Production of L-asparaginase free L-glutaminase from bacterial isolate *Pseudomonas stutzeri* Glu-11

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***ABSTRACT*: L-Glutaminas, belongs to amino hydrolyase family, hydrolyses L-glutamine to glutamic acid and ammonia. This activity of enzyme is very useful in cancer treatment by selective killing of cancer cells by starving. In current work soil sample was collected from different locations of Baijnath, Palampur and Shimla (HP). After enrichment of samples, specific medium was used for isolation. Total 16 isolates were obtained out of which Glu-11 was selected on the basis of highest enzyme activity (5.95 U/ml) after primary and secondary screening. Selected isolate was identified as *Pseudomonas stutzeri* by biochemical characterization. The enzyme production was optimized by screening of different production conditions and highest enzyme activity 25.44 U/mg protein was recorded in medium consist of 0.9% xylose, 0.24% yeast extract, 1% L-glutamine, 1.3% NaCl at 30^{o}C after 24 hours. For characterization of enzyme crude enzyme was used in which it has been found that at reaction temperature enzyme was stable for more than 3 hours. Obtained results from the present work suggest that the obtained isolate can be used for industrial applications.**

***Keywords*: L-glutaminase, *Pseudomonas*, anticancer**

INTRODUCTION

Enzyme plays important role in various industrial processes such as food, pharmaceutical, textile etc (Saptarshi and Lele, 2011). L-glutaminase is enzyme with diverse industrial applications. L-Glutaminase (L glutamine amidohydrolase E.C 3.5.1.2) belongs to hydrolase family that deaminates L-glutamine to glutamic acid and ammonia. L-glutaminase activity is required for normal physiological process as it is important for ammonia synthesis in Kidney (Goldstein and Schooler, 1967). L-glutaminase enzyme gets much attention due to its amino acid depleting nature which helps in cancer and HIV treatment (Roberts *et al*., 1972; Holcenberg, 1982; Binod *et al.,* 2017) and taste development in soy sauce (Kumar *et al*., 2003; Sinha and Nigam, 2016). Other promising application of L-glutaminase is development of biosensors to monitor L-glutamine level in mammalian and hybridoma cell lines (Mulchandani and Bassi, 1996).

The demand of L-glutaminase is continuously rising in various industrial sectors hence it is necessary to produce L-glutaminase to reach its demand in various fields. The enzyme is in world trend is about 500 million US dollar while it is limited to just 20 crore in India. The predicted growth rate of enzyme market is 20-30X annually and the enzymes in food industries alone present about Rs.300 crores. Hence to match the market requirement the commercial production of enzymes using microbes is economically important (Nair, 1995; Bhotmange and Shastri, 1994). Both plants and animals can be used as source for L-glutaminase along with microbes. For large scale production, microbes are preferred due to economic production, constancy, facility of modulation and optimization ways (Al-wasify and Hamed, 2014). L-Glutaminase is produced by most of microorganisms like bacteria, filamentous fungi, yeast mould etc (Kashyap *et al*.; 2002; Weingand *et al*.; 2003; Iyer and Singhal, 2008). *Escherichia coli, Pseudomonas stutzeri, Acinetobacter stutzeri, Bacillus stutzeri, Proteus morganni, Candida and Aspergillus oryzea,* etc are some terrestrial microbes already reported for their Glutaminlytic or glutaminolysis ability (Sabu, 2003) including some marine microbes *i.e. Pseudomonas flurosence, Micrococcus luteus, Beuveria bassiana* (Chandranshekkeran 1997). The optimization of physical and chemical conditions of growth medium is the effective method for better production and improvement for the industrial application, informed by many researches. Optimum production of enzyme resulting due to interaction between all the factors like constituents of growth medium and their concentrations, surroundings growth conditions (Sathish and Prakasham, 2010; Mahalaxmi *et al.;* 2009). For industrial application is in search of new isolate with high production capacity, optimum production conditions are some important factors which govern the production of L-glutaminase enzyme (Mulchandani and Bassi, 1996; Kiruthika and Saraswathy, 2013; Unissa *et al*., 2014).

Materials and Methods

Material: All the chemicals and reagents were of analytical grade, and supplied by Himedia and SRL chemicals.

Sample collection and isolation: Soil samples were collected from different sites *i.e.* Public toilets, slaughter houses of Palampur, Baijnath and Shimla of Himachal Pradesh and processed in Department of Biotechnology HPU Shimla. All samples were enrichment with L-glutamine in saline water and incubated in shaker at 30 ^{o}C (fungi) and 35^{o}C (bacteria) and 160rpm (Sathish and Prakasham, 2010). Isolation was done on selective media by spreading and consecutive streaking on selective medium (0.5% soya peptone, 0.2% yeast extract, 0.1% beef extract, 1.0% L-Glutamine, 0.5% NaCl, 1.5% agar and pH was adjusted to 7.0).

Screening of Isolates: After incubation, colonies of their different shapes, size and color were obtained which were screened by primary and secondary screening.

Primary screening (Phenol red agar plate method): Primary screening of bacterial isolates was done by phenol red agar plate method using Zobell's agar media (ZM). In this method, phenol red was used as pH indicator which turned yellow to

pink due to ammonia production by L glutaminase from L-glutamine as substrate. Isolates were aseptically transferred and incubated at 37ºC for 24 hours (Gulati *et al.*, 1997; Sinha and Nigam, 2016).

Secondary screening: For secondary screening enzyme activity of all the isolates was measured by following Fawcett and Scott method (1960) and enzyme activity was determined according to the formula:

$$\text{Enzyme activity } \left(\frac{U}{ml}\right) = \frac{O.D.TEST}{O.D.STD} \times \frac{Conc.Std.}{Incubation\ time} \times \frac{1}{Volume\ of\ Enzyme(ml)} \times Dilution\ Factor$$

"One unit of L-glutaminase activity was defined as the amount of enzyme that liberated 1µ mole of ammonia per minute under standard assay condition".

Protein estimation: The concentration of protein was estimated by dye binding method (Bradford, 1976). Specific enzyme activity was determined by using formula:

Specific enzyme activity (U/mg protein)=Enzyme activity (U/ml)/Protein content (mg/ml)

Biochemical characterization: On the basis of highest enzyme activity microbial isolate was selected for further work and characterized. Preliminary identification of isolate was done by gram staining followed by biochemical characterization.

Growth profile: For the analyses of bacterial and enzyme production physiology growth profile was prepared. 1ml sample was decanted after every 2 hours under aseptic conditions upto 48 hours. Absorbance (660nm), dry cell weight and enzyme activity was recorded of each sample.

Production of L-glutaminase: For L-glutamine production from selected isolate, complex medium consist of Soya peptone: 0.5%, Yeast extract: 0.2%, beef extract: 0.1%, NaCl: 0.5%, L-glutamine: 1.0% (Padma and Singhal, 2011); was used after screening of 09 different production media. 5% of 24 hrs old inoculum, prepared in nutrient broth was transferred to 100ml production medium and incubated at 30^0C for 24 hours at 160 rpm.

Optimization of process parameters for maximum production of L-glutaminase: The optimization of different process parameters were carried out by OVAT (one variable at a time). For optimization, production conditions such as production medium components, pH, incubation temperature and other production conditions were screened.

Characterization of L-glutaminase from *Pseudomonas stutzeri* Glu-11: As isolate from selected isolate was characterized for different reaction conditions such as presence of inhibitors, metal ions and stability at different temperature.

Results and Discussion

Total 16 bacterial isolates were obtained from soil samples from which Glu-11 was selected on the basis of highest enzyme activity (Table 1).

As BG11 showed the highest enzyme activity hence it was selected for further study. Selected isolate was also characterized microscopically (Figure 1) and biochemically (Table 2).

Growth study of L-glutaminase producing *Pseudomonas stutzeri* Glu-11: Every microorganism has specific phase for metabolite production. To find out the nature of L-glutaminase or in which phase of growth it will be produced, growth curve was plotted and OD (660nm), biomass and enzyme activity was estimated (Figure 2).

From the growth profile of *Pseudomonas stutzeri* Glb-11 for L-glutaminase it was suggested that L-glutaminase was produced in late exponential or early stationary phase.

Effect of different parameters on L-glutaminase production: Effect of production medium components, their concentrations and other physiochemical parameters is determined by the genetic nature of the organisms (Al-Wasify and Hamed, 2016). In current work effect was studied by "one variable at a time" and obtained optimum conditions in each case were used for further tests.

Effect of production medium components of L-glutaminase production: Different concentrations of all the medium components were screened and it has been observed that not only medium components but also their concentration has a significant impact on enzyme production. While optimization varying in concentration of medium components resulted in significant enhance in enzyme activity (Table 3).

Characterization of L-glutaminase enzyme

Specificity substrate: Many reports have been claimed in earlier work that in some cases L-glutaminase enzyme also has L-asparaginase activity. So the find the nature and specificity of enzyme, three different substrates were used in enzyme assay. Results from the experiments suggested that L-glutaminase from *Pseudomonas* Glu-11 was L-asparaginase free.

Optimization of buffer: Enzyme need appropriate buffer system for activity. To analyze the impact of different buffer systems, different buffers such as sodium acetate, sodium citrate, potassium phosphate, Glycin NaOH and Tris-HCl of varying buffer strength 0.025-0.25M was used. Highest enzyme activity was recorded in 0.025 M potassium phosphate buffer with highest enzyme activity of 35.08 U/mg (Figure 4).

Effect of metal ions and solvents: Mostly divalent metal ions inhibited enzyme activity except Mg^{2+}which stimulated enzyme activity while monovalent ions enhance activity. Similar results have been observed in current work while all the solvents were reported to suppress the enzyme activity (Figure 5).

Stability towards pH: pH has direct effect on enzyme activity. To analyze the stability of enzyme at different pH, potassium phosphate buffer of varying pH was used. Results from current work showed highest enzyme activity at pH 8 (Figure 6).

Thermo-stability: Enzyme was stored at different temperature and enzyme activity was recorded upto 2 hours at the interval of 20 minutes. As temperature increased, enzyme activity was continuously dropped. In present work half life of the enzyme was expected to be more than 3hrs at reaction temperature (Figure 7). In earlier work, thermostability of L-glutaminase from *Bacillus* was analyses at 37 ^{o}C and it was found that the half life of enzyme was 3 hrs (Sinha and Nigam, 2016).

Discussion

L-glutaminase is a commercial enzyme has application in food as well as pharmaceutical industries. Literature from previous works reported the production of L-glutaminase from microbes and this exploration is continues till date. Carbon and nitrogen sources are an important factor for microbial growth and enzyme production. In comparison to present work Hamed and Al-wasify (2016) was found maximum activity was recorded in sucrose with enzyme activity 1465.44 U/ml. While in another work lactose was found to be supporting optimum enzyme production from *Bacillus* KK2S4 with enzyme activity of 0.315 U/ml (Makky et al., 2013) while El Sayed found glucose as the best carbon source for L-glutaminase activity that was 35 U/ml (El-Sayed, 2009). In case of nitrogen sources, various nitrogen sources have been reported by researchers with different microbes such as for *Bacillus* KK2S4: sodium nitrate with enzyme activity of 0.430 U/ml (Makky *et al.*, 2013), 2% soyabean meal for Vibrio azureus JK-79 of 2% showing enzyme activity of 300 U/ml (Kiruthika and Saraswathy, 2013). For L-glutaminase production L-glutamine is used as enhancer. In earlier work 1% was used glutamine was used for enzyme production. 0.025% concentration of L-glutaminase was found to be suitable for optimum activity from *Fusarium ozysporum* (Hamed and Al-wasify, 2016) while concentration reached upto 4% in case of *Streptomyces griseus* with enzyme acivity of 45 IU/ml (Kumar *et al.*, 2013). Some literatures have suggested 7 days and 2 days for enzyme production from *Fusarium oxysporum* (49.33 U/ml) and *Aspergillus oryzae* respectively (Prasanna and Raju, 2011, Kashyap *et al.*, 2002).While in present work it was only 24 hours which is quite less.

Appropriate substrate and its concentration are necessary for optimum activity otherwise low and high concentration beyond optimum leads to decrease in enzyme activity due to decrease in binding efficiency of substrate to active site on enzyme. Effect of different concentrations of L-glutaminase and L-asparaginase suggest that it was asparaginase free and optimum activity was recorded with 1.4% L-glutamine while 40mM concentration of L-glutamine was found to be optimum for highest enzyme activity (Singh and Banik, 2014). Like present work, phosphate buffer (pH8) was also reported as best buffer for enzyme activity from *Bacillus* sp, phosphate buffer but with lower pH (Sinha and Nigam, 2016, Bulbul and Karakus, 2013). Even higher reaction temperature 50^{o}C was reported for enzyme reaction (Bulbul and Karakus, 2013).

In case of metal ions and solvents results from present work was little bit similar to earlier reports as Nathiya *et al.*, (2011) and El-Gendy et al (2016) also found the highest activity in presence of Mg^{+2}. L-glutaminase from *Bacillus* was reported to be stable at reaction temperature 37 ^{o}C with half-life of 3 hrs (Sinha and Nigam, 2016) while in present work enzyme was forum to be thermally stable for more than 3 hours at reaction temperature.

Summary

L-glutaminase produced by diverse group of microorganisms including fungi and bacteria. L-glutaminase is presently used in the treatment of leukemia, HIV and also as flavor enhancing agent in food industries. Although L-glutaminase activity was reported in mostly of microorganisms, in our current research is focused on isolation screening and optimization of bacterial isolates for L-glutaminase. For isolates, soil samples were collected from different location of Shimla, Palampur and Baijnath. After enrichment, total 16 isolates were obtained out of which isolate BG-11 was showing highest enzyme activity of 5.95 U/ml which was later identified as *Pseudomonas* sp. by biochemical characterization. For optimum enzyme production M1 medium with the composition (%): Soya peptone: 0.5%, Yeast Extract: 0.2%, Beef extract: 0.1%, NaCl, 0.5%, L-Glutamine: 1.0% was selected on the basis of highest enzyme activity. After optimizing the production medium and other production conditions highest enzyme activity was reported in optimized medium comprise of 0.9% xylose, 0.24% yeast extract, 1% L-glutamine, 1.3% NaCl at 30^{o}C with 8% 24 hours old inoculum. Appropriate substrate concentration is necessary for better activity otherwise low and high concentration beyond optimum leads to decrease in activity due to decrease in binding efficiency. 1.4% of substrate with enzyme concentration volume of 80 µl (21.23 µg) of enzyme gave highest enzyme activity of 41.59 U/mg. While optimizing the buffer system, 0.025M phosphate buffer was found to be best for enzyme activity with highest enzyme activity of 44.77 U/ml. Later effect of metal ions and inhibitors and solvents were studied and it has been found that among metal ions mostly divalent metal ions and solvents inhibit the enzyme activity while monovalent ions and Mg^{+} enhance the enzyme activity upto some extant. While analyzing the thermal stability of L-glutaminase, it has been observed that low temperature is suitable for enzyme storage but still enzyme retain its activity at higher temperature (reaction temperature) upto some extant for more than 3 hours. Further rise in temperature resulted in decrease in enzyme activity.

Figures

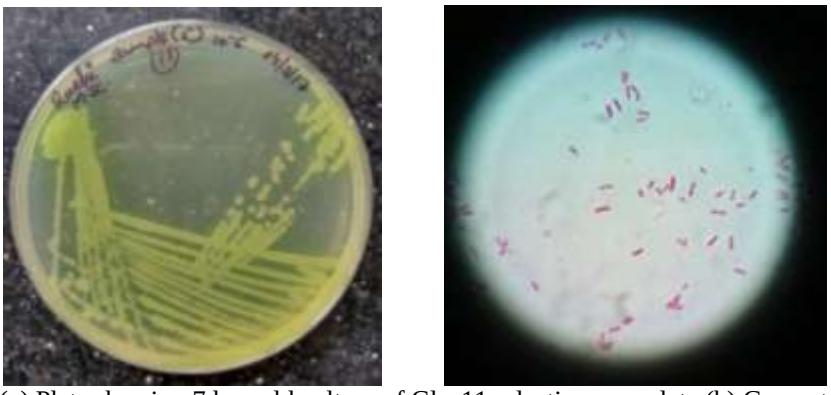

Figure 1 (a) Plate showing 7days old culture of Glu-11 selective agar plate (b) Gram staining of Glu-11

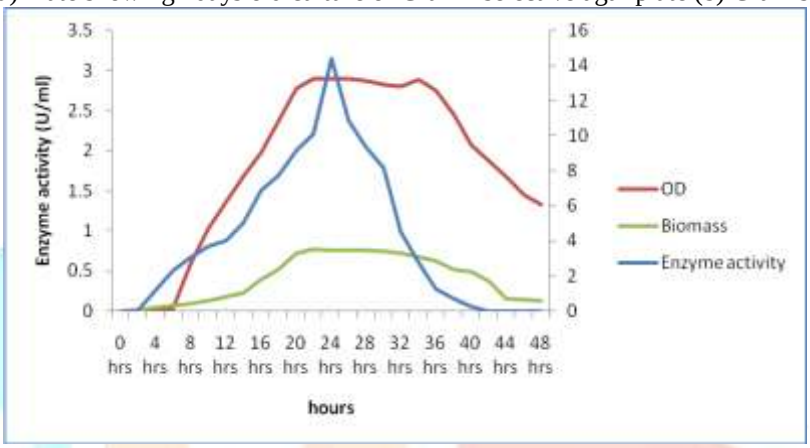

Figure 2: Growth curve between enzyme activity, optical density (OD) and biomass

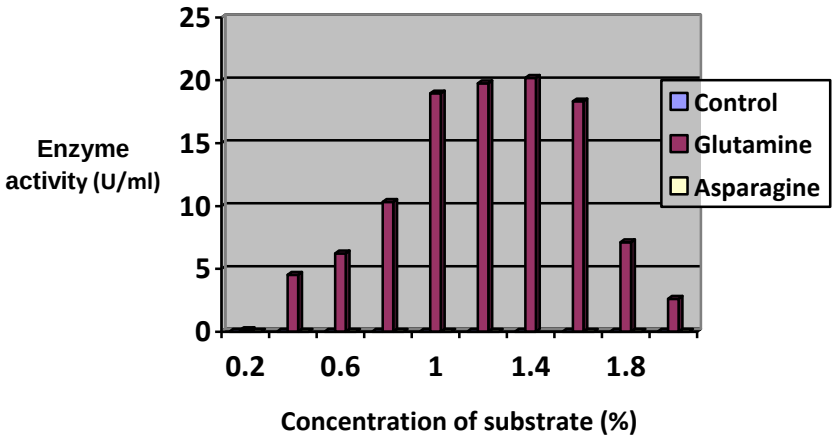

Figure 3: Effect of substrate concentration on enzyme production

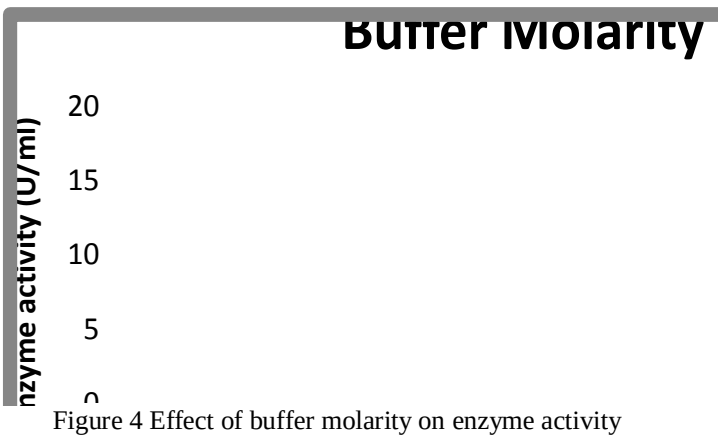

Figure 4 Effect of buffer molarity on enzyme activity

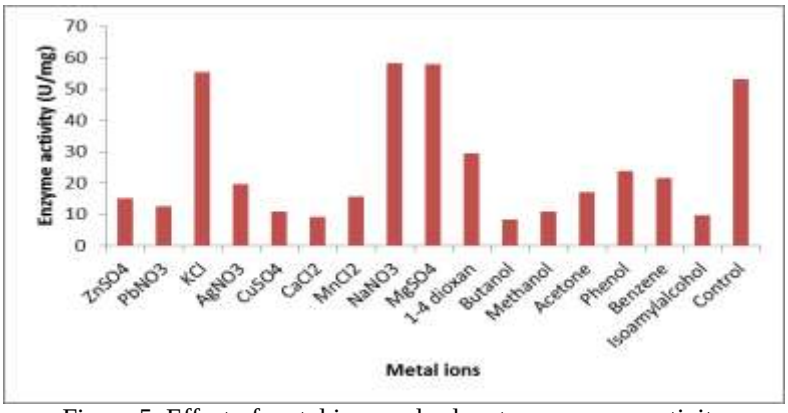

Figure 5: Effect of metal ions and solvents on enzyme activity

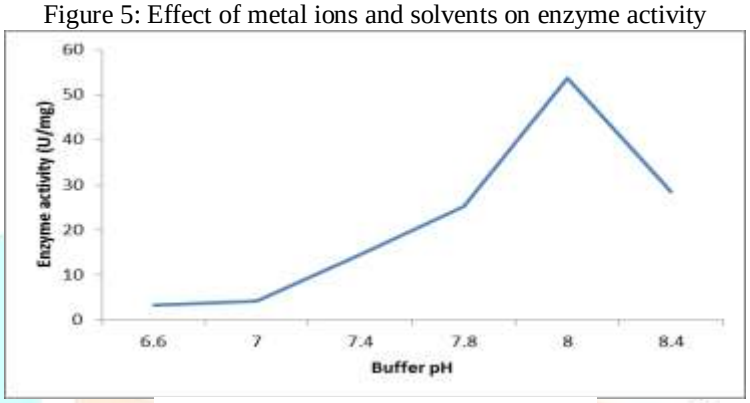

Figure 6: Effect of pH of enzyme activity

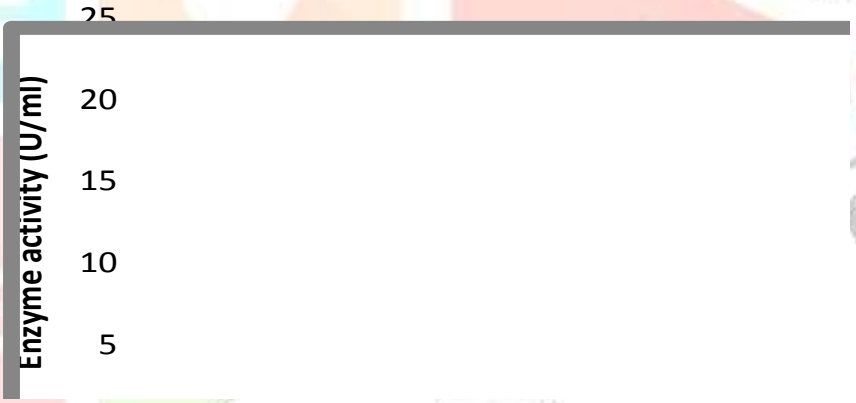

Figure 7: Effect of storage temperature on activity of L-glutaminase

Tables

Table 1: Isolates showing their enzyme activity by three different enzyme assay methods

Sr. No.	Location	Isolate	Enzyme activity (U/ml) Fawcett and Scott 1960
1.	Shimla	Glu-1	0.38
2.		Glu-2	1.12
3.		Glu-3	0.138
4.		Glu-4	2.16
5.		Glu-5	1.60
6.		Glu-6	0.84
7.	Palampur	Glu-7	2.48
8.		Glu-8	0.46
9.		Glu-9	2.56
10.		Glu-10	5.26
11.		**Glu-11**	**5.95**
12.	Baijnath	Glu-12	2.14
13.		Glu-13	0.47
14.		Glu-14	1.18
15.		Glu-15	0.37
16.		Glu-16	0.44

Table 2: Biochemical characterization of bacterial isolate

Characteristics	Glu-11	
	Observation	Interpretation
Pigment	Yellowish, green	Yellowish, green
Gram Staining	Pinkish	Negative
Shape	Rods	Rods
Capsule (Capsulated/Non-Capsulated)	No capsule visualized	Non-Capsulated
Spore (Sporing/Non-Sporing)	No	Non-Sporing
Catalase	Bubbling	Positive (+ve)
Oxidase	Blue pigmentation	Positive (+ve)
MR	Yellow	Negative (-ve)
VP	Yellow	Negative (-ve)
OF (Oxidative/Fermentative)	Y/G*	Oxidative
Indole	No color change	Negative (-ve)
Citrate	Blue	Positive (+ve)
Urease	Yellow	Negative (-ve)
Nitrate Reduction	Red color	Positive (+ve)
H_2S	Yellow	Negative (-ve)
Gas	Crack in slant	Positive (+ve)
Gelatin Hydrolysis	Liquefication	Positive (+ve)
Fermentation of sugars		
Arabinose	Red	Negative (-ve)
Fructose	Red	Negative (-ve)
Glucose	Red	Negative (-ve)
Maltose	Red	Negative (-ve)
Mannitol	Yellow	Positive (+ve)
Sorbitol	Red	Negative (-ve)
Sucrose	Red	Negative (-ve)
Lactose	Red	Negative (-ve)
Interpretation	*Pseudomonas stutzeri*	

Table 3: Effect of medium components on enzyme production

Medium components	Earlier value	Optimized value	Specific enzyme activity (U/mg)
Carbon source	NA	Xylose	11.85
Nitrogen source	yeast extract, beef extract and soya peptone	yeast extract	12.09
C-source concentration	0.5%	0.9%	14.31
N-source concentration	0.2+0.1+0.5	0.24%	15.24
L-glutamine concentration	1%	1%	15.17
NaCl concentration	0.5%	1.3%	17.49
Inoculum age	24	24	17.13
Inoculum size	5%	8%	25.51
Time course of production	24	24	25.44
Incubation time	30°C	30°C	25.27
pH	7	7	25.04

References

Al-Wasify, R.S. Hamed, S.R. 2014. Bacterial biodegradation of crude oil using local isolates. International Journal of Bacteriology, 2014, 8 (doi:10.1155/2014/863272).

Bhotmange, M.G. Shastri, P.N. 1994. SSF in relation to food enzymes, In: Solid State Fermentation, Pandey, A (ed). Wiley Eastern Ltd, New Delhi pp. 105-113.

Binod, P. Sindhu, R. Madhavan, A. Abraham, A. Mathew, A.K. Beevi, U.S. Sukumaran, R.K. Singh, S.P. Pandey, A. 2017. Recent Developments in L-Glutaminase Production and Applications - An Overview. *Bioresource Technology*, 245:1766-1774 DOI: 10.1016/j.biortech.2017.05.059.

Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, 72: 248-254.

Bülbül, D. Karakuş, E. 2013. Production and Optimization of L-glutaminase enzyme from *Hypocrea jecorina* pure culture. Preparative Biochemistry & Biotechnology, 43:385–397.

Chandrasekaran, M. 1997. Industrial enzymes from marine microorganisms- Indian scenario. Journal of Marine Biotechnology, 5:86-89.

El-Gendy, M.M.A.A. Taha, T.M. Abo-Dahab, N.F. Hassan, F.S.M. 2016. Process optimization of L-glutaminase production; a tumour inhibitor from marine endophytic isolate *Aspergillus stutzeri* ALAA-2000. International Journal of PharmTech Research, 9: 256-267.

El-Sayed, A.S.A. 2009. L-glutaminase production by *Trichoderma koningii* under solid state fermentation. *Indian Journal of* Microbiology, 49:243–250.

Fawcett, J.K. Scott, J.E. 1960. A rapid and precise method for the determination of Urea. *Journal of clinical pathology*, 13: 156.

Gluati, R. Saxena, R.K. Gupta, R. 1997. A rapid plate assay for screening L-asparaginase producing micro-organisms. Letters in Applied Microbiology, 24: 23-26.

Goldstein, L. Schooler, J.M. 1967. Regulation of ammonia production in the rat kidney. Advances in Enzyme Regulation, 5: 71-86.

Hamed, S.R. Al-wasify, R.S. 2016. Production and optimization of L-glutaminase from a terrestrial fungal *Fusarium oxysporum. International Journal of PharmTech Research*, 9(4): 233-241.

Holcenberg, J.S. 1982. Enzyme Therapy: Problems and Solutions. *Annual Review of Biochemistry*, 51: 795–812.

Iyer, P. Singhal, R.S. 2008. Production of glutaminase (E.C.3.2.1.5) from *Zygosaccharo mycesrouxii*: Statistical optimization using response surface methodology. Bioresource Technolology, 99: 4300–4307.

Kashyap, P. Sabu, A. Pandey, A. Szakacs, G. Soccr, C.R. 2002. Extracellular L-glutaminase production by *Zygosaccharomyces raxuii* under solid state fermentation. *Process Biochemistry*, 38: 307-312.

Kiruthika, J. Saraswathy, N. 2013. Production of L-glutaminase and its optimization from a novel marine isolate *Vibrio azureus* JK-79. African Journal of Biotechnology, 12: pp. 6944-6953.

Kumar, R.S. Chandransekaran, M. 2003. Continuous production of L-glutaminase by an immobilized marine *Pseudomonas* spp. BTMS-51 in a packed bed reactor. *Process Biochemistry*, 38: 1431-1436.

Kumar, S.S. Muthuvelayudham, R. Viruthagiri, T. 2013. Medium optimization for production of L-Glutaminase (EC 3.5.1.2) by *Streptomyces griseus* under submerged fermentation. *International Journal of Science and Engineering Applications*, 2(1): 2319-7560.

Mahalaxmi, Y. Sathish, T. Prakasham, R.S. 2009. Development of balanced medium composition for improved rifamycin B production by isolated *Amycolatopsis stutzeri* RSP-3. *Letters in Applied Microbiology*, 49: 533-538.

Makky, E.A. Ong, J.J. Karim, M.R. Lee, C.M. 2013. Production and optimization of L-asparaginase by *Bacillus stutzeri* KK2S4 from corn cob. African Journal of Biotechnology, 12: 2654-2658.

Mulchandani, A. Bassi, A.S. 1996. Determination of glutamine and glutamic acid in mammalian cell cultures using tetrathiafulvalene modified enzyme electrodes. *Biosensors and Bioelectronics*, 11:271-80.

Nair, S.R. Rakshit, S.K. Panda, T. 1995. Effect of carbon sources on the synthesis of pectinase by *Aspergilli.* Bioprocess and Biosystems Engineering. 13: 37-40.

Nathiya, K. Nath, S.S. Angayarkanni, J. Palaniswamy, M. 2011. Optimised production of L-glutaminase: A tumour inhibitor from *Aspergillus flavus* cultured on agroindustrial residues. *African Journal of Biotechnology*, 10: 13887-13894.

Padma, I. Singhal, R. 2010. Isolation, screening, and selection of an L-glutaminase producer from soil and media optimization using a statistical approach. Biotechnology and Bioprocess Engineering, 15: 975. doi:10.1007/s12257-009-0187-8.

Prasanna, K.L. Raju, K.J. 2011. Production of l-glutaminase by *Aspergillus oryzae* NCIM 1212 under solid state fermentation using agro–residues. Journal of Chemical, Biological and Physical Sciences, 2: 261-269.

Roberts, J. John, S. William, D.C. 1972. Isolation, Crystallization and properties of *Achromobacter* Glutaminase-asparaginase with antitumour activity. The Journal of Biological Chemistry, 247: 84-90.

Sabu, A. 2003. Sources, properties and applications of microbial therapeutic enzymes. Indian Journal of Biotechnology, 2:334-341.

Saptarshi, S.D. Lele, S.S. 2011. Localization and disruption kinetics of L-asparginase from *Erwinia carotovora* cells by high power ultrasound. Chemical and Biochemical Engineering Quarterly Journal, 25: 65–73.

Sathish, T. Prakasham, R.S. 2010. Enrichment of glutaminase production by *Bacillus subtilis* RSP-GLU in submerged cultivation based on neural network - genetic algorithm approach. Journal of Chemical Technology and Biotechnology, 85:50-58.

Hamed, R.S. Al-wasify, H.R.S. 2016. Production and optimization of L-glutaminase from a terrestrial fungal *Fusarium oxysporum.* International Journal of PharmTech Research, 9: 233-241.

Sinha, S. Nigam, V.K. 2016. Production and Characterization of L-Glutaminase by Bacillus *stutzeri*. International Journal of Pharmaceutical Sciences and Research, 7: 1620-1626.

Unissa, R. Sudhakar, M. Reddy, A.S.K. Sravanthi, K.N. 2014. A review on biochemical and therapeutic aspects of glutaminase. International Journal of pharmaceutical sciences and research, 5: 4617-4634.

Weingand-Ziade, A. Gerber-Decombaz, C. Affolter, M. 2003. Functional characterization of a salt and thermo tolerant glutaminase from *Lactobacillus rhamnosus. Enzyme and Microbial Technology*, 3: 862-867.