https://doi.org/ 10.33472/AFJBS.6.9.2024.4329-4342

L - glutaminase production and optimization from saline soil isolate *Bacillus halotolerans* PHN 1.

S. H. Nadaf[1], N. H. Nadaf[2], P. B. Dandge[1]*

[1] Department of Biochemistry, Shivaji University, Vidyanagar, Kolhapur (MS) India.

[2] Department of Microbiology, Shivaji University, Vidyanagar, Kolhapur (MS) India.

*Corresponding author:

Dr. (Mrs.) Padma B. Dandge

Associate Professor Department of Biochemistry, Shivaji University, Vidyanagar, Kolhapur (MS) 416004.

India. E mail: pbd_biochem@unishivaji.ac.in

Article History: Volume 6,Issue 9, 2024

Received: 27 Apr 2024

Accepted : 14 May 2024

doi: 10.33472/AFJBS.6.9.2024.4329-4342

Abstract:

In this study, screening and isolation of potent L- glutaminase producing halotolerant bacteria was done. This strain was identified as *Bacillus halotolerans* PHN1. It was observed that organisms have the ability to synthesize and produce L-glutaminase enzyme at 3% NaCl concentration and can withstand with 7% of NaCl concentration. Maximum enzyme production was at 96 h of its growth. Partial purification of this enzyme was carried out at 60 % ammonium sulphate precipitation followed by dialysis and then Ion exchange chromatography. The overall yield obtained after purification is 4.68% with 1.56 U/mg of specific activity. The molecular weight of the enzyme was shown 60 kDa confirmed by SDS-PAGE electrophoresis. The enzyme has optimum activity at pH 7.0 with 30ºC. It is strongly inhibited by various metal ions included Hg^{+2}; Ag^{+2}, Zn^{+2}, (percentage inhibition obtained was 60-70% with a concentration of metal ion taken was 25µg, 50µg and 75µg/ ml respectively) Mg^{2+} and Mn^{2+} concentration dependent manner. Km and V_{max} for enzyme are 0.014 mM and 0.07U/ml respectively.

Keywords: Halophiles, L-glutaminase, Anticancer, *Bacillus*,

1. Introduction:

There is a great importance of microbial products and enzymes in industrial, agriculture, and ecological sectors which have economic applications. Out of such enzymes, one is (EC 3.5.1.2) L-glutaminase which catalyses amido-hydrolase reaction and converts L - glutamine to L-glutamate and ammonia. L-glutaminase is one of the crucial enzymes for nitrogen metabolism and nearly found among all types of leaving cells and animals [1]. Recently, because of its explored anticancer properties this enzyme got major attention by medical as well as pharmaceutical fields [2]. It can also be utilized as a catalyst in the production of the flavour enhancers and helps to improvise tastes of different food like soy sauce and other fermented foods [3]. It can also serve as an analytical tool in the form of biosensors to detect residual concentration of glutamine and synthesized theanine [4]. Due to its measure applications, this enzyme can be treated as one of the important economic enzymes in terms of industrial microbiology. Therefore, it became essential to find out efficient, sustainable, eco-friendly cost-effective source for L-glutaminase production [5,6].

As L-glutaminase is a common enzyme which is ubicates in nature so present in all living beings including plants, animals and different type of microorganism like fungi, yeast, bacteria, actinomycetes [7, 8]. Among all these sources more development for the enzyme production was made in terms of microorganism because, they are simple sources for manufacturing of any product, microorganisms having ability to produce valuable by products as well, they are well known biological sources for designing of affordable fermentation processes like down streaming, medium optimization for enhanced recovery [9, 10].

Reports suggested that bacterial species like *Pseudomonas, Bacillus, E. coli* and *Actinobacterium* are known as L-glutaminase producers and synthesized enzyme extracellularly and intracellularly [5] whereas in moulds *Aspergillus sp*. and *Trichoderma sp*. were commonly recognised for L-glutaminase production [11]. Major attempt was taken to isolate these microorganisms from marine ecosystem and few from soil [12]. Since, there are limitations for L-glutaminase which are produced by normal terrestrial microorganisms like incapability with human physiology basically with blood, induction of various side effects in humans and they are unable to withstand with specified conditions [13]. Halophiles can be recognised as a good source of extremophilic enzyme also recognised as a halozyme. These enzymes can tolerate at broad ranges of salts, pH and temperatures etc. Moreover, there is a need of increased research for obtaining a high quality of such extremozymes (Halozyme) like L-glutaminase and their exploitation for various applications [14, 15].

By considering all these features of L-glutaminase and halophilic microorganism, present work is aimed for the evaluation of halophilic microorganism isolated from saline soil habitat and improvisation of L-glutaminase enzyme productivity to used it in therapeutic and other economic purpose

2. Materials and methods:

2.1 Sampling:

For screening of halophilic bacterial strains, sample was collected from various locations of agriculture saline soil from the Sangli district of Maharashtra state, India, because of higher use of chemical fertilizers soil became saline and unfertile. However, by assuming the presence of high salt tolerating microorganism these saline agricultures were selected for sampling.

2.2 Enrichment of halophilic L- glutaminase producers:

About 1.0 gm of soil samples were kept for the enrichment in 100 ml medium containing 3.0 g $NaNO_3$, 0.5 g KCl; 0.5 g $MgSO_4$; 1.0 g KH_2PO_4; 0.1 g $FeSO_4$; 30 g NaCl; 10 g glutamine at g/L concentration. Flask was kept on shaker for incubation up to 24h at 30ºC temperature. After incubation dilutions of enriched broth was prepared by taking 1ml broth and diluted serially up to 10^{-10} dilutions out of these 10^{-4} was used of each enriched soil sample for isolation of microorganism.

2.3 Isolation of halophilic L - glutaminase producers:

0.1 ml of 10^{-4} dilution was spreads on plates containing solid medium having same composition as used for the enrichment of soil sample. Phenol red is added in this medium as pH indicator at 0.002 g/l concentration. Incubation of plates was carried out for the 48h at 37ºC. After incubation grown plates were checked for the development of visible growth of colony with pink coloured zone. The pink coloured is an indication of L-glutaminase activity which is detected by pH indicator. L-glutaminase acted on glutamine and liberate ammonia this ammonium ion accumulation at the time of utilization of glutamine changes the pH and hence detected. A colony showing larger pink coloured zones was purified and preserved by agar slope method carried for the further screening studies.

2.4 Fermentative production of L - glutaminase:

From previous enrichment and screening 4 isolated colonies were monitored to check higher enzyme production ability to screen a potent L - glutaminase producer. For this each isolates suspension was prepared then Inoculation was done in 2 different of 1L capacity Erlenmeyer flasks containing 500 ml production media having same composition like enrichment medium. These flasks were kept incubated in shaking incubator for 120 hours at 30ºC. Shaking revolution was adjusted at 120 rpm. At the time of incubation at regular interval of 24h, 10 ml sample was collected aseptically were checked for the growth (bacterial cell density checked by optical density) and then centrifuged at 6000 rpm15 min at 4ºC and checked for crude enzyme activities. Out of these 4 isolated one strain is continued for further studies. Isolate is regularly sub-cultured at an interval of every 2 weeks.

2.5 Identification of bacterial stain:

DNA isolation from bacteria was carried out using phenol chloroform method and its overall quality was determined by Agarose gel (1%) electrophoresis. After electrophoresis a single high molecular weight band was observed which confirms its purity. Gene sequencing of sample was done at Eurofins Genomics India Pvt Ltd., Maharashtra India. Amplification of separated 16S rDNA was done using 27F and 1492R primers. Obtained separate singular PCR amplicon band of 1500 bp with the help of DNA electrophoresis. Removal of other contaminating fragments were being purified in this process to obtained this amplicon. Furthermore, this amplicon was monitored for DNA sequencing using forward and reverse primers with the help of BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. This amplified 16S rDNA gene's sequencing was carried out by using BLAST algorithm with other sequences of the NCBI gene bank database. Depending on higher identity score amongst screened sequences, first ten sequences were screened and monitored further for multiple sequence alignment using Clustal WB and based on that using MEGA 7

phylogenetic tree constructed. High L- glutaminase producing microorganism was isolated for further genomic identification using 16S rRNA sequencing [16].

2.6 Enzyme extraction, purification:

L - glutaminase was purified by using 1L cell free broth obtained by cold centrifugation (at 10000 RPM and 4ºC) after fermentation of 96h as described in previous section. For extraction of L- glutaminase firstly cell free broth saturated with 60 % ammonium sulphate and kept for residual protein precipitation in refrigerator for overnight at 4ºC. The obtained precipitate from previous step was then dissolved by using 25 mL of 50 mM sodium phosphate buffer pH 7 and recognized as crude enzyme solution. This crude enzyme solution was subjected to dialyze against same buffer for overnight at 4ºC. After dialysis the enzyme solution was passed through Ion exchangers for further purification. For Ion exchange chromatography approximately 20 mL of crude enzyme solution (obtained after dialysis) was pooled in pre-activated and equilibrated column of DEAE-Cellulose resin. Total 30 fractions (each of 5ml) were collected. 0.5 ml min^{-1} flow rate was maintained for the fractions collection. For elution was carried out by increasing concentration of NaCl gradient (0.025 M, 0.05 M, and 0.2 M NaCl) prepared in same buffer. After completion of elution, protein contents of each eluted sample (fractions) were recorded by taking absorbance at 280 nm and enzyme activities of each fraction was also determined. Fractions showing L - glutaminase activities were then selected and collected together. Mixed fractions obtained from ion exchange were then concentrated up to 1mL by using sucrose and used for SDS-PAGE analysis for molecular weight determination.

2.7 Enzyme activity:

The activity of L - glutaminase was calculated using Nesslerization principle put forth by Jesuraj et al., (2013) [17] with some modification. The enzyme activity was calculated calorimetrically by detecting relative liberated ammonium ions by adding Nessler's reagent in reaction mixture. The reaction cocktail contains, 0.5mL enzyme, 0.5mL of 0.2M glutamine. The enzyme and substrate were prepared in phosphate buffer 50mM and 7 pH. The enzyme reaction is carried out for the ½ h after then activity stopped by adding (10% TCA) 100 µl. After stopping the reaction, the activity was measured by preparing a mixture of 0.1 ml of reaction cocktail, 3.7ml distilled water and 0.2 ml of Nessler's reagent. The activity was calculated by recording absorbance at 450 nm.

2.8 pH and Temperature effect on enzyme activity:

Effective pH and temperature of L-glutaminase was checked by using a protocol reported by [18] with some modification. The pH range was selected from 4.0 to 10.0, by using buffers with different pH like citrate-buffer for pH 3-5; Sodium- phosphate buffer for pH 6-8; for pH 9-10 glycine-NaOH buffer is used. 10ºCto 90ºC temperature range was selected to determine the temperature optima of L-glutaminase. To check impact of selected pH and Temperature, extracted enzyme was pre incubation for 30 mins in each pH containing buffers and at different temperatures. Enzyme activities were also recorded with same pH and temperature.

2.9 Effect of metal ions:

Analysis of metal ions effect on L-glutaminase activities was checked by carrying prior incubation of enzyme in presence of metal ion salts like Hg^{2+}, Ag^{3+}, Zn^{2+}, Mg^{2+}, Mn^{2+} at their 1 mM and 10 mM concentration prepared in phosphate buffer with 7 pH and subsequently the activity of L - glutaminase was carried out as stated earlier.

2.10 Enzyme kinetics:

By Lineweaver-Burk plot method using reciprocal reaction velocities against reciprocal substrate concentrations was used for determination of K_m and V_{max} of the L - glutaminase. An enzyme reaction accomplished by using increasing substrate concentration of glutamine ranging from 0.1 to 1 ml and other protocol remains same [18].

2.11 Protein estimation:

For determination of specific activity of enzyme protein concentration was estimated by using Lowry method [19].

2.12 Statistical analysis:

Graph Pad software (Graph Pad Instat version 3.00, Graph Pad software, San Diego, CA, USA) was used to obtained results where, obtained result for each experiment is the mean of three or more determinants. ANOVA was done for all data at $P< 0.05$ using

3. Result and discussion:

3.1 Enrichment and screening of L - glutaminase isolates:

Halophiles were known for their countless uses in different industrial sectors like detergent, baking, leather and dairy as they possess capacity of versatile enzymes production with higher stability [20, 21, 22, 23]. Among all reported application of these enzymes some are concern with therapeutic application. L-glutaminase is one of such enzymes which has great application in food, therapeutics as well as other industries too.

The primary screening of L-glutaminase producers was done by using a specialized mineral base media containing glutamine as a carbon and nitrogen source and phenol red as a pH indicator. De-amination reaction was detected by change in colour of the medium which is resulted due to increased pH because amines accumulation. Near about 4 bacterial colonies were shown the pink colour zone. Out of this a single colony which showing prominent zone in short time was selected for further studies. Different kind of strategies has been adopted by the various scientists to screen, isolate and produce this enzyme from microbial resources. Most applied methods used are submerged and solid-state. These processes can be improved for high efficiency as well as for controlled efficiency. However, for commercial production of L - glutaminase mostly submerged fermentation is used [24].

3.2 Identification of bacterial stain:

16S rRNA sequence of the bacteria was identified out of its entire isolated genome. This gene was then amplified up to 1500bp (Figure 1.A).

The 16S rDNA sequence was determined and then BLAST with other sequences of NCBI gene-bank databases. Using multiple sequence alignment based on maximum similarity. After sequence alignment with NCBI database, isolated bacterial strain showed maximum percent similarities with *Bacillus sp.* especially in close relation with its halotolerance. So isolated strain sequence was identified and sequence deposited in database (Accession no.: SUB11221004 _ ON059646) as *Bacillus halotolerance* PHN 1. The phylogeny-based tree constructed using distance matrix with help of MEGA 7 software (Figure 1.B). It was observed that the maximum L-glutaminase producing strain were belongs to halotrophic group of microorganisms. These organisms were normally isolated from marine environment.

In this study, isolated strain *Bacillus halotolerans* PHN1 shows good tolerance against salt concentration and has L - glutaminase activity. Bacterial L-glutaminase are popular amongst all other salt tolerant L - glutaminase producing organisms. *M. luteus* K-3 isolated

from the sea water, exclusively seen to synthesize L-glutaminase I and II, in presence of 3% NaCl and retained these activities optimally at 8–16% [25]. Other bacterium like *Bacillus sp.* LKG-01 (MTCC 10401) shown high concentration of active L-glutaminase even at 20% NaCl concentration. Higher L - glutaminase activity at 20% NaCl concentration was reported in *P. Brevicompactum* NRC 829 but this activity was seen to reduce up 80% at 25% NaCl concentration [26]. Whereas L-glutaminase produced by *B. amyloliquefaciens* retained 68% of its total activity at similar 80% concentration of NaCl [27].

3.3 Fermentative production of L - glutaminase:

It was observed that the *Bacillus halotolerance* PHN 1 grown in a media containing glutamine as a C and N_2 source. Maximum growth with enzyme activity was seen at 96 h. where culture was supposed to be in mid logarithmic phase. Residual enzyme activity was seen at 96 h measured up to 0.956 U/ml, activity remains nearly constant after this point. Therefore, this point of fermentation was taken as a point of extraction.

3.4 Enzyme extraction and purification:

The overall 19.25mg of protein was obtained after ammonium sulfate precipitation of 500 ml cell free medium having 16.65 U/mL enzyme activity. Ion exchange chromatography shows peaks having protein content as well as enzyme activities were confirmed the presence of enzyme in these fractions (Figure 2 A). Fractions showing enzyme activity were mixed together for calculation of enzyme activity. Activity of purified fractions was observed near about 1.56 U/mg of protein. This purified enzyme was stored and further used for enzymes biochemical characteristics. The steps involved in purification of L - glutaminase as purification profile were shown in Table 1. Amongst all studied bacterial sp. production yield obtained of *Bacillus halotolerance* PHN1 shown to have slightly less than another reported *bacillus sp.* like *Bacillus cereus; Bacillus amyloliquefaciens; B. licheniformis* have 19.64 U/gds; 69.1 U/ml; 69.6 U/ml individually [27, 28, 29]. SDS-PAGE investigation revealed that enzyme has a 60 kDa molecular weight (Figure 2 B). Klein et al., (2002) [30] reported 55 kDa L - glutaminase from *B. pasteurii* which is nearly similar with *Bacillus halotolerance* PHN 1. Whereas a higher molecular weight L - glutaminase (175kDa) enzyme was reported from *Bacillus cereus* MTCC 1305 [11].

3.5 Temperature and pH effect:

A temperature and pH optima of L - glutaminase enzyme was 30^{o}C and 7 pH (Figure 3 A, B) Utmost L - glutaminase isolated from various bacteria showed mesophilic range (20–45^{o}C) of temperature for their optimal production [31, 29] some L - glutaminase also shows high optimum temperature. L - glutaminase of *Bacillus sp. LKG-01*(MTCC 10401) shows activity at 70^{o}C [29]. Wakayama et al., (2005) [32] reported 60^{o}C optimum temperature of L - glutaminase in case of *Stenotrophomonas maltophilia*.

3.6 Heavy metals effect:

Effect of heavy metal on L - glutaminase enzyme was assessed and results were recorded as shown in Figure 4. It was observed that Mg^{2+} andMn^{2+} showed minimum inhibition of enzyme at their increasing concentration in comparison of Hg^{+2}; $Ag^{+2}$and Zn^{+2} salts.

It was observed that the Hg^{+2}; Ag^{+2} as well as Zn^{+2} has nearby similar inhibitory impact of inhibition on L - glutaminase but Ag^{+2}showed higher enzyme inhibitory activity at higher concentration (75µg/ml); whereas at its lower concentration Hg^{+2} (25µg/ml) is seen to be more effective act on enzymes inhibition than Ag^{+2}. Though, *Bacillus amyloliquefaciens and Bacillus*

sp. LKG-01(MTCC 10401) in presence of Mg^{2+} and Mn^{2+} activated production of L - glutaminase [27, 29]. Subsequently L - glutaminase production of *Bacillus halotolerance* PHN1 with Mg^{2+} and Mn^{2+} was act as an activator at its lower concentration but at its increased concentration, it was observed comparatively less activity. However, repressive impact of Hg^{+2}; as well as Zn^{+2} metals was observed previously in case of *B. cereus MTCC 1305* reported by Brown et al., (2008) [33].

3.7 Enzyme Kinetics:

The K_m of L - glutaminase is found 0.014 mM whereas the V_{max} was found to be 0.07U/ml, respectively (Figure 5). The Km of purified enzyme was seen to be less than other bacterial sp. less Km indicated that the organism high substrate utilization capacity. Different microorganism has diversity in glutamine utilization according to their kinetic properties [5].

4. Conclusion:

This investigation carried out to study screening production and purification of L - glutaminase by isolated halophilic bacterium *Bacillus halotolerans* PHN 1. From our study we conclude that our saline soil isolate *Bacillus halotolerans* PHN 1 will be recognised as a nice option to produce optimum concentration of L - glutaminase. Enzyme having optimum pH 7.0 and 30°C temperature in addition to that this enzyme is active in 3% salt concentration. These all properties make this enzyme to be prefer versatile role in different industries included food, medical, pharmaceutical etc. this enzyme has lower km so would have capacity to work at higher scale promisingly due to its salt tolerating ability as well as stability. Complete inhibition of enzyme was not observed at various inhibitors that again make this enzyme capable to work in high metallic ion concentration. In conclusion, by considering previous work and recent technologies it is needed to develop different tools and technique to make this enzyme suitable for the various industries and to exploit more applications of this enzyme which are yet to be not explored.

Authors contribution statement:

Salama Nadaf: Design and execution of all experiments writing and preparation of manuscript. **Naiem Nadaf:** Contributed for the completion of some part of experimental work. **Padma Dandge:** Directed research work, analysis and authentication of results, helps in editing of manuscript**.**

Conflicts of interest: authors declare no any conflict of interest.

References:

References:

1. D. Bülbül, and E. Karakuş, Production and optimization of L - glutaminase enzyme from *Hypocrea jecorina* pure culture. Prep. Biochem. Biotechnol. *43*(4) (2013) 385-397. DOI: 10.1080/10826068.2012.741641.
2. J. Roberts, J.S. Holcenberg, and W.C. Dolowy, Antineoplastic activity of highly purified bacterial L - glutaminases. *Nature*, *227*(5263) (1970)1136-1137. DOI: 10.1038/2271136a0
3. T. Yokatsuke, Fermented protein foods in the Orient, with emphasis on shoyu and miso in Japan. In. Microbiol. Ferment. foods. (1985)197-247.
4. T. Sathish, and R.S. Prakasham, Isolation and identification of L -glutaminase an antileukemic enzyme producing mico-organism from Godavari River bank soils in Andhra Pradesh. Int. Res. J. Pharm. 1 (2010) 367-373.

5. A. Amobonye, S. Singh, and S. Pillai, Recent advances in microbial glutaminase production and applications—a concise review. Crit. Rev. biotechnol. 39 (7) (2019) 944-963. DOI: 10.1080/07388551.2019.1640659 PMID: 31327254
6. J.M. Janda and S.L. Abbott, The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin Microbiol Rev. 23 (1) (2010) 35 - 73. https://doi.org/10.1128/CMR.00039-09 PMID: 20065325
7. P. Binod, R. Sindhu, A. Madhavan, A. Abraham, A.K. Mathew, U.S. Beevi, A. Pandey Recent developments in L - glutaminase production and applications–An overview. Biores. Technol. 245 (2017) 1766-1774.
8. M.S. Ardawi, and E.A. Newsholme, Glutamine metabolism in lymphocytes of the rat. Biochem. J. 212(3) (1983) 835-842. DOI: 10.1042/bj2120835 PMID: 6882397 DOI: 10.1016/j.biortech.2017.05.059 , PMID: 28549811
9. D.D. Teja, V.S. Devi, N. Harsha, S.S. Vishala, and P.S. Lakshmi, Production of L-glutaminase from marine ecosystems and optimal conditions for maximal production by actinomycetes. Int. J. Adv. Res. 2 (1) (2014) 485-491.
10. L.V. Aishwariyaa, L. Sheeja, and M.S. Devi, Screening and identification of asparginase and L - glutaminase producing halophilic bacteria from natural saline habitats. Int. J. Rec. Adv. Biotechnol. Nanotechnol. 3 (2020) 34-53.
11. P. Singh, and R.M. Banik, Biochemical characterization and antitumor study of L-glutaminase from *Bacillus cereus MTCC* 1305. App. Biochem. Biotechnol. 171 (2) (2013) 522-531. DOI: 10.1007/s12010-013-0371-3, PMID: 23873638
12. P. Iyer, and R.S. Singhal, Production of L - glutaminase (EC 3.2. 1.5) from *Zygosaccharomyces rouxii* in solid-state fermentation and modeling the growth of Z. rouxii therein. J. Microbiol. Biotechnol. 20 (4) (2010) 7 PMID: 20467247
13. A. Sabu, Sources, properties and applications of microbial therapeutic enzymes. Ind J Biotechnol. 2 (2003) 334–341. ISSN: 0975-0967 (Online); 0972-5849 (Print)
14. R. Balagurunathan, M. Radhakrishnan, and S.T. Somasundaram, L-Glutaminase producing actinomycetes from marine sediments–selective isolation, semi quantitative assay and characterization of potential strain. Aust. J. Basic. Appl. Sci. 4(5) (2010) 698-705. ISSN : 1991-8178, URL : http://www.insipub.com/.../698-705.pdf
15. E. Chasanah, U.S.F. Tambunan, and T. Yulianti, Screening and characterization of L-glutaminase produced by bacteria isolated from Sangihe Talaud Sea. Squalen Bull. Mar. Fish. Posth. Biotechnol. *7*(3) (2012) 115-122. DOI:10.15578/SQUALEN.V7I3.6
16. P.A. Rochelle, J.A. Will, J.C. Fry, G.J. Jenkins, R.J. Parkes, C.M. Turley, Weightman AJ(1995). In: Trevors JT, van Elsas JD (eds) Nucleic acids in the environment. Springer, Berlin, pp 219–239. DOI: 10.1007/978-3-642-79050-8
17. S.A.V.M. Jesuraj, J. Praya, R. Kingsley, L. Dinesh Kumar, and M. Ravikumar, Bio-processing of Ar isolates for economical production of l-glutaminase by solid-state fermentation. Int. J. Chem. Tech. Res. 5 (2013) 1428-1436. ISSN : 0974-4290
18. N.H. Nadaf, and J.S. Ghosh, Purification and characterization of catechol 1, 2-dioxygenase from *Rhodococcus sp*. NCIM 2891. Res. J. Environ. Earth. Sci. 3(5) (2011) 608-613. ISSN: 2041-0492
19. O.H. Lowry, N.N. Rosebrough, A.L. Farr, R.Y. Randall, Protein measurement with the Folin Phenol reagent. J Biol Chem. 193 (1951) 265–275. PMID: 14907713

20. L.S. Izotova, A.Y. Strongin, L.N. Chekulaeva, V.E. Sterkin, V.I. Ostoslavskaya, L.A. Lyublinskaya, and V.M. Stepanov, Purification and properties of serine protease from *Halobacterium halobium*. J. Bacteriol. 155(2) (1983) 826-830. DOI: 10.1128/jb.155.2.826-830.1983, PMID: 6348027
21. M.J. Coronado, C. Vargas, E. Mellado, G. Tegos, C. Drainas, J.J. Nieto, and A. Ventosa, The α-amylase gene amyH of the moderate halophile *Halomonas meridiana*: cloning and molecular characterization The EMBL accession number for the sequence reported in this paper is AJ239061. Microbiol. 146 (4) (2000) 861-868. DOI: 10.1099/00221287-146-4-861, PMID: 10784044
22. M. Müller-Santos, E.M. de Souza, F.D.O. Pedrosa, D.A. Mitchell, S. Longhi, F. Carrière, F., and N. Krieger, First evidence for the salt-dependent folding and activity of an esterase from the halophilic archaea *Haloarcula marismortui*. Biochim. et Biophys. Acta (BBA)-Mol. and Cell Biol. of Lipids, 1791(8) (2009) 719-729. DOI: 10.1016/j.bbalip.2009.03.006, PMID: 19303051
23. M. Vidyasagar, S. Prakash, C. Litchfield, and K. Sreeramulu, Purification and characterization of a thermostable, haloalkaliphilic extracellular serine protease from the extreme halophilic archaeon *Halogeometricum borinquense* strain TSS101. Archaea, 2(1) (2006) 51-57. DOI: 10.1155/2006/430763, PMID: 16877321
24. D. Mousumi, A. Dayanand, Production and antioxidant attribute of L-glutaminase from *Streptomyces enissocaesilis* DMQ-24. Int J Latest Res Sci Technol. (2013) 2 1–9. ISSN (Online):2278-5299
25. M. Moriguchi, K. Sakai, R. Tateyama et al., Isolation and characterization of salt-tolerant glutaminases from marine *Micrococcus luteus* K-3. J. Ferment. Bioeng. 77 (1994) 621–625. DOI: 10.1016/0922-338X(94)90143-0
26. L. Kumar, B. Singh, D.K. Adhikari et al., A temperature and salt-tolerant L-glutaminase from Gangotri region of Uttarakhand Himalaya: enzyme purification and characterization. Appl. Biochem. Biotechnol. 166 (2012) 1723–1735. DOI: 10.1007/s12010-012-9576-0 PMID: 22367638
27. M. Ye, X. Liu, L. Zhao, Production of a novel salt-tolerant L-glutaminase from *Bacillus amyloliquefaciens* using agro-industrial residues and its application in Chinese soy sauce ferment. Biotechnol. 12 (2013) 25–35. DOI: 10.3923/biotech.2013.25.35
28. S.S. More, R. Swamy, N. Mohan et al., Purification and characterization of anti-cancer L-glutaminase of *Bacillus cereus* strain LC13. Proc. Natl. Acad. Sci. India Sect. B Biol. Sci. 88 (2018) 695–705 DOI: 10.1007/s40011-016-0808-x
29. S. Sinsuwan, J. Yongsawatdigul, S. Chumseng, et al. Efficient expression and purification of recombinant glutaminase from *Bacillus licheniformis* (GlsA) in *Escherichia coli*. Protein Expr. Purif. 83 (2012) 52–58. DOI: 10.1016/j.pep.2012.03.001, PMID: 22433447
30. M. Klein, H. Kaltwasser, T. Jahns, Isolation of a novel, phosphate-activated glutaminase from *Bacillus pasteurii*. FEMS Microbiol. Lett. 206 (2002) 63–67. DOI:10.1111/j.1574-6968.2002.tb10987.x
31. S.R.K. Pandian, V. Deepak, S.D. Sivasubramaniam, et al. Optimization and purification of anticancer enzyme L-glutaminase from *Alcaligenes faecalis* KLU102. Biologia. 69 (2014) 1644–1651. DOI: 10.2478/s11756-014-0486-1

32. M. Wakayama, T. Yamagata, A. Kamemura, N. Bootim, S. Yano, T. Tachiki, K. Yoshimune, M. Moriguchi, Characterization of salt-tolerant glutaminase from *Stenotrophomonas maltophilia* NYW-81 and its application in Japanese soy sauce fermentation. J. Ind. Microbiol. Biotechnol. 32 (2005) 383-390. DOI: 10.1007/s10295-005-0257-7, PMID: 16012776
33. G. Brown, A. Singer, M. Proudfoot et al., Functional and structural characterization of four glutaminases from *Escherichia coli* and *Bacillus subtilis*. Biochem.;47 (2008) 5724–5735. DOI: 10.1021/bi800097h, PMID: 18459799

Figure legends:

Figure. 1: (A) Isolated 16S PCR amplicon (B) Constructed phylogenetic tree by using isolate (named as *Bacillus halotolerance* PHN 1) and related *Bacillus* sp. (Bar = 0.00050 substitutions per site)

Figure. 2: (A) Purification profile of L - glutaminase enzyme from *Bacillus halotolerans* PHN 1. (B) SDS PAGE analysis of L - glutaminase where, M-molecular weight markers, C- after ammonium sulphate P-protein after ion exchange chromatography.

Figure. 3: (A) Effect of pH on L - glutaminase enzyme activity error bars indicates standard deviation. (B) Effect of Temperature enzyme activity error bars indicates standard deviation.

Figure. 4: Effect of Heavy metals on L - glutaminase enzyme activity error bars indicate standard deviation

Figure. 5: Km of L - glutaminase enzyme

Tables:

Table 1. Purification profile of L - glutaminase

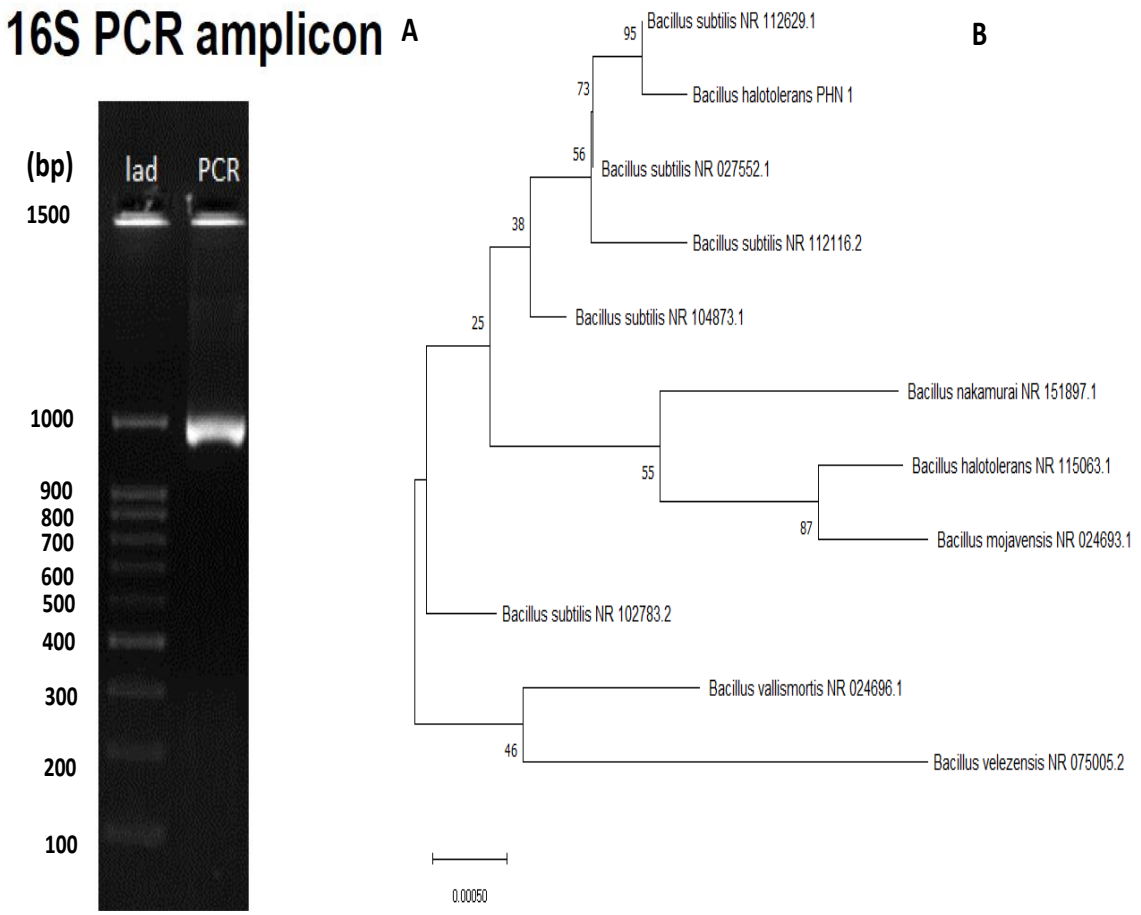

Figure. 1. (A) Isolated 16S PCR amplicon (B) Constructed phylogenetic tree by using isolate (named as *Bacillus halotolerance* PHN 1) and related *Bacillus* sp. (Bar = 0.00050 substitutions per site)

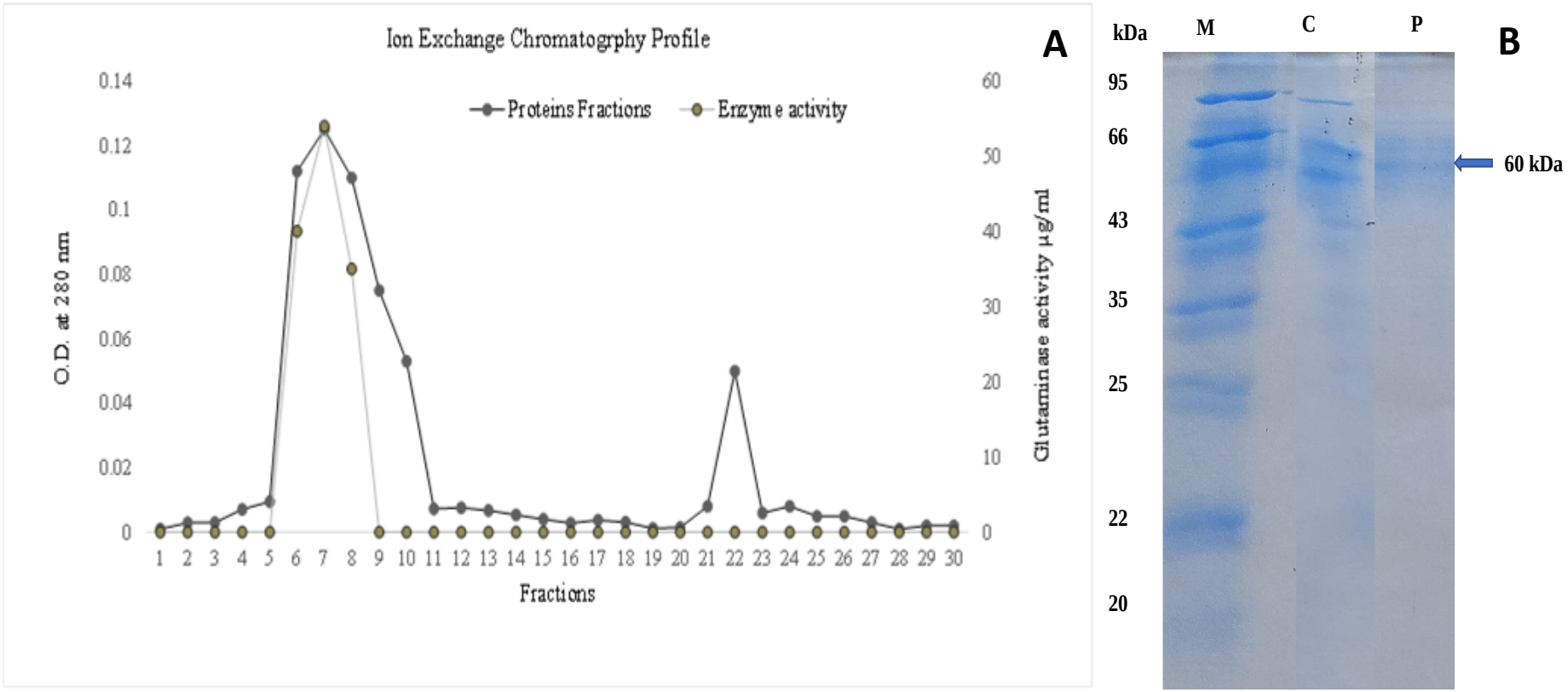

Figure. 2: (A) Purification profile of glutaminase enzyme from *Bacillus halotolerans* PHN 1. (B) SDS PAGE analysis of glutaminase where, M-molecular weight markers, C- after ammonium sulphate P-protein after ion exchange chromatography.

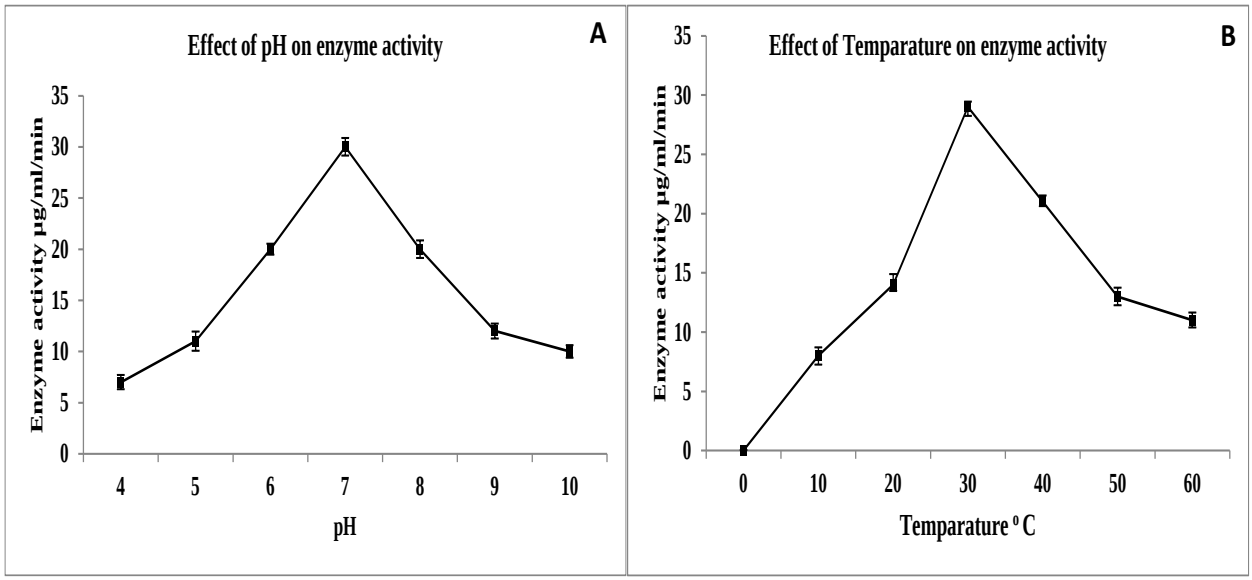

Figure 3: (A) Effect of pH on Glutaminase enzyme activity error bars indicates standard deviation. (B) Effect of Temperature enzyme activity error bars indicates standard deviation.

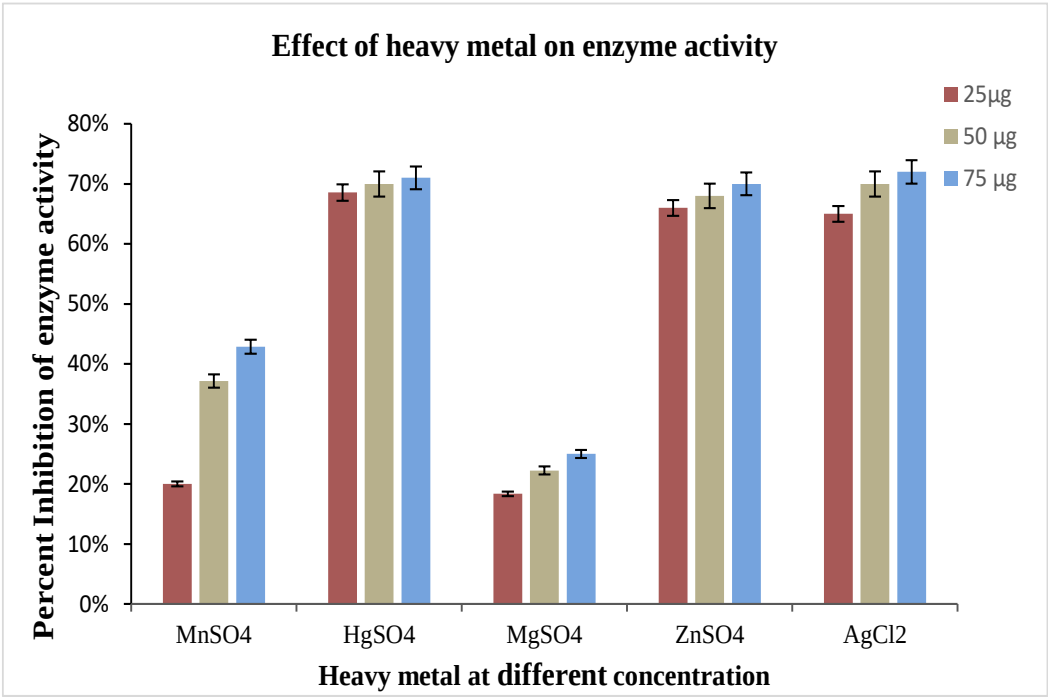

Figure. 4: Effect of Heavy metals on glutaminase enzyme activity error bars indicate standard deviation

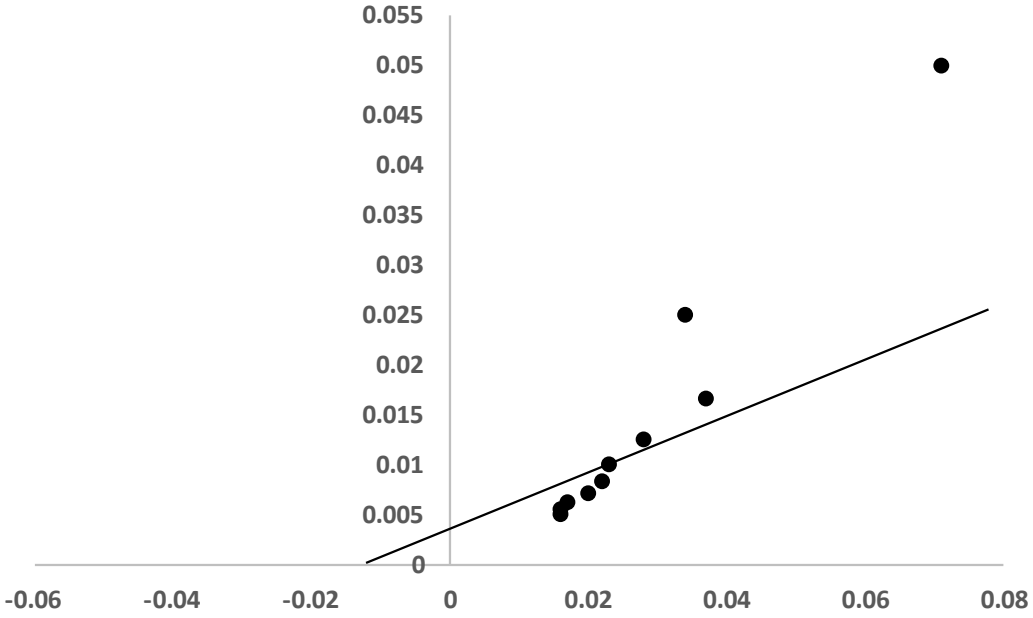

Figure. 5: Km of glutaminase enzyme

Table 1. Purification profile of glutaminase

Purification step	Volume (ml)	Total activity U	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	500	16.65 ± 0.036	31.5 ± 02	0.528 ± 0.064	100	1
After $(NH_4)_2SO_4$ Precipitation	350	12.25 ± 0.089	19.25 ± 0.082	0.766 ± 0.034	73.57	1.45
DEAE Ion exchange	20	0.78 ± 0.043	0.500 ± 0.072	1.56 ± 0.024	4.68	2.95

P values are significant at $P < 0.05$.