**Research Article** **Biotechnology**

**International Journal of Pharma and Bio Sciences** **ISSN 0975-6299**

# CONDITION OPTIMIZATION AND PRODUCTION OF EXTRACELLULAR L-GLUTAMINASE FROM *PSEUDOMONAS FLUORESCENS*

**CHITANAND M.P.*AND SHETE H.G.**

*Post Graduate Department of Microbiology Netaji Subhashchandra Bose College, Nanded, Maharashtra- 431602.*

## ABSTRACT

L-Glutaminase [L-Glutaminase amido-hydrolase, EC-3.5.1.2] is widely distributed in microorganisms including bacteria, yeast, and fungi. The enzyme has received greater attention and significance in pharmaceutical industry since it is recognized as having potent antitumor activity. The enzyme also has applications in food industries. In the present work an extra cellular L-glutaminase was produced from *Pseudomonas fluorescens*. The screening of extra cellular L-Glutaminase was done by dye based procedure on minimal agar medium. The conditions for production of glutaminase were optimized by studying the effect of incubation period and different carbon and nitrogen sources. The effect of temperature, pH and substrate concentration on L-glutaminase activity was studied. Maximum activity of enzyme was observed at 37 $^{0}$C and at pH 8.

**KEY WORDS : L-Glutaminase, L-Glutamine, *Pseudomonas fluorescens***

**CHITANAND M.P**
**Post Graduate Department of Microbiology Netaji Subhashchandra Bose College, Nanded, Maharashtra- 431602**

# INTRODUCTION

The L- glutaminase (L-glutamine amido hydrolase E.C. 3.5.1.2) is the cellular enzyme which catalyses the hydrolysis of L-glutamine to L-Glutamic acid and ammonia. The enzyme acts as proteolytic endopeptidase, which hydrolyses the peptide bonds present in the interior of the protein molecules. Although L-glutaminase can be derived from plant as well as animal sources, microbial enzymes are generally used for industrial purposes.[1,2,3] L- glutaminase has received significant attention recently owing to its potential applications in medicine as an anticancer agent and in food industries.[4,5] Microbial glutaminases have found applications in several fields. It has been tried as therapeutic agent in the treatment of cancer and HIV.[6] Another important application of L- glutaminase is in biosensors for monitoring glutamine levels in mammalian and hybridoma cell cultures without the need of separate measurement of glutamic acid. It is also used as an analytical agent in determination of glutamine and glutamate.[7] However, one of the major uses of microbial glutaminase is in the food industry where it is used as a flavor enhancing agent.[8] L-Glutaminase is generally regarded as a key enzyme that controls the delicious taste of fermented foods such as soy sauces.

A major work of recent research was focused on mammalian glutaminase, its biochemistry, genetic make up and its role in mammalian metabolism. Glutaminases derived from microbial sources have promising applications in the pharmaceutical and food industry however still not much work was done on the L- glutaminase from microbial origin. In the present work, therefore different bacteria were screened for L- glutaminase production and optimized production of L-glutaminase from *Pseudomonas fluorescens* was reported.

# MATERIALS AND METHODS

### *Screening of L-Glutaminase producing bacteria*

The cultures of *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, and *Bacillus subtilis* procured from National chemical laboratory Pune, were subcultured on nutrient agar slants and incubated at $37^0$C for 24 hrs. All four cultures were used for the detection of L-Glutaminase production by dye based procedure.[9] All bacterial cultures were spot inoculated on glutamine containing minimal agar plates incorporated with phenol red pH indicator. The plates were incubated at $37^0$C for 48 hrs. The colour change from yellow to pink around colony indicated positive response. The culture giving strong positive test was used further for L- glutaminase production. The culture was maintained on nutrient agar slant.

### *Inoculum preparation*

A loopful of culture from 24 hr. old nutrient agar slant was inoculated in 10 ml of inoculum medium containing ( g/L of distilled water) : peptone-5, yeast extract -1 and NaCl-2.45 .The inoculated medium was incubated at $37^0$C on rotary shaker at 120 rpm for 48 hrs.

### *Production of L- glutaminase*

10 ml of inoculum was transferred aseptically to 100 ml of production medium. The production medium contained (g/L distilled water): L-glutamine -20, D-glucose -10 supplemented with minerals, and yeast extract. The pH of resulting medium was adjusted to 8. Inoculated production medium was incubated at $37^0$C on rotary shaker at 120 rpm for 144 hrs. After every 24 hours, 10ml of sample was removed aseptically and centrifuged at 2000 rpm for 30 min. The supernatant was used for enzyme estimation.

### *Glutaminase assay*

The glutaminase activity was measured by estimating the amount of ammonia liberated from glutamine. The method of Imada et al[10] (1972) was followed. 0.5 ml of enzyme extract was added to 0.5ml of 0.04M L-glutamine and 0.5ml of distilled water. To this 0.5ml of 0.1M phosphate buffer solution of pH 7 was added. The reaction mixture was incubated at $37^0$C for 30 min. The reaction was stopped by adding 0.5ml of 1.5M tricloroacetic acid (TCA). Blank was

prepared similarly without adding enzyme preparation. About 0.5ml of above mixture was taken and added to 3.7ml of distilled water. About 0.2ml of Nessler's reagent was added to it. The absorbance was recorded at 450 nm on spectrometer. The standard graph was constructed by treating 1ml of various concentrations (0.5mM, 1.0mM, 1.5mM, 2.0mM etc) of ammonium sulphate with phosphate buffer, TCA and Nessler's reagent. One unit of Glutaminase was defined as amount of enzyme that liberates one micromole of ammonia under optimum conditions. The enzyme activity was calculated by the formula

$$\text{Units / ml / min} = \frac{\mu \text{ moles of } NH_3 \text{ liberated} \times 2.50}{0.1 \times 30 \times 0.5}$$

Where, 2.50 = volume of step 1
0.1 = volume of step 1 used in step 2
30 = time of assay in minutes,
0.5 = volume of enzyme used
(1 mole of ammonium sulphate corresponds to 2 moles of ammonia.)

***Optimisation of conditions for glutaminase production***

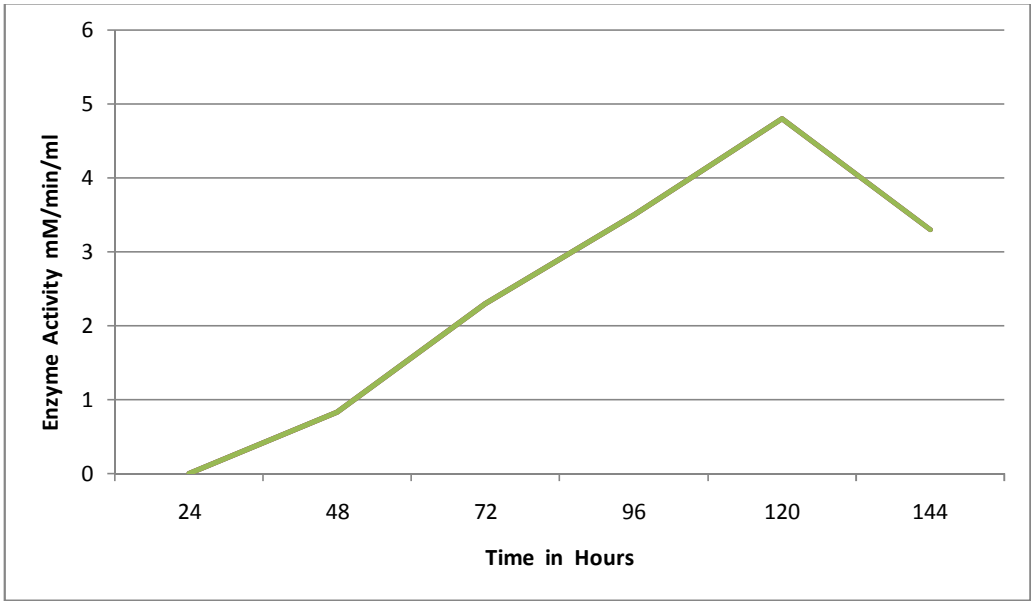
Various parameters that enhance the yield of L- glutaminase were investigated. The effect of incubation period (0-144 hrs.) and the effect of different carbon and nitrogen sources on L-glutaminase production were studied. Different carbon sources used were glucose, sucrose, maltose, lactose and mannitol while amino acids L-tyrosine, L-lysine, L-asparagine and L- glutamic acid were substituted at the place of L- glutamine in the production medium.

***Effect of different parameters on L-glutaminase activity***

The effect of different parameters like, pH of the reaction mixture (pH 7, 7.5, 8, 8.5 and 9), temperature, (20 $^0$ C, 30$^0$ C, 40 $^0$C, 50$^0$ C, and 60$^0$ C) and effect of different concentrations of substrate i.e. L-glutamine (1%, 2%, up to 6%) on L- glutaminase activity were studied.

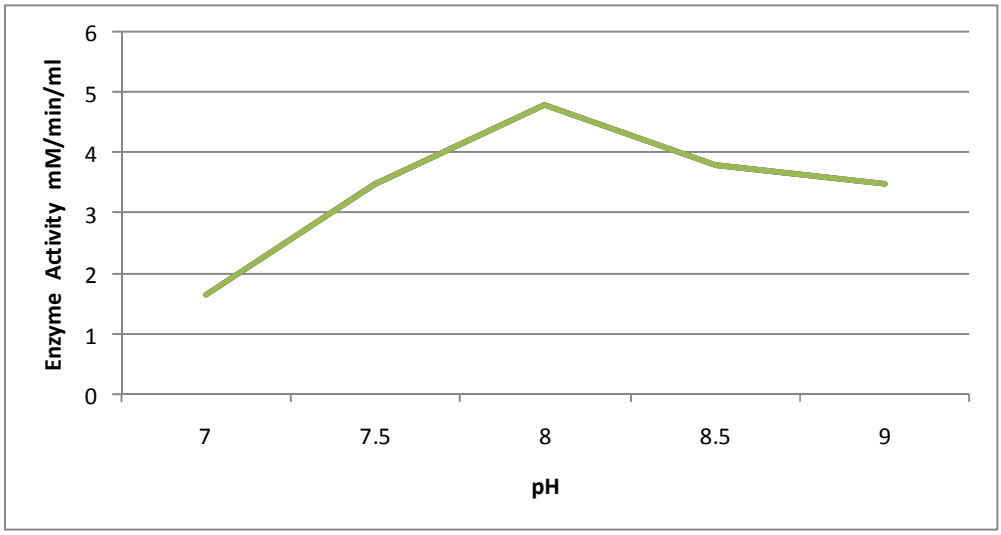
## RESULTS AND DISCUSSION

Out of four cultures, *Pseudomonas fluorescens* was selected for glutaminase activity as the organism showed positive results in the dye based procedure. The organism produced pink coloured zones around the colony on phenol red minimal agar medium. The medium used for screening was selective for L- glutaminase producer as it contained L-glutaminase as the only carbon and nitrogen source which helped in the direct selection of L-glutaminase producer. The organism produced L- glutaminase in the L-glutamine, glucose containing production medium .The enzyme production showed growth relatedness as the incubation period progressed and the maximum enzyme production was observed after 120 hrs (5days) of incubation (Graph 1). After 5 days the enzyme production decreased as the growth of microorganisms might have reached a stage where the organisms could no longer remained in the balanced state of the growth with the available nutrients in the medium or enzymes might be inactivated.[11]



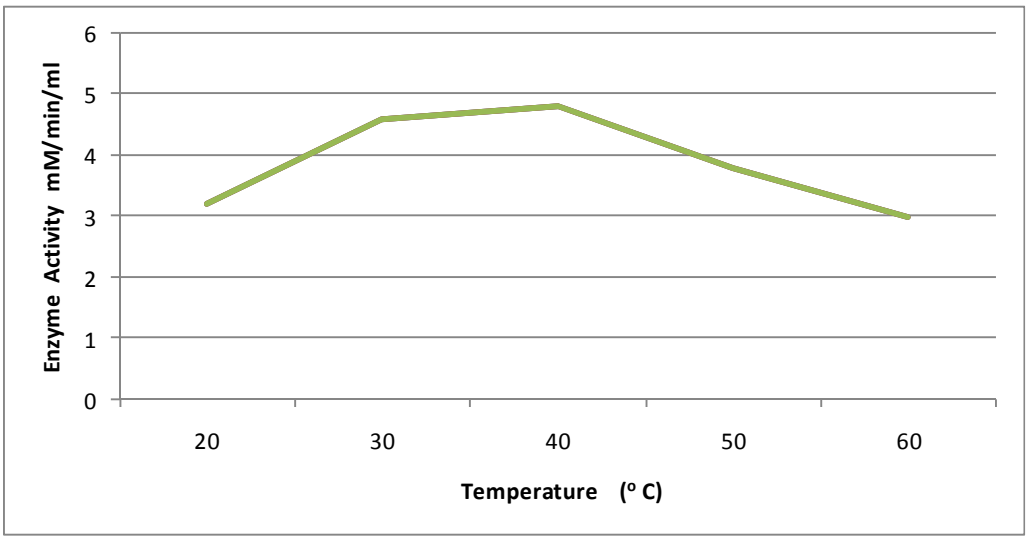

**Graph 1**
***Effect of incubation period on Glutaminase production***

The carbohydrates are essential constituents in the cultivation of media being important for formation of cell constituents. Among the various sugars tested for its effect on the L- glutaminase production (Graph 2), glucose was found to be the best carbon source yielding maximum L-glutaminase production (4800 µmoles /ml/min). The effect of different carbon sources on L- glutaminase production was also studied by Chanakya et al[12] (2010). In their work glucose and maltose were found to be the preferred carbon sources. Amino acids were common growth factor required for the synthesis of protein as major nitrogen source.[13] The yield of L-glutaminase was varied, when the amino acid was changed. Substitution of L-glutamine by other




**Graph 2**
***Effect of pH on Glutaminase production***

Amino acids did not show much induction effect on extracellular L-glutaminase production. Maximum glutaminase production was observed with L- glutamine only, *while* minimum production was observed with L-glutamic acid (Graph 3). Similar results were reported by Prakash et al[14] (2010). In their work, L-*g*lutamine and L-asparagine resulted in high yield of L-Glutaminase but in the present work L-tyrosine and L- lysine had better induction effect on L- glutaminase production than L- asparagine.

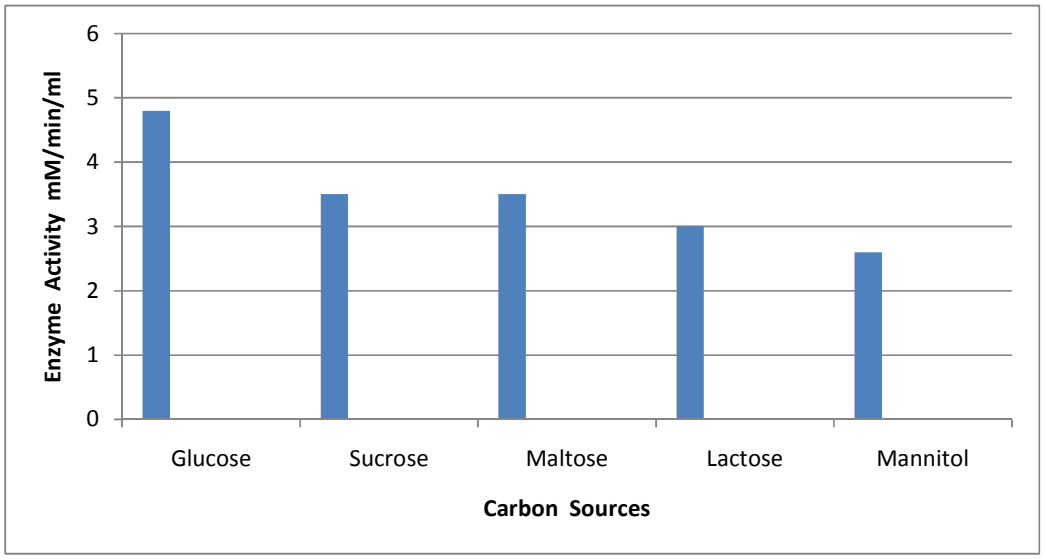



**Graph 3**
***Effect of temperature on Glutaminase production***

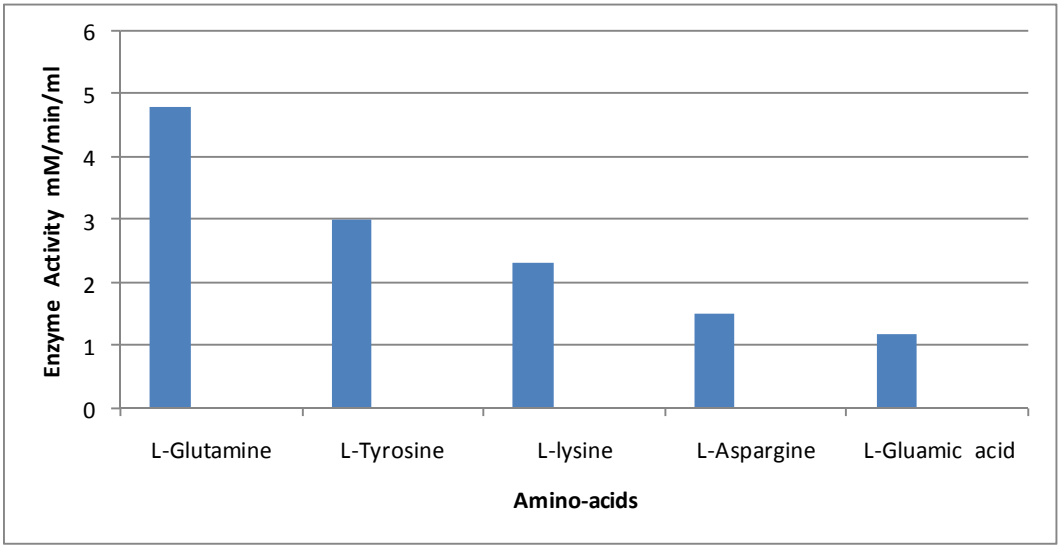
***Effect of different parameters on enzyme activity***

The conditions for enzyme-substrate reaction were optimised by studying parameters like pH of the reaction mixture, temperature and effect of substrate concentration. pH of the medium strongly affects growth and activity of enzymes. The enzyme shows maximum activity at particular pH. When pH of the reaction mixture was adjusted to varying pH, the maximum activity of L-glutaminase was observed in the alkaline range i.e. at pH 8 (Graph 4). Above pH 8, the activity decreased. Incubation temperature influenced the microbial metabolism both with respect to rates at which cellular processes run and the rates at which the enzymatic reactions occur. On incubated in different temperature the reaction mixture showed the increased rate of enzymatic reaction from 20°C to 40°C (Graph 5) and after that the enzyme activity was decreased from 40°C to 60°C.The optimum temperature was $40^0$C. The effect of substrate concentration i.e. L-glutamine concentration is very significant in determining the rate of reaction, the rate of reaction increased with increase in the L-glutamine concentration from 1% to 4%. Further increase in the concentration did not increase the rate of reaction. L glutaminase activity showed the maximum activity with 4% concentrations of L-glutamine (Graph 6). Km and Vmax values were determined on the basis of Michaelis-Menten graph. Km for L- glutaminase obtained was 14.2 mM while Vmax was 23.3 mM/ml/min. Lower Km and higher Vmax indicates that enzyme has higher efficiency.

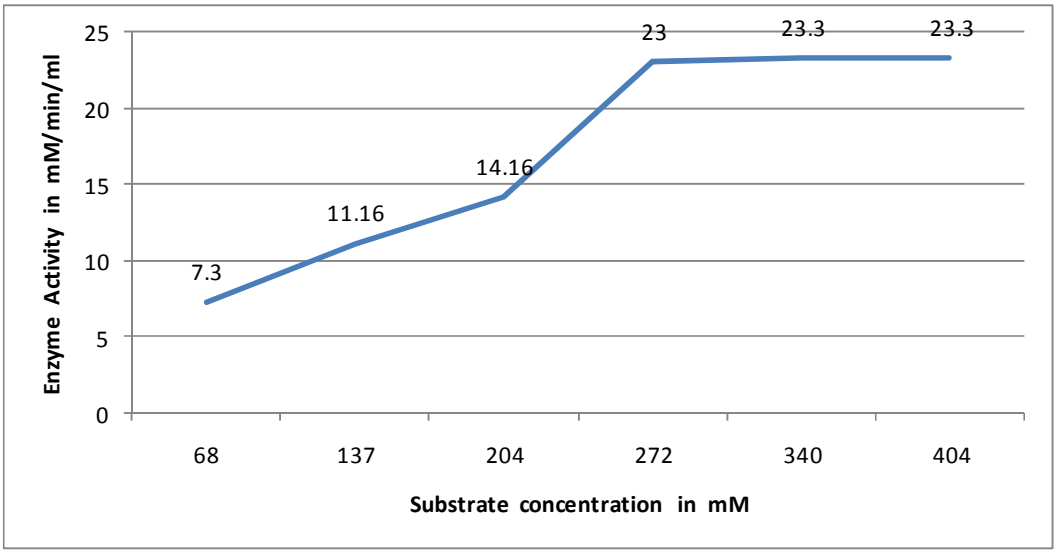
So the present work concludes that *Pseudomonas fluorescens* is a very good source of L-glutaminase enzyme which produces L-glutaminase in glucose containing medium at 37°C and at pH 8.




**Graph 4**
***Effect of different carbon sources on Glutaminase production***




**Graph 5**
***Effect of different amino-acids on Glutaminase production***




**Graph 6**
***Effect of glutamine concentration on Glutaminase production***

## REFERENCES


1. Sabu A, Kumar S. R and Chandrasekran M, Continuous production of extracellular L-glutaminase by Ca-alginate immobilised marine *Beauvina bassiavia* BTMF S-10 in packed bed reactor: Appl. Biochem. Biotechnol.102-103(1-6)71-79, (2002).
2. Pandey A, Soccol C.R, Selvakumar P and Nigam P., Solid-state fermentation for production of industrial enzymes, Current Science: 77.149-162(1999).
3. Prakash, P. J, E. Poorani, P. Anantharaman and T., Balasubramaniam, L-glutaminase production and the growth of marine bacteria. Res. J. Microbiol., 4: 168-172, (2009).
4. Pal S, and Maity P, Antineoplastic activities of purified bacterial glutaminase on transplanted tumour systems, Indian J .Cancer. Chemother. 13, 73-76. (1992).
5. Thadikamala S. and Reddy S. P, Enrichment of Glutaminase production by *Bacillus subtilis* RSP-GLU in submerged cultivation based on neural network- genetic algorithm approach, J.Chem Technol Biotechno 2010; 85:50-58. (2010).
6. Roberts J. and McGregor W.G.; Inhibition of Mouse retroviral disease by bioactive glutaminase-asparaginase, J.Gen.Virol.72 (2)299-305(1991).
7. Lund P, L-Glutamine and L-Glutamate: UV-Method with Glutaminase and Glutamate Dehydrogenase. Methods of Enzymatic Analysis,; Volume 8, H. U. Bergmeyer, (Ed). , pp 357-363. (1986).
8. Loeliger J, Function and importance of glutamate for savoury foods; J., Nutr.130, 915-920 (2000).
9. Gulati R, Saxena R.K.and Gupta R, A rapid plate assay for screening of L-asparaginase producing microorganisms, Lett. Appl. Microbiol.; 24, 23-26(1997).
10. Imada A, Igarasi S, Nakahama K.and Isono M, Asparaginase and glutaminase activities of microrganisms., J Gen Microbiol ; 76:85 -99. (1973).
11. Kashyap P., Sabu A, Pandey A, Szakacs G. and Soccrl C. R, Extracellular L-glutaminase production by *Zygosacharomyces rauxii* under solid

state fermentation; Process Biochem. 38: 307-312,(2002).
12. Chanankya P, Manipati S, and Somlanka S.R, Process optimisation of L-glutaminase production by *Trichoderma koningi* under solid state fermentation, IJABPT 1(3) 1168-1174. (2010).
13. CruzSoto R, Muhammed S.A, Newbold C.J, Steward C.S. and Wallace R.J.; Influence of peptides amino acids and urea on microbial activity in sheep receiving grass hay and on the growth of rumen bacteria in vitro" Animal feed Sci, Tech.49 pp151-161. (1994)
14. Prakash P.J, Poorani E. and Ananthraman P, Effect of media composition on L-glutaminase production from lagoon *Vibrio* Spp.SFL-2; Int, J. Biotech.Biochem 6(5) 769-782. (2010).