ammonia. This enzymatic activity is widespread, being observed across a spectrum from microorganisms to mammals [2]. Glutamic acid is recognized as a significant amino acid,

*Correspondence concerning this article should be addressed to Duni Chand, Department of Biotechnology, Shimla, Himachal Pradesh, India. E-mail: dunichand_2000@yahoo.com

contributing not only to the pleasant taste of "Umami" but also to the nutritional qualities of food. Consequently, the incorporation of safe starter cultures with glutaminase activity into fermented sausages is desirable. This enzyme acts on L-glutamine, which is present in relatively high amounts in the fresh mix, generating ammonia as a neutralizer of acidity and L-glutamate as a flavour enhancer [3]. In soil, L-glutaminase facilitates the release of ammonium into the inorganic nitrogen pool through the breakdown of L-glutamine [4].

The quest for L-glutaminase with properties suitable for application as an antileukemic and/or flavour-enhancing agent remains ongoing [5]. The therapeutic and industrial significance of L-glutaminase necessitates not only the discovery of new and more productive microbial strains but also the development of economically viable bioprocesses for its large-scale production [6]. The utilization of L-glutaminase enzymes as a drug has gained considerable importance [7]. L-Glutamine serves as an efficient carrier for the transport of nitrogen and carbon skeletons among various tissues within a living organism. Tumour inhibition operates through the suppression of both nucleic acid and protein synthesis in tumour cells. One potential strategy to impede tumour growth involves the specific inhibition of glutamine uptake by tumour cells. L-Glutaminase induces selective death in glutamine-dependent tumour cells by depriving them of L-glutamine, making it an effective agent in the treatment of acute lymphocytic leukaemia and HIV [8].

L-glutaminase also finds applications as a biosensor and analytical agent. It is utilized for analysing the L-glutamine content in culture mediums and determining reaction rates in threonine synthesis [9]. Moreover, the enzyme plays a role in regulating cerebral concentrations of glutamine and glutamate, crucial in processes like ammonia detoxification [10]. Microbial enzymes contribute significantly to the diagnosis, treatment, biochemical investigation, and monitoring of various diseases [11]. The production of enzymes for pharmaceutical use constitutes a pivotal aspect of today's pharmaceutical industry [12].

Microorganisms stand out as an excellent source of numerous therapeutic enzymes due to their substrate specificity, diversity, and amenability to genetic manipulation. The majority of enzymes presently employed in industry originate from microbial sources. L-glutaminases are widespread in the biological realm [13]. In microorganisms, glutaminases have been identified in numerous species, encompassing both gram-positive and gram-negative bacteria, as well as yeasts and fungi [14]. Nonetheless, bacteria exhibit the capability to produce higher amounts of L-glutaminase during their minimum growth, making them preferred candidates for large-scale industries as highly potent strains [15]. The synthesis of L-glutaminase has been documented in various bacterial genera, with a notable presence in terrestrial sources such as E. coli, *Pseudomonas* sp., *Acinetobacter*, and *Bacillus sp.* However, reports on the screening of bacteria as potential sources of L-glutaminase producers are currently limited to specific localities [16].

L-glutaminase has garnered significant attention due to its broad applications in pharmaceuticals, serving as an anti-leukemic agent and an efficient anti-retroviral agent. Additionally, it is employed for the production of specialty chemicals like theanine through c-glutamyl transfer reactions [17]. In *Pseudomonas* sp., recombinant glutaminase has been patented for its activity against cancer and HIV virus therapy [18]. Cancer, particularly leukaemia, remains a global challenge, and despite sincere efforts in the past, the search for efficient drugs to address this issue continues worldwide.

The advancement of biotechnology has opened up new applications for microbial L-glutaminases in clinical settings and the production of metabolites. Notably, L-glutaminase has emerged as a substitute for Monosodium Glutamate (MSG) in enhancing the taste of Chinese foods. Concurrently, microbial L-glutaminases have garnered significant attention for their role in food flavouring, particularly in industries related to soy sauce in the orient. This interest has sparked a quest for industrial sources of enzymes [19]. In present study, we reported production, purification and characterization of L-Glutaminase from Pseudomonas sp.

II. MATERIALS AND METHODS

A. *Chemicals*

All chemicals employed in the experiments were of analytical grade. The media components and other fine chemicals used throughout the experiments were acquired from HI-Media, Mumbai, India and Sigma, USA.

B. *Microorganisms and Cultural Conditions*

Microorganisms producing L-glutaminase were isolated from various regions of Himachal Pradesh, India. Isolates were further screened quantitatively for L-glutaminase production through submerged fermentation and assessing enzyme activity. In this process, soil samples were added to enrichment medium (Soya Peptone 0.5%, Yeast Extract 0.2%, Beef Extract 0.1%, NaCl 0.5%, L-glutamine 1.0% w/v, pH 7.0) and after incubation each sample were serially diluted and spread over nutrient agar (NA) plates at pH 7.0 and potato dextrose agar (PDA) plates at pH 5.5 to isolate bacterial and fungal

strains, respectively. Colonies exhibiting diverse shapes, sizes, and colours were selected, and pure cultures were established by streaking single colonies on NA and PDA. Various bacterial and fungal strains were cultured in nutrient broth and incubated at 30^{o}C for 24 to 74 hours at 150 rpm. Samples were harvested after every 24 hours and centrifuged at 10,000 g for 10 minutes at 4^{o}C. Both the pellet and supernatant were assayed for enzyme activity, and the isolate demonstrating the highest activity was chosen for further study.

C. Enzyme Production

The selected isolate GPB-06 was cultivated in production medium comprised Soya Peptone 0.5, Yeast Extract 0.2, Beef Extract 0.1, NaCl 0.5, and L-glutamine 1.0 (%, w/v), with a pH of 7.0 [20]. Filter sterilized L-glutamine was added to medium after autoclaving. Twenty-four hr aged seed culture (8% v/v) was added to production medium and incubated at 30^{o}C for 24 hrs at 160rpm. After incubation, contents of individual flasks were then centrifuged at 10,000 rpm for 15min. at 4^{o}C [16]. Supernatant and cell pellets were thus collected and used as crude enzyme and were further assayed for enzyme activity as per protocol mentioned below.

D. Enzyme Assay

The assay for L-glutaminase enzyme was conducted utilizing a colorimetric method, wherein ammonia formation was quantified through spectrophotometric analysis. The Fawcett and Scott method, 1960 [21] was used for determination of enzyme activity with certain modifications.

The reaction mixture contained Potassium Phosphate Buffer, 0.1M, pH 8.0 (900μl), enzyme (whole resting cells suspensions) 50l, substrate (0.1M L-glutamine) 50μl. This reaction mixture was incubated at 35^{o}C for 5 min in a water bath shaker and the reaction was stopped by the addition of 1 ml of 0.1N HCl. A control was also prepared by omitting the enzyme (cell suspensions), which was added after the reaction was stopped to consider the auto hydrolysis of substrate. The reaction mixture was centrifuged at 10,000 g for 10 min, discarded the pellet and clear supernatant was collected for estimation of ammonia. To 1 ml of centrifuged reaction mixture added 2.5 ml each of solution A [Phenol (10g) + Sodium nitroprusside (50mg) in 1000 ml distilled water] and B [Sodium hydroxide (5.0g) + Sodium hypochlorite (20ml) in 1000ml distilled water] and thoroughly mixed and kept in boiling water bath for 5 minutes for maximum development of colour. The absorbance was read at 640 nm.

E. Dry Cell Weight Standard

One millilitre of resting cells with various optical densities (O.D.) was placed in pre-weighed Eppendorf tubes and centrifuged at 10,000 g for 15 minutes. The supernatant was carefully poured off, and the pellet was dried at 80^{o}C until three concordant weights were obtained. A graph was generated by plotting the dry weight of cells against their absorbance at 600 nm.

F. Protein Estimation

The protein concentration was determined using the dye-binding method, commonly known as the Bradford method in 1976 [22]. This method relies on the interaction of Coomassie Brilliant Blue G-250 with proteins. The binding of the dye to protein induces a shift in the absorption maximum of the dye from 465 to 595 nm, and the increase in absorption at 595 nm is then measured. This assay is highly reproducible and rapid, with the dye binding process essentially completed in approximately 2 minutes, and it exhibits good color stability for up to 1 hour. Fifty microliters (50 $^o\mu$L) of the sample (enzyme) were taken, and 950 $^o\mu$L of buffer was added if necessary. To this, 1 mL of Bradford working reagent was added and mixed thoroughly. The absorbance of the reaction mixture was measured at 595 nm after 2-5 minutes. A standard curve for protein was constructed using various concentrations of BSA (Bovine Serum Albumin).

G. Optimization of Physic-Chemical Parameters for L-glutaminase Production

The optimal conditions necessary for achieving maximal growth and L-glutaminase production by the selected strains were determined through subjecting them to various experimental conditions. These conditions included different incubation temperatures, pH levels, substrate concentrations, NaCl concentrations, additional carbon and nitrogen sources along with their respective concentrations, inoculum size in the growth media, and varying incubation periods.

H. Enzyme Purification

The purification of L-glutaminase from *Pseudomonas* sp. GPB-06 was undertaken to investigate its biochemical characteristics. All enzyme purification experiments were conducted at 4^{o}C, employing a potassium phosphate buffer (0.1 M). The whole resting cells were disrupted by using BeadBeaterTM (BioSpec Products, Inc., USA). The cell slurry (100 ml, 2.50 g dcw) was added to bead beater chamber (350 ml) half filled with zirconium beads (0.1 mm). A single beating cycle involved 1 min agitation and 1 min rest. The beating chamber was jacketed with ice to

absorb the heat produced due to high speed rotation of the impeller. After every cycle, samples were withdrawn and centrifuged to remove cell debris. The amount of protein released and acyltransferase activity in the membrane free extract was determined to check the disintegration efficiency of the method. The cell debris from this mixture were removed by centrifugation at 20,000 g at 4^{o}C for 30 min. Clear supernatant was collected and filtered through 0.45 m PVDF Durapore membrane (Millipore) using vacuum pump. The cell free extract (CFE) was used for the further purification of enzyme.

The cell free extract was subjected to various saturation/concentration of ammonium sulphate (10-100%). The ammonium sulphate precipitation protocol (Scopes 1982) was followed to calculate the required amount of ammonium sulphate to be added in cell free extract. It was added with continuous stirring and stored at 4^{o}C for 45 min. The precipitated proteins were recovered by centrifugation at 20,000 g for 30 min. and suspended in 0.1 M potassium phosphate buffer (pH 7.5). Protein concentration and L-glutaminase activity were estimated in each protein samples. Based on the result of the above experiment, an initial cut of 30% was employed to cell free extract (30 ml) in order to precipitate the contaminating proteins and the supernatant was given final cut of 80% ammonium sulphate to precipitate the protein of interest which was recovered by centrifugation and suspended in 0.1 M potassium phosphate buffer (pH 7.5) and was taken for further purification of enzyme.

Anion exchange chromatography was performed using 20 ml of pre-packed DEAE Sepharose column (HiPrepTM 16/10 DEAE FF, GE Healthcare) at a flow rate of 1.0 ml/min. The sample obtained after ammonium sulphate fractionation (8 ml) from the above step was dialyzed and then applied to the column pre-equilibrated with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 M NaCl. After loading the protein sample, the column was washed with 100 ml of above buffer to remove the unbound protein and then the bound proteins were eluted with a linear gradient of NaCl from 0-1.0 M in 0.1 M potassium phosphate buffer. The length of the gradient was 200 ml, the flow rate was 1.0 ml/min and the fraction size was 2.0 ml. The protein concentrations in the samples were determined by the Bradford method (1976) [22] with BSA used as a standard. SDS-PAGE [23] analysis of the enzyme-rich fractions obtained from the DEAE ion exchange chromatography steps was conducted to assess the purification status of the enzyme.

I. Characterization of Purified Enzyme

The purified L-glutaminase from *Pseudomonas* sp. GPB-06 underwent biochemical characterization to compare its properties with those of other reported L-glutaminases. Various biochemical properties, including molecular weight, pH and temperature optima, buffer molarity, Km, and Vmax, were investigated for the purified enzyme.

J. Optimization of Substrate Concentration and Determination of Km and Vmax of Purified Enzyme

The optimal substrate concentration is crucial for achieving maximal enzyme activity. Deviating from this optimum range, either with low or high concentrations can lead to a decrease in activity. This is attributed to variations in the binding efficiency of the substrate to the active site on the enzyme. Therefore, the effect of substrate concentration plays a significant role in influencing enzyme activity, with both increases and decreases observed based on the concentration. To optimize the substrate concentration and determine the Km and Vmax of the purified L-glutaminase from Pseudomonas sp. GPB-06, the concentration of L-glutamine was systematically varied within the range of 25-200 mM. The enzymatic reaction was conducted at 30^{o}C in a 100 mM potassium phosphate buffer (pH 7.0) and allowed to proceed for 5 minutes. Storage stability of enzyme was investigated both at 4^{o}C and 25^{o}C for 20 days (at an interval of 2 days).

III. RESULTS AND DISCUSSION

A. Screening of L-Glutaminase Producers

By submerged fermentation sum total of 11 bacterial and 5 fungal isolates showed enzyme activity. Following the preliminary screening, the identified positive isolates underwent further cultivation in nutrient broth. The cultures were incubated at 30^{o}C for 24 hours in an orbital shaker at 150 rpm. Among the isolates, bacterial strain GPB-06 exhibited the highest L-glutaminase production (12.12 Units/mg dcw). In contrast, the enzyme production was relatively low in the case of fungal isolates when compared to bacterial isolates.

B. Identification and Characterization of Selected Isolate

The bacterial isolate GPB-06 was identified as *Pseudomonas* sp. at the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (INDIA). Subsequent production and characterization of L-glutaminase were conducted with *Pseudomonas* sp. GPB-06, as this isolate exhibited the highest enzyme activity. On nutrient agar plates, *Pseudomonas* sp. GPB-06 forms cream-colored colonies after 24 hours of incubation (Figure 1).

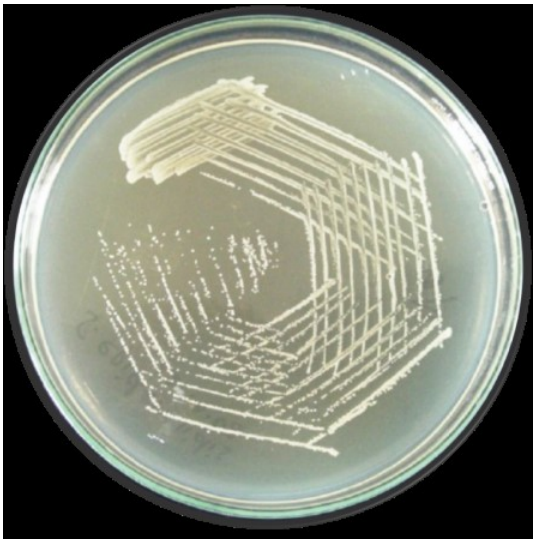

Fig. 1. Morphological characteristic from Pseudomonas sp. GPB-06 on nutrient agar plate (24 h).

C. *Optimization of Various Production Parameters*

Various physico-chemical parameters, including the selection and optimization of the growth medium, optimization of production pH, temperature, inoculum size, incubation time and the role of an inducer, were systematically optimized to achieve the optimum growth and production of L-glutaminase from Pseudomonas sp. GPB-06. The bacterium was cultivated in eight different media known for L-glutaminase production. The results indicate that the medium with a pH of 7.0, comprising soya peptone (0.5% w/v), L-glutamine (0.2% w/v), D-maltose (0.5% w/v), and a 2% (w/v) inoculum, was found to be the most suitable for L-glutaminase production at 25^{o}C. The investigation focused on assessing the impact of various carbohydrates (Sucrose, Fructose, Glucose, Xylose, Galactose, Lactose, and Maltose) on the production medium to determine the carbon source that yields maximum activity. Notably, D-Maltose emerged as the sole optimized carbon source, with highest activity at 16.39 U/mg dcw. Interestingly, findings by Sahu and Gupta, 2017 [24] diverged from the current study, as they identified fructose as the best carbon source, yielding 156.025 U/ml, while sucrose resulted in 13.375 U/ml. In contrast, the present study reported the lowest yield when lactose was used as a sole source of carbon, with an enzyme activity of 10.646 U/ml. This differs from the work of Makky et al., 2013 [25], where lactose supported optimum enzyme production from *Bacillus* KK2S4, resulting in an enzyme activity of 0.315 U/ml. These variations underscore the complexity of carbon source influence on enzyme activity and highlight the need for a nuanced understanding of such interactions in diverse microbial systems.

The optimization process for L-glutaminase production involved supplementing the medium with various concentrations of D-Maltose, revealing that at 0.07% (w/v), the highest activity was achieved at 17.2 U/mg dcw. In a study by Hiremath et al., 2011 [16], glucose supplementation at a concentration of 10g/l resulted in the highest L-glutaminase activity, reaching 58.4 U/ml. Similarly, El-Sayed, 2009 [26] identified glucose as the optimal carbon source for L-glutaminase activity, reporting a maximum activity of 35 U/ml. These findings underscore the significance of carbon source concentration in influencing L-glutaminase production, with D-Maltose at 0.07% (w/v) demonstrating promising results in the present study compared to glucose concentrations used in other investigations.

Initially, nitrogen sources such as yeast extract, soya peptone, and beef extract were employed. To explore the impact of alternative organic nitrogen sources, various nitrogen sources were introduced. It was revealed that Soya peptone emerged as the most effective, yielding the highest activity at 18.2 U/mg dcw. In contrast, for *Streptomyces sp.*, Desai et al., 2016 [27] identified the substrate L-glutamine as the nitrogen source providing the maximum enzyme activity of 33.1 U/ml. This demonstrates the diversity in nitrogen source preferences among different microorganisms and underscores the importance of tailoring the nitrogen component based on the specific requirements of the organism under study.

In the investigation of various concentrations of soya peptone, *Pseudomonas* sp. GPB-06 exhibited the highest enzyme activity of 21.3 U/mg dcw when soya peptone was utilized at a proportion of 0.6% (w/v). Contrastingly, Kiruthika and Saraswathy, 2013 [28] reported soyabean meal as an optimized source at a concentration of 2%, resulting in an enzyme activity of 300 U/ml from *Vibrio*

azureus JK-79. These findings highlight the significance of both the nitrogen source and its concentration in influencing enzyme activity, showcasing the variability in requirements among different microbial strains.

During the exploration of various concentrations of L-glutamine for L-glutaminase production from Pseudomonas sp. GPB-06, the maximum enzyme activity of 22.7 U/mg dcw was observed at a concentration of 0.2%. In contrast to the current findings, Hamed and Al-wasify, 2016 [29] reported that a lower concentration of L-glutamine, specifically 0.025%, was found to be optimal for achieving maximum activity from *Fusarium oxysporum*. These results underscore the importance of fine-tuning nutrient concentrations to meet the specific requirements of individual microorganisms, emphasizing the need for a tailored approach in optimizing culture conditions for enzyme production.

In the context of L-glutaminase production from *Pseudomonas* sp. GPB-06, the investigation revealed that the maximum enzyme activity, reaching 25.27 U/mg dcw, was achieved at a 0.2% NaCl concentration. In a similar study, Desai et al., 2016 [27] have also reported the optimal L-glutaminase production from *Streptomyces sp.*, with a 0.1% NaCl concentration. These findings highlight the nuanced nature of salt concentration optimization, emphasizing the need to tailor such conditions based on the specific microbial strains under consideration.

Following optimization, it was determined that incubation at 25^{o}C (pH 7.0) for 24 hours was optimal for *Pseudomonas* sp. GPB-06, exhibiting the highest enzyme activity of 26.4 U/mg dcw among different temperature ranges. According to [2] the isolate *Streptomyces pratensis* NRC 10 demonstrated improved growth and enzyme production at 25^{o}C, pH 7.5, after 4 days of incubation. Similarly, another investigation on L-glutaminase production from *Pseudomonas* VJ-6, conducted by [16] identified the optimum temperature as 25^{o}C, resulting in a yield of 79.23%. These findings collectively underscore the significance of temperature optimization and its impact on enzyme production, showcasing the preference for 25^{o}C in different microbial systems.

In the present study, the optimized pH range was determined to be 7.0 (neutral pH), resulting in the maximum activity of 29.24 U/mg dcw. In contrast [30] reported that the optimal activity of L-glutaminase from *Pseudomonas aeruginosa* was observed at an alkaline pH range of 7.5-9.0. These differing pH preferences highlight the diversity in enzymatic characteristics among microbial strains and underscore the importance of pH optimization based on the specific requirements of the organism under investigation.

The highest L-glutaminase production by *Pseudomonas* sp. GPB-06 was achieved with a 2% (v/v) inoculum size, resulting in a maximal activity of 32.72 U/mg dcw, as indicated in Table 1. According to [31] there is little evidence that maximal L-glutaminase production at a 2% (v/v) inoculum size when utilizing wheat bran and sesamum oil cake. These consistent results highlight the importance of inoculum size as a critical parameter influencing L-glutaminase production across different studies. Production profile of L-glutaminase revealed that enzyme yield increases with increase in incubation period but up to 28 h (37.58 U/mg dcw) only thereafter decline in enzyme was observed (Figure 2).

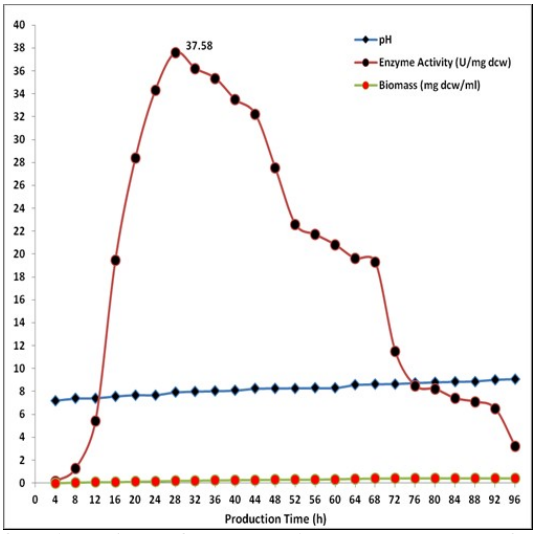

Fig. 2. Grwoth and production prifile of L-glutaminase from *Pseudomonas* sp. GPB-06.

TABLE 1
OPTIMIZATION OF VARIOUS PRODUCTION PARAMETERS FOR L-GLUTAMINASE FROM *PSEUDOMONAS* SP. GPB-06

Optimized Parameter	Enzyme Activity (U/mg dcw)
Carbon source (Maltose)	16.39
Carbon source concentration (0.07%)	17.2
Organic Nitrogen source (soya peptone)	18.2
Nitrogen source concentration (0.6%)	21.3
Substrate concentration (0.2%)	22.7
NaCl concentration (0.2%)	25.27
Production temperature (25)	26.4
Production pH (7)	29.24
Inoculum size (2%)	32.72

D. Enzyme Purification

The purification of L-glutaminase from *Pseudomonas* sp. GPB-06 was achieved through a two-step process. Initially, the cells were disintegrated using Bead-Beater up to five cycles. Highest specific activity (36.02 U/mg protein) was obtained after five beating cycles after which decline in specific activity was observed. Later beating cycles resulted in an increase in the protein release from the cells but at the same time decline in enzyme activity was also observed due to denaturation of enzyme. Thus five beating cycles were performed for the disruption of the *Pseudomonas* sp. GPB-06 cells. The results of the optimization of cell disruption by Bead BeaterTM are shown in (Figure 3). The resulting membrane-free enzyme extract underwent an initial cut of 30% ammonium sulphate saturation to eliminate contaminating proteins, followed by a final cut of 80% ammonium sulphate to precipitate the protein of interest. Subsequently, after dialysis, the enzyme was further purified through DEAE ion exchange chromatography. The purification process yielded a purification fold of 1.66 with a 41.15% yield. The purified protein was identified as a monomer with a subunit size of 41 kDa as shown in (Figure 4). Following the loading of the protein sample, a thorough wash was conducted using 100 ml of potassium phosphate buffer to eliminate any unbound proteins. Subsequently, the bound proteins were eluted by applying a linear gradient of NaCl (ranging from 0 to 1.0 M) in a potassium phosphate buffer (0.1 M). The gradient spanned a length of 200 ml, with a flow rate of 1.0 ml/min and fractionation at 2.5 ml intervals. The resulting protein elution profile and the corresponding L-glutaminase activity within the collected fractions are depicted in (Figure 5). Although a very high specific activity was shown by fractions 35-39, maximum activity was recorded in 37th fraction (60.08 U/mg proteins). Purification profile of L-glutaminase from *Pseudomonas* sp. GPB-06 is summarized in Table 2.

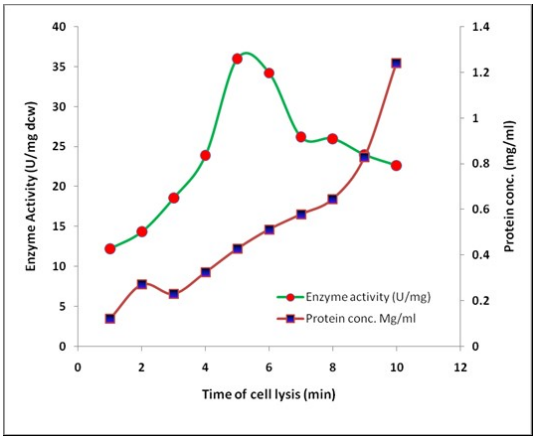

Fig. 3. Profile of protein release and L-glutaminase activity in the membrane free extract during cell disintegration

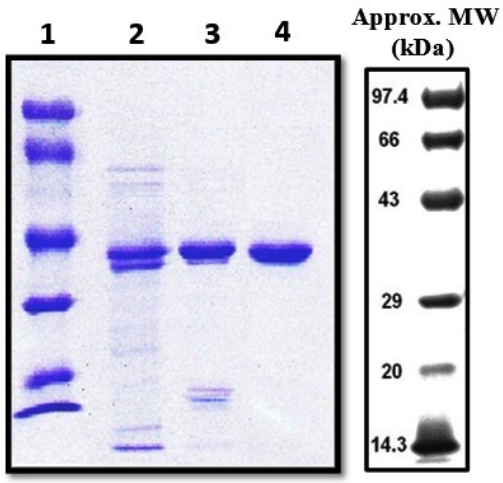

Fig. 4. SDS PAGE anlysis for L-glutaminase from *Pseudomonas* sp. GPB-06 [Lane 1: Molecular Weight Markers; Lane 2: Crude protein; Lane 3: Dailyzed sample of ammonium sulphate precipitates; Lane 4: Pooled fractions of DEAE]

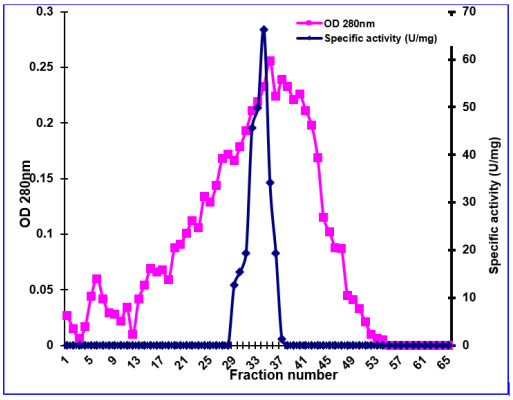

Fig. 5. Protein elution and *L-glutaminase* activity profile of DEAE Ion exchange chromatography

TABLE 2
PURIFICATION OF L-GLUTAMINASE FROM PSEUDOMONAS SP. GPB-06

Purification	Total activity	Total protein (mg)	Specific activity	Fold purification	Yield (%)
Crude	1901.25	39	36.02	1	100
ASP (dialyzed)	943.56	15.99	48.75	1.35	49.62
DEAE-Sepharose	269.15	4.48	60.08	1.66	41.15

E. Optimization of Reaction Parameters and Characterization of L-glutaminase

The purified enzyme derived from *Pseudomonas* sp. GPB-06 underwent thorough characterization to establish the optimal reaction parameters. These parameters included the choice of buffer and its pH, buffer molarity, reaction temperature, substrate concentration, and the influence of metal ions and solvents, as well as an assessment of shelf-life.

The investigation involved utilizing the following buffers to determine the pH optimum for the reaction: Potassium Phosphate buffer (pH 6.0 - 8.5), Citrate buffer (pH 3.5 - 6.0), Sodium Phosphate buffer (pH 5.5 - 8.0), and Acetate buffer (pH 3.0 - 5.5) [Figure 6]. The reaction was carried out at 30^{o}C for 2 minutes in a 2.0 ml reaction mixture comprising 0.1 M of the specified buffer, 75 mM L-glutamine, and the purified glutaminase (diluted as per requirement). Among the various buffers tested, potas-

sium phosphate buffer at pH 7.0 yielded the maximum activity of 68.45 U/mg. These results closely resemble those obtained from thermostable L-glutaminase from *Bacillus circulans* [32]. The selection of an optimal buffer is crucial for maintaining the enzyme's stability and activity under specific reaction conditions. The impact of buffer molarity on L-glutaminase activity was also investigated by varying the buffer concentration from 25 to 200 mM in the reaction. The highest specific activity, reaching 84.67 U/mg, was observed when using 100 mM potassium phosphate buffer. This finding emphasizes the importance of selecting an appropriate buffer concentration to optimize the enzymatic activity, and the specific activity achieved with 100 mM potassium phosphate buffer highlights its effectiveness in supporting L-glutaminase activity.

To assess the impact of reaction time on the activity of purified L-glutaminase from *Pseudomonas* sp. GPB-06, the reaction was terminated at various time intervals ranging from 1 minute to 10 minutes. The enzyme activity was found to be maximized with a 3-minute incubation period, reaching 86.35 U/mg. This contrasts with the findings of [33] who observed a reaction time of 30 minutes for Bacillus sp. YJ6. These differences highlight the variations in optimal reaction times among different L-glutaminase enzymes and microbial sources. The temperature optimum for the purified L-glutaminase of *Pseudomonas* sp. GPB-06 was determined by varying the reaction temperature from 25-50^{o}C. The optimal temperature for L-glutaminase activity was found to be 30^{o}C, resulting in an activity of 92.88 U/mg. According to [34], where the temperature optimum for L-glutaminase was reported to be 32^{o}C. This agreement emphasizes the consistency of optimal temperature ranges for L-glutaminase activity across different studies.

The enzyme reaction was conducted with varying doses of L-glutaminase in the reaction mixture, ranging from 10μg to 100μg. The maximum activity, reaching 110.46 U/mg, was recorded in the presence of 20μg of the enzyme (Figure 7). Interestingly, a decrease in activity was observed upon increasing the concentration beyond 25μg, which aligns according to [35]. This observation suggests that there might be an optimal enzyme dose beyond which further increases do not contribute to a proportional enhancement in activity. None of the tested metal ions showed any positive effect on the activity of L-glutaminase from *Pseudomonas* sp. GPB-06 compared to the control shown in (Figure 9). Enzyme activity was strongly inhibited by Ag and Hg ions which ascertained the presence of cysteine residues at the active site of enzyme. The enzyme reaction was also performed in biphasic solvent systems but no enhancement in L-glutaminase activity was observed as shown in (Figure 10).

The purified L-Glutaminase from *Pseudomonas* sp. GPB-06 displayed a Vmax of 144.92 IU/mg protein, with a Km value of 0.60 mg/ml, as determined by the Line Weaver Burk plot using L-glutamine as the substrate (Figure 11). The enzyme reaction was conducted using various concentrations of L-glutamine in the reaction mixture, spanning from 25 to 200 mM. The maximum activity observed was 118.88 U/mg (Figure 8), and it occurred when the reaction mixture contained 100 mM of L-glutamine. Notably, these findings align with previous studies conducted by Muthezhilan et al. 2007 and Sanghi et al. 2010 [36]. Comparatively, earlier reports on L-glutaminase from A. fischeri indicated a Km of 4.88 mg/ml and a Vmax of 588 mmol/min/mg [37]. These findings highlight the differences in substrate affinity and catalytic efficiency between L-glutaminases from *Pseudomonas* sp. GPB-06 and *A. fischeri*, providing insights into the enzymatic characteristics of these microbial sources. Storage stability of enzyme was also investigated for both at 4^{o}C and 25^{o}C for 20 days (at an interval of 2 days) as shown in (Figure 12).

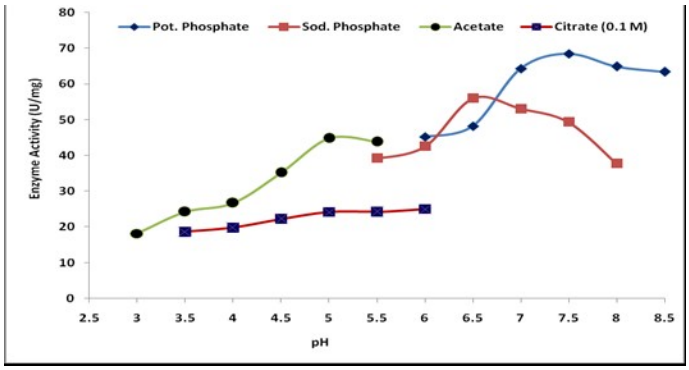

Fig. 6. Optimization of buffer system for purified L-glutaminase of *Pseudomonas* sp. GPB-06.

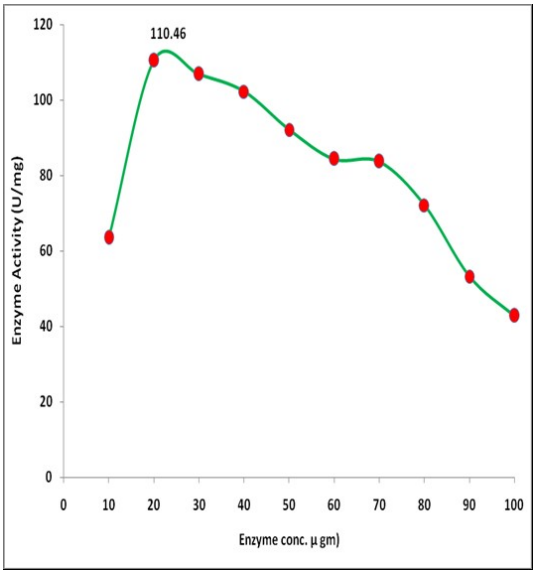

Fig. 7. Optimization of enzyme dose

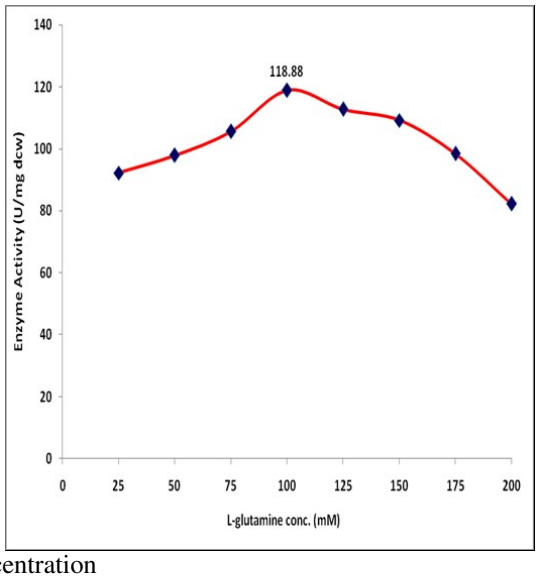

Fig. 8. Optimization of substrates concentration

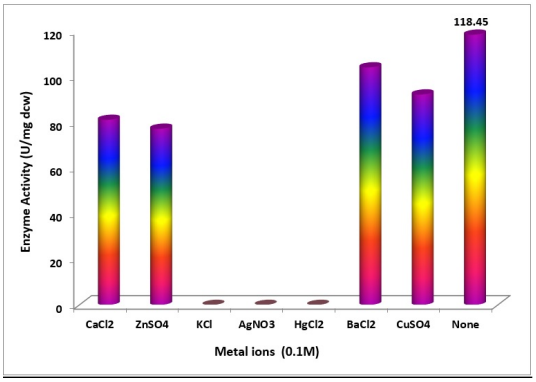

Fig. 9. Effect of different metal ions on L-glutaminase activity

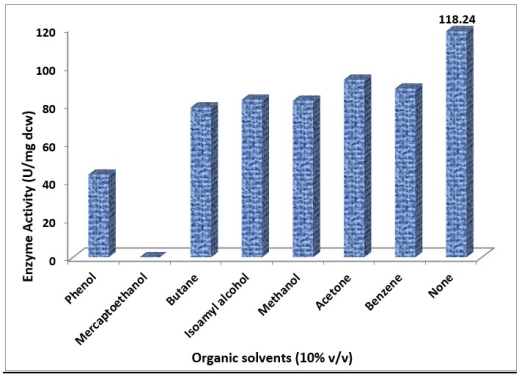

Fig. 10. Effect of different solvents on L-glutaminase activity

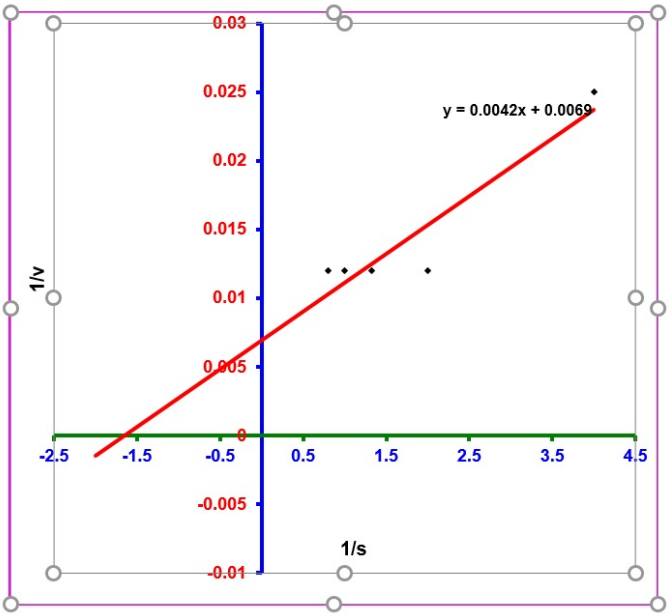

Fig. 11. Determinations of Km and Vmax

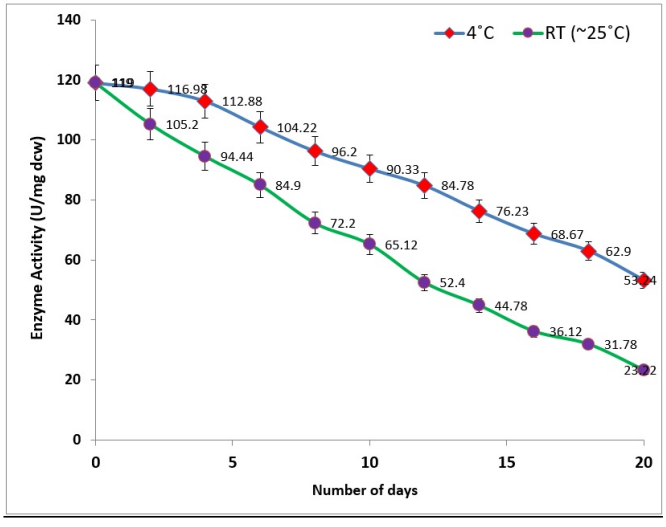

Fig. 12. Storage stability of L-glutaminase from *Pseudomonas* sp. GPB-06.

IV. CONCLUSION

In the current investigation, various microorganisms sourced from soil underwent quantitative screening for L-glutaminase production through submerged fermentation, and the enzyme activity was assessed. A promising L-glutaminase-producing microorganism was chosen and subsequently identified as *Pseudomonas* sp. at the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh (INDIA), using morphological and biochemical characteristics. Subsequent studies examined the impact of different parameters on enzyme production by *Pseudomonas* sp. The L-glutaminase enzyme was then subjected to a series of purification steps, including dialysis, ion exchange chromatography, and SDS-PAGE, followed by characterization of its various properties.

REFERENCES

[1] P. Binod, R. Sindhu, A. Madhavan, A. Abraham, A. K. Mathew, U. S. Beevi, R. K. Sukumaran, S. P. Singh, and A. Pandey, "Recent developments in l-glutaminase production and applications–an overview," *Bioresource technology*, vol. 245, pp. 1766–1774, 2017.

[2] S. E. Tork, M. M. Aly, and O. Elsemin, "A new l-glutaminase from streptomyces pratensis nrc 10: Gene identification, enzyme purification, and characterization," *International journal of biological macromolecules*, vol. 113, pp. 550–557, 2018.

[3] M. Dura, M. Flores, and F. Toldrá, "Purification and characterisation of a glutaminase from debaryomyces spp." *International Journal of Food Microbiology*, vol. 76, no. 1-2, pp. 117–126, 2002.

[4] P. Pavani, Chandrasekhar Rao and N. Kumar, "Effect of soil temperature, moisture and ph on soil l-glutaminase activity," *International Journal of Current Microbiology and Applied Sciences*, vol. 6, no. 8, pp. 3081–3087, 2017.

[5] G. N. Prabhu and M. Chandrasekaran, "Purification and characterization of an anti-cancer enzyme produced by marine vibrio costicola under a novel solid state fermentation process," *Brazilian Archives of Biology and Technology*, vol. 42, pp. 363–368, 1999.

[6] P. V. Iyer and R. S. Singhal, "Screening and selection of marine isolate for l-glutaminase production and media optimization using response surface methodology," *Applied biochemistry and biotechnology*, vol. 159, pp. 233–250, 2009.

[7] J. Bertino, P. O'brien, and J. McCullough, "Inhibition of growth of leukemia cells by enzymic folate depletion," *Science*, vol. 172, no. 3979, pp. 161–162, 1971.

[8] P. Singh and R. Banik, "Biochemical characterization and antitumor study of l-glutaminase from bacillus cereus mtcc 1305," *Applied biochemistry and biotechnology*, vol. 171, pp. 522–531, 2013.

[9] S. A. V. Jesuraj, M. M. R. Sarker, L. C. Ming, S. M. J. Praya, M. Ravikumar, and W. T. Wui, "Enhancement of the production of l-glutaminase, an anticancer enzyme, from aeromonas veronii by adaptive and induced mutation techniques," *PloS one*, vol. 12, no. 8, p. e0181745, 2017.

[10] L. Olalla, J. C. Aledo, G. Bannenberg, and J. Márquez, "The c-terminus of human glutaminase l mediates association with pdz domain-containing proteins," *FEBS letters*, vol. 488, no. 3, pp. 116–122, 2001.

[11] G. F. Karim and K. M. Thalij, "Determination of l-glutaminase activity by some bacterial species," *Int. J. Curr. Microbiol. Appl. Sci*, vol. 5, pp. 218–225, 2016.

[12] S. Saptarshi and S. Lele, "Localization and disruption kinetics of l-asparaginase from e. caratovora cells by high power ultrasound," *Chemical and Biochemical Engineering Quarterly*, vol. 25, no. 1, pp. 65–73, 2011.

[13] J. Roberts, J. S. Holcenberg, and W. C. Dolowy, "Antineoplastic activity of highly purified bacterial glutaminases," *Nature*, vol. 227, no. 5263, pp. 1136–1137, 1970.

[14] R. Nandakumar, M. Wakayama, Y. Nagano, T. Kawamura, K. Sakai, and M. Moriguchi, "Overexpression of salt-tolerant glutaminase frommicrococcus luteusk-3 inescherichia coliand its purification," *Protein expression and purification*, vol. 15, no. 2, pp. 155–161, 1999.

[15] P. Prakash, E. Poorani, P. Anantharaman, and T. Balasubramaniam, "L-glutaminase production and the growth of marine bacteria," *Res J Microbiol*, vol. 4, pp. 168–172, 2009.

[16] H. Jyothi, S. Kumar, and R. Vandana, "Production of l-glutaminase by pseudomonas vj-6," 2011.

[17] R. Nandakumar, K. Yoshimune, M. Wakayama, and M. Moriguchi, "Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry," *Journal of Molecular Catalysis B: Enzymatic*, vol. 23, no. 2-6, pp. 87–100, 2003.

[18] A. Sabu, T. Keerthi, S. R. Kumar, and M. Chandrasekaran, "L-glutaminase production by marine beauveria sp. under solid state fermentation," *Pro-*

cess Biochemistry, vol. 35, no. 7, pp. 705–710, 2000.

[19] R. Athira, T. Elizebeth, T. Narendra, T. A. Sheik, S. K. Gupta, M. Chaudary, K. Siddalingeshwara, and T. Pramod, "Investigation on the production of l-glutaminase from pseudomonas stutzeri strain under solid state fermentation using various agro residues," *Journal of Drug Delivery and Therapeutics*, vol. 4, no. 2, pp. 81–85, 2014.

[20] I. Padma and R. Singhal, "Isolation, screening, and selection of an l-glutaminase producer from soil and media optimization using a statistical approach," *Biotechnology and Bioprocess Engineering*, vol. 15, pp. 975–983, 2010.

[21] J. Fawcett and J. Scott, "A rapid and precise method for the determination of urea," *Journal of clinical pathology*, vol. 13, no. 2, p. 156, 1960.

[22] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[23] P. J. Blackshear, "[12] systems for polyacrylamide gel electrophoresis," in *Methods in enzymology*. Elsevier, 1984, vol. 104, pp. 237–255.

[24] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage t4," *nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[25] B. Daniel, J. Stuart, and S. Edelstein, "Protein concentration determination," *Protein Methods*, pp. 45–55, 1991.

[26] D. Greenberg *et al.*, "Studies on the mechanism of inhibition of tumor growth by the enzyme glutaminase." 1966.

[27] S. S. Desai, S. J. Chopra, and B. S. Hungund, "Production, purification and characterization of l-glutaminase from streptomyces sp. isolated from soil," *Journal of Applied Pharmaceutical Science*, vol. 6, no. 7, pp. 100–105, 2016.

[28] J. Kiruthika and N. Saraswathy, "Production of l-glutaminase and its optimization from a novel marine isolate vibrio azureus jk-79," *African Journal of Biotechnology*, vol. 12, no. 50, p. 6944, 2013.

[29] S. Hamed and R. S. Al-wasify, "Production and optimization of l-glutaminase from a terrestrial fungal fusarium oxysporum," *Int J PharmTech Res*, vol. 9, no. 4, pp. 233–241, 2016.

[30] K. Soda, M. Ohshima, and T. Yamamoto, "Purification and properties of isozymes of glutaminase from pseudomonas aeruginosa," *Biochemical and biophysical research communications*, vol. 46, no. 3, pp. 1278–1284, 1972.

[31] A. K. Kashyap, R. Rajan, and J. C. Stein, "Banks as liquidity providers: An explanation for the coexistence of lending and deposit-taking," *The Journal of finance*, vol. 57, no. 1, pp. 33–73, 2002.

[32] A. Dhillon and S. Khanna, "Production of a thermostable alkali-tolerant xylanase from bacillus circulans ab 16 grown on wheat straw," *World Journal of Microbiology and Biotechnology*, vol. 16, pp. 325–327, 2000.

[33] H. Yin, X. Zhao, and Y. Du, "Oligochitosan: a plant diseases vaccinea review," *Carbohydrate Polymers*, vol. 82, no. 1, pp. 1–8, 2010.

[34] A. Gessesse and B. Gashe, "Production of alkaline xylanase by an alkaliphilic bacillus sp. isolated from an alkalinesoda lake," *Journal of Applied Microbiology*, vol. 83, no. 4, pp. 402–406, 1997.

[35] G. Sanghi, M. R. Dogra, P. Das, A. Vinekar, A. Gupta, and S. Dutta, "Aggressive posterior retinopathy of prematurity in asian indian babies: spectrum of disease and outcome after laser treatment," *Retina*, vol. 29, no. 9, pp. 1335–1339, 2009.

[36] R. Muthezhilan, R. Ashok, and S. Jayalakshmi, "Production and optimization of thermostable alkaline xylanase by penicillium oxalicum in solid state fermentation," *African Journal of Microbiology Research*, vol. 1, no. 2, pp. 020–028, 2007.

[37] K. C. Raj and T. Chandra, "Purification and characterization of xylanase from alkali-tolerant aspergillus fischeri fxn1," *FEMS Microbiology Letters*, vol. 145, no. 3, pp. 457–461, 1996.