CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1134384

Available online at: http://www.iajps.com Research Article

PRODUCTION AND PARTIAL PURIFICATION OF L-ASPARAGINASE ENZYME FROM BACTERIA

E. Poongothai, N.Siddharthan, N. Hemalatha* and R.Balagurunathan

Department of Microbiology, Periyar University, Periyar Palkalai Nagar, Salem – 11

Abstract:

L-asparaginase is a potential anti-leukemic enzyme. It does not affect normal cells in the human body but target only cancerous cells. L- asparaginase had been isolated from many different microbial sources. The demand for the enzyme worldwide especially in health sector had driven researches focusing on enzyme yield per substrate quantity by applying optimization techniques. In the current study soil microbial isolates were screened for potential producers of L-asparaginase using phenol red indicator growth medium. The isolate was characterized by biochemical tests and was found to belong to Bacillus sp. The enzyme was mass produced by submerged fermentation method. Different concentrations of nitrogen and carbon sources, like peptone and lactose were used for optimizing the enzyme production. The enzyme was partially purified by ammonium sulphate precipitation. Dialysis was carried out to remove the excess salt. The dialysis purified enzyme exhibited maximal enzyme activity at a pH of 7 and temperature of 38°C. The activity was found to be 75% greater than the activity of crude enzyme in broth culture. Protein profiling by SDS-PAGE revealed the molecular weight of the protein to be 42 kDa.

Keywords: *Bacillus sp, Enzyme, L-asparaginase, partial purification.*

Corresponding author:

N. Hemalatha,

Department of Microbiology,
Periyar University, Periyar Palkalai Nagar,
Salem – 11
Email Id: aarthihema2004@yahoo.com

QR code

*Please cite this article in press as E. Poongothai et al., **Production and Partial Purification of L-Asparaginase Enzyme from Bacteria**, Indo Am. J. P. Sci, 2017; 4(12).*

INTRODUCTION:

Enzymes are delicate protein molecules necessary for life. Enzymes are well known biocatalysts that perform a multitude of chemical reactions in the metabolism of almost all organisms' viz., plants, animals, fungi, bacteria and viruses [1] and are commercially exploited in the detergent, food, leather processing, pharmaceutical, diagnostics, and fine chemical industries.

L-Asparaginase is an enzyme of high therapeutic importance which has been used in acute lymphoblastic leukemia (ALL) & Mast cell tumor. It's a most important medication needed in a basic health system (WHO). The demand for this enzyme is expected to increase in coming years owing to its potential industrial applications in food industries besides its clinical applications. The enzyme which converts L-asparagine to L-aspartic acid and ammonia, has been used as a chemotherapeutic agent. It has received increased attention in recent years for its anti carcinogenic potential [2]. The clinical action of this enzyme is attributed to the reduction of L-asparagine; tumour cells unable to synthesise this amino acid are selectively killed by L-asparagine deprivation. On a large industrial scale, productions of secondary metabolites by microbes are mostly carried out by using submerged fermentation system and the overall cost of enzyme is one of the major challenges against the cost-effective industrial application of enzymes.

The cost of the enzyme production and downstream processing is of major concern for successful application in the industry. An increase in either the enzyme activity in terms of substrate conversion rate or increase in enzyme stability can facilitate enzyme utilization in varied and extreme conditions. The present study was designed to mass produce the enzyme in a much easier microbial way without compromising for the quality of the product. The enzyme efficiency is checked under varied physical factors like temperature and pH that mostly influences the enzyme activity.

MATERIALS AND METHODS:

Microorganism and culture condition

Bacterial strains were isolated from the magnesite mine soil sample collected from Salem, using M9 medium by serial dilution method. The inoculated agar plates were incubated at 37ºC in an incubator. The microbial strains were isolated, based on phenol-red zone in the medium. After purification the culture was characterized using standard biochemical tests for identifying the organism. The slant cultures maintained at 4°C were used for the present study.

Preparation of inoculum

The selected bacterial strain was inoculated in nutrient agar plates and incubated at 37°C for 1 - 2 days. After incubation, a loopful of bacterial growth from Nutrient agar plate was inoculated into 50 ml of nutrient broth in 250 ml flask and kept in rotary shaker with 120 rpm at 37ºC for 48 hours. The broth culture of selected bacterial strain was used as inoculum for enzyme production following the method of Pallavi and Audipudi, 2015[3].

Production of L-asparaginase enzyme

Production of Asparagine was carried out by adopting submerged fermentation method. Media composition (g/l): KH_2PO_4 2.0, L-asparagine 6.0, $MgSO_4.7H_2O$ 1.0, $CaCl_2.2H_2O$ 1.0, peptone 3.0 and lactose 3.0 [4]. Each 50 ml of modified M9 medium was prepared in two 250 ml conical flask. After sterilization, about 10 % of bacterial inoculum was aseptically transferred into one of the production medium flasks and the flasks were kept in rotary shaker adjusted to 120 rpm at 37ºC for 48 hours. The uninoculated flask serves as negative control. After incubation, both flasks were added with few drops of phenol red solution [0.012 gm/litre]. A colour change of phenol red to pink colour observed in test flask indicates positive result for production of L-asparaginase enzyme.

Enzyme assay for L-asparaginase

The L-asparaginase activity was determined by hydrolysis of L-asparagine to release the ammonia which was measured by using Nessler's reaction. A mixture of 0.1 ml of enzyme extract, 0.2 ml of 0.05 M Tris HCl buffer (pH 8.6), and 1.7 ml of 0.01 M L - asparagine was incubated for 10 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1.5 M trichloroacetic acid (TCA). After centrifugation at 1000 rpm, 0.5 ml of the supernatant was diluted to 7 ml with distilled water and treated with 1 ml of Nessler's reagent. The colour reaction was allowed to develop for 10 minutes and the absorbance read at 480 nm with a spectrophotometer [5].

The enzyme activity of crude and purified enzymes were checked in different physical conditions of varying pH ranges (4,5,6,7,8,9,10) and temperatures (25,30,37,40,47,50,57).

Crude enzyme preparation

After fermentation the flasks were removed from the shaker and the fermentation broth was separated by centrifugation at 10,000 rpm for 30 minutes to separate the biomass. The clear supernatant was collected in a screw cap tube and stored at 4°C until

further use. The clear supernatant was used as crude enzyme.

Partial purification of enzyme

Ammonium sulphate precipitation and Dialysis

During this process, the crude enzyme was brought to 85 % saturation with ammonium sulphate at pH 8.4 and kept overnight in a refrigeration condition at 4°C. It was there after subjected to centrifugation at 10,000 rpm for 20 minutes. The obtained precipitate was resuspended in a minimal volume of 1 M Tris HCl pH 7.5. Finally the enzyme activity of dialysate was quantitatively assayed. The precipitate from this step was collected and stored at 4°C [6].

Estimation of Protein

The amount of protein was estimated by the method of Lowry *et al.*, (1951)[7] using bovine serum albumin as the standard.

Molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (1970) [8], with a separating 10% of acrylamide in a Tris-borate buffer pH 7.1containing 0.1% SDS. The gels were stained with 0.025 Coomassie brilliant blue R-250 and destained with a solution of methnol, acetic acid and water.

RESULTS AND DISCUSSION:

The bacterial strain was isolated from magnesite mine soil sample collected from Salem. Microbial strains producing L-Asparaginase were identified by a pink coloured zone formation around colonies on M9 agar medium with phenol red as indicator for detection of L-Asparaginase producing organisms [9]. Among the isolated bacterial colonies, the bacterial strain S8 produced maximum pink coloured zone and this strain was selected for further studies **(Fig.1)**. The isolated strain S8 was characterized by morphological and biochemical tests. The strain was gram positive thick rod arranged Singly/ Diplo/ Patterns/ Short chains, endospores present, motile, catalase – negative, oxidase – positive and OF test - negative. According to the morphological and biochemical characteristics of the strain, S8 was classified to be a species belonging to the *Bacillus* genus.

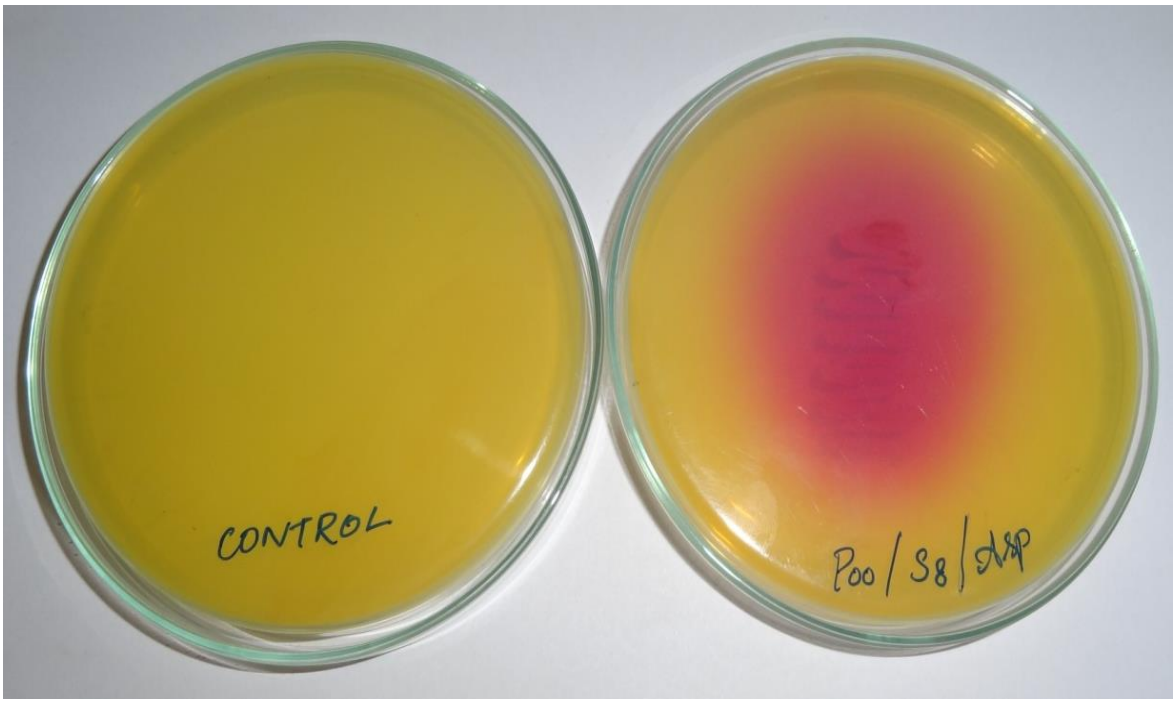

Fig.1: Pink colony production by L-Asparaginase Producing *Bacillus*

Table: 1 Purification steps of L-Asparaginase from Bacillus Strain (S8)

Purification steps	**Enzymes** U/ml	**Protein mg/ml**	**Specific Activity U/mg**	**Recovery (%)**
Crude extract	48.6	38	1.08	100
Ammonium sulphate precipitation	30	20	1.26	71
Dialysis	26.1	12	2.15	52

Production and Partial purification of enzyme

The selected bacterial strain (S8) showed maximum growth at 48 h of submerged fermentation. Enzyme activity and specificity was calculated for the crude and partial purified sample of the organism. The maximum enzyme activity and specific activity was observed in *Bacillus* by using L-Asparagine as substrate showed in **Table: 1**.

Enzyme production was determined in terms of enzyme activity. The protein content in the broth culture was found to be 48 mg/ml. The crude enzyme activity of S8 strain showed a maximum of 48.6 U/ml L-Asparaginase enzymes with Asparagine as substrate in the test solution. The specific activity of L- Asparaginase enzyme from the centrifuged culture broth in crude state and after purifications has been observed, using their total protein concentration measurements at each purification level. An increase in the specific activity of L- Asparaginase from the isolate and a decrease in the total protein concentration after partial purification of the enzyme have been observed. A higher fold increase and decrease in the enzyme specific activity and total protein content respectively were observed in the bacterial enzyme counterpart after purification with dialysis technique.

The enzyme activities of purified enzymes in the two levels were calculated in terms of total protein content as specific enzyme activities which were found to be greater than the crude enzyme's specific activity. The specific activity of dialysed enzyme was 44% greater than the specific activity of the crude enzyme and 27 % greater than the specific activity of the ammonium sulphate precipitated purified enzyme.

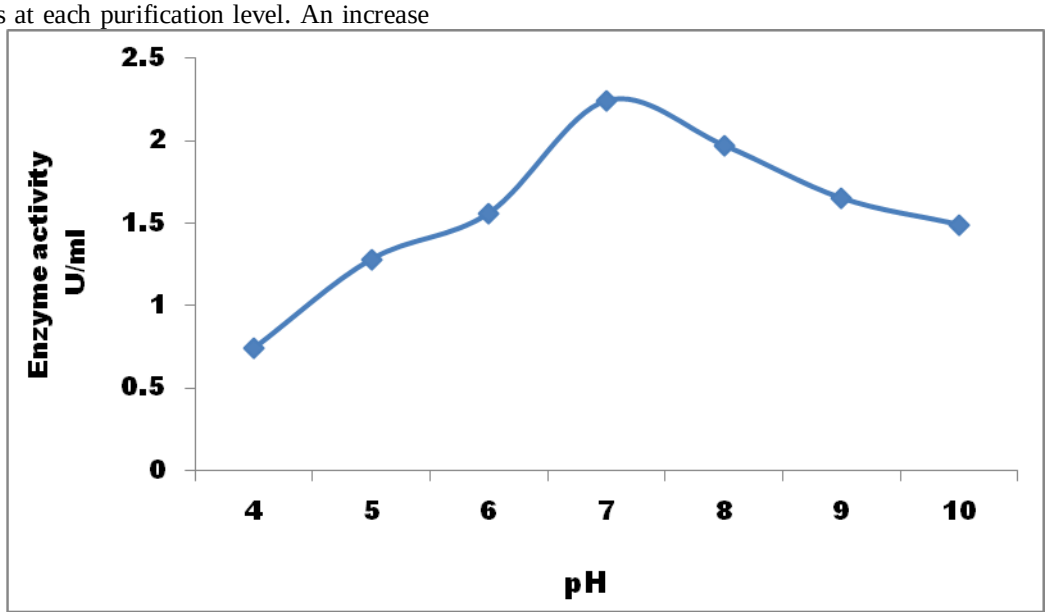

Fig. 2: Effect of different pH on enzyme activity

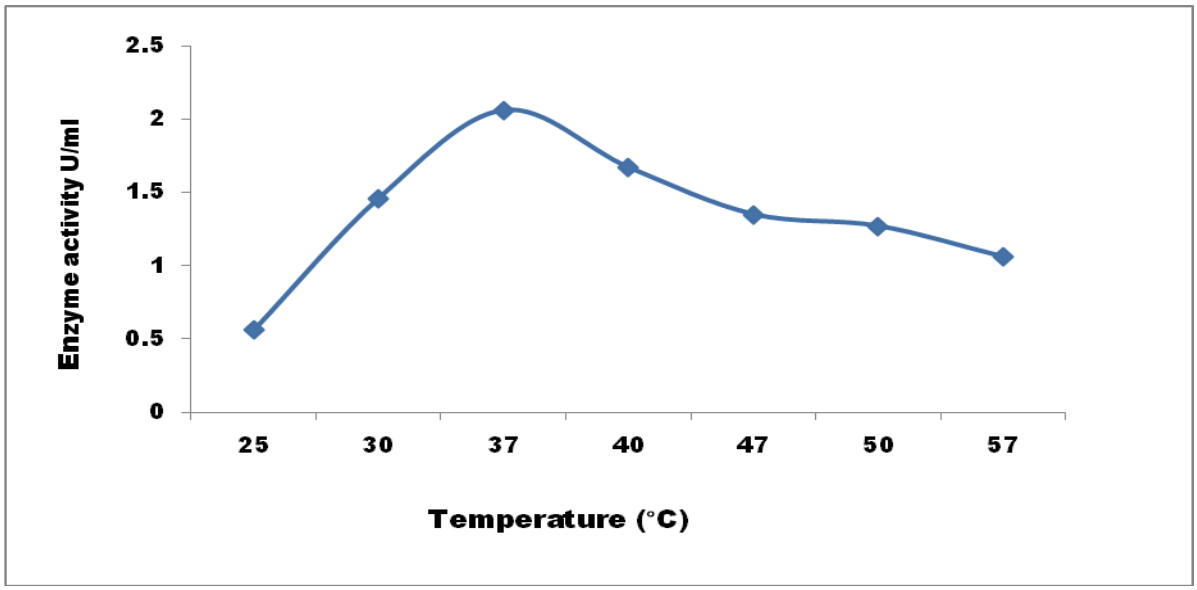

Fig. 3: Effect of different Temperature on enzyme activity

The enzyme stability check results showed that the activity was stable with very little fluctuations at variable pH ranges and temperature with the optimum asparaginase activity of 2.24 U/ml & 2.06 U/ml **(Fig.2 & 3)** at a temperature and pH of 37°C & 7 respectively when tested with dialysed enzyme fraction. The isolate S8 showed 2.15 U/ml of specific activity with a total protein concentration of 12 mg.

Similar results were obtained by Kothari and Deshmukh, 2014 [14]. The isolate ASP-I showed 1.79 U/mg of specific activity with a total protein concentration of 1.33 mg, while 1.85 U/mg of activity with a total protein of 0.75 mg was shown by ASP-II. Moorthy *et al.*, 2010[10], reported that a total protein concentration of 2.60 mg the activity of the crude extract was 0.10, while after ammonium sulphate precipitation and dialysis the an increase in the specific activity of the enzyme was about 1.09 U/mg. Sunitha *et al.*, 2010 [11] has used *Bacillus cereus* MNTG-7 for the production of L-Asparaginase. Roberts *et al.*, 1968 [12], has achieved 0.950 U/mg of activity from *E.coli*. HAP strain. When compared to the earlier reports, our study more 50% of specific activity to be increased by using *Bacillus sp.* (S8).

Bacillus sp. (S8) showed effective production with the medium containing peptone and lactose as sole nitrogen and carbon source at 37°C and pH 7.0. In a previous report, the maximum L-asparaginase activity was found to be at 37°C and pH 8.0 [13] (Mohapatra *et al.*, 1995). The purified enzyme was protein profiled by SDS PAGE for determination of molecular weight of the enzyme. The result thus obtained was a protein band of a molecular weight of approximately 42 kDa **(Fig. 4)**.

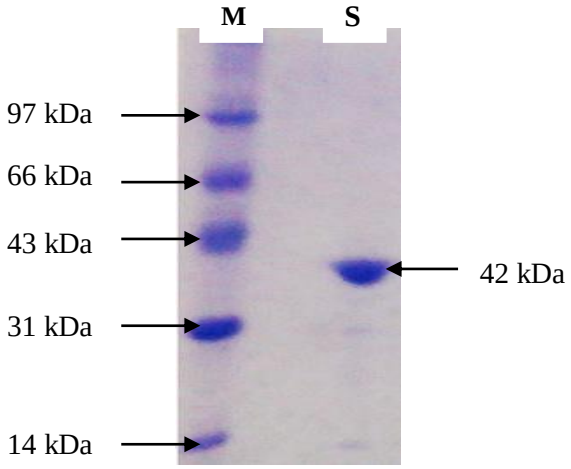

Fig.4: Molecular weight determination – SDS PAGE

CONCLUSION:

The present study concluded that, L-Asparaginase production by submerged fermentation from soil isolate of *Bacillus* (S8). The enzyme was partially purified by ammonium sulphate precipitation and dialysis was carried out to remove the excess salt. The largest quantity of L-asparaginase was produced when *Bacillus* was grown in the modified M-9 medium for 48 hrs and the maximum enzyme activity and specific activity was observed in *Bacillus*. Protein profiling by SDS-PAGE revealed the molecular weight of the protein to be 42 kDa. Furthermore high catalytic activity of the enzyme over a wide range of pH and temperature and its considerable stability makes it highly favorable for use as potent anticancer agent and for other application in healthcare industry.

ACKNOWLEDGEMENT

The authors are thankful to Head of the Department, Department of Microbiology, Periyar University, Salem for their encouragement and support for the research activity. And also we thank to ICMR [Ref.no.5/8/5/26/TF/2013/ECD-I Dt.24.03.2015] and DST-FIST [SR/FST/LSI-640/2015(c)3 Dt.30/05/2016] to the Department of Microbiology, Periyar University for financial and instrumental support.

REFERENCES:

1.Narayana, K. J. P., Kumar, K. G., & Vijayalakshmi, M. L-asparaginase production by Streptomyces albidoflavus. Indian Journal of Microbiology, 2008;48(3):331-336.

2.Manna, S., Sinha, A., Sadhukhan, R. & Chakrabaty, S. L. Purification, characterization and antitumor activity of L-asparaginase isolated from Pseudomonas stutzeri MB-405. Current Microbiology,1995;l30:291-298.

3.Pallavi Rudrapati & Amrutha Audipudi. Characterization and Bioprocessing of Oncolytic Enzyme - L-asparaginase isolated from Marine bacillus AVP 14. International Journal of Pharmaceutical Science,2015; 36:195-201.

4.Vidhya Moorthy., Aishwarya Ramalingam., Alagarsamy Sumantha & Rajesh Tippapur Shankaranaya. Production, purification and characterisation of extracellular L-asparaginase from a soil isolate of Bacillus sp. African Journal of Microbiology Research, 2010;4(18): 1862–1867.

5.Kishor, D., Nimkande., Zia Khan, H., Shankar Mular, M. & Sangita S. Isolation, Purification & Characterization of LAsparaginase from dry seeds of Pisum sativum and Vigna radiate. International Journal of Applied Research,2015; 1(7):628-631.

6.Basha, S., Rekha, R., Komala, M. & Ruby, S. Production of Extracellular Anti- leukaemic Enzyme L-asparaginase from Marine Actinomycetes by Solid state and Submerged Fermentation: Purification and Characterisation. Tropical Journal of Pharmaceutical Research.2009; 8 (4): 353-360.

7.Lowry, O. H., Rosebrough, H. J., Farr, A. L., & Randall, R. J. Protein measurement with the Folin Phenol Reagent. Journal of Biological Chemistry, 1951;193: 265- 275.

8.Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 1970;227: 680-685.

9.Prakasham, R. S., Hymavathi, M., Subba Rao, C. H., Arepalli, S. K., Venkateswara Rao, J., Kannady Kavin, P., Nasarruddin, K., Vijaykumar, J. B., & Sarma, P.N. Evaluation of antineoplastic activity of extracellular asparaginase produced by isolated Bacillus circulans. Applied Biochemistry and Biotechnology,2010; 160: 72-80.

10.Moorthy, V., Ramalingam, A., Sumantha, A., & Shankarnarayan, R. Production, purification and characterization of extracellular L-asparaginase from a soil isolate of Bacillus sp. African Journal of Microbiology Research.2010; 4(18):1862-1867.

11.Sunitha, M., Ellaiah, P., & Devi, R. Screening and optimization of nutrients for L asparaginase production by Bacillus cereus MNTG-7 in SmF by plackett-burmann design. African Journal of Microbiology Research, 2010;4 (4): 297-303.

12.Roberts, J., Burson, G., & Hill, J.New Procedures for Purification of L-Asparaginase with High Yield from Escherichia coli Journal of bacteriology. 1968;95(6): 2117-2123.

13.Mohapatra, B. R., Sani, R. K., & Banerjee, U.C. (1995). Characterization of L-asparaginase from Bacillus species isolated from an intertidal marine alga (Sargassum sp.) Letters in Applied Microbiology, 21, 380-383.

14.Kothari, P., & Deshmukh, D.V. Isolation and Purification of Anti-Tumor Enzyme L-Asparaginase from Soil isolates. International Journal of Pharmacognosy,2014; 1(6): 384-388.