

## Media Optimization, Isolation and Purification of L-Asparaginase from Marine Isolate

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### ABSTRACT

L-Asparaginase is an enzyme based drug used for the treatment of Acute Lymphoblastic Leukemia (ALL). In the current study, a bacterial strain was isolated from marine sediment, and screened for L-asparaginase production. On the basis of biochemical tests and 16S rDNA sequencing the organism was identified as *Bacillus pumilus*. The nutritional parameters affecting the L-asparaginase production by the strain were optimized. Maximum yield of L-asparaginase ( $157.03 \pm 2.81$  IU/ml) was obtained after 48h of incubation of medium supplemented with Galactose (2%) and asparagine (0.1%) as carbon and nitrogen sources, respectively. The enzyme extracted from *Bacillus pumilus* was partially purified by ammonium sulphate fractionation (70%), and DEAE cellulose chromatography. The apparent molecular weight of the enzyme and its subunit was 136 kDa and 71 kDa, respectively. The glutaminase activity of the enzyme was found to be 3.5 times lower than its asparaginase activity.

**Keywords:** 16S rDNA, *Bacillus pumilus*, enzyme purification, glutaminase side activity, L-asparaginase, marine ecosystem

### Introduction

L-Asparaginase (L-asparagine amino hydrolases (EC 3.5.1.1)) is a potent antineoplastic agent, widely exploited in the treatment of certain cancers, especially acute lymphoblastic leukemia [1]. L-Asparaginase catalyzes the hydrolysis of the amino acid asparagine into aspartic acid and ammonia. Lymphocytic leukemia cells are not capable of synthesizing L-asparagine and rely on the exogenous pool of this amino acid for their growth and survival. L-Asparaginase exploits this dependency of the leukemia cells on L-asparagine to kill them. L-Asparaginase hydrolyses L-asparagine to aspartic acid and ammonia, thus; depleting the amino acid supply to leukemia cells and starves them to death [2]. On the contrary, normal cells are protected from L-asparagine starvation due to their ability to produce this essential amino acid [3]. Since the observation that L-asparaginase from *E. coli* has an antitumour activity similar to that of the guinea pig serum, there has been considerable interest in asparaginase from various sources specially microorganisms [4]. Although various microorganisms like *Escherichia coli* [5, 6], *Erwinia carotovora*[7], *Pseudomonas acidovorans*, *Pseudomonas aeruginosa*[8], *Erwinia chrysanthemi*[9],

*Enterobacter aerogenes*[10], *Thermus thermophilus*[11], *Nocardia levis*[12], *Bacillus circulans*[13], *Bacillus cereus*[14], and *Bacillus brevis*[15] have a potential for the enzyme production, just the purified enzyme from *E. coli* and *Erwinia sp.* are currently in medical use as efficient as drugs in the lymphocytic leukemia, because of high substrate affinity [16].

The therapeutic use of L-asparaginase from above two sources was limited due to immunological responses. L-Asparaginase from bacterial origin has several issues like hypersensitivity due to long-term use leading to allergic reactions and anaphylaxis [17], asparaginase resistance [18], leukemogenicity [19], and glutaminase activity [20]. So, due to all these factors, there is a need for newer asparaginases with new immunological properties.

Microorganisms from extreme environments like marine water and hypersaline lakes are expected to have halophilic proteins and enzymes with modified structure and different immunological properties [1]. The L-asparaginase from these halophilic organisms may be used in the treatment for hypersensitive patients. In the current study, an L-asparaginase producing organism was isolated from marine sediment of Tarapur region, Maharashtra, India. The soil sample was provided by Central Institute of Fisheries Education, located in Mumbai, India.

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## Materials and Methods

### Isolation of microorganisms from marine soil

The soil sample was collected from Tarapur region, located in Maharashtra, India and was provided by Central Institute of Fisheries Education, Mumbai, India. The soil was enriched in Glucose Asparagine broth, Starch Casein broth and Sea water complex broth, appropriately diluted and inoculated on respective agar plates. The plates were incubated at room temperature for 24 hours. A total of 11 isolates were obtained from the plates, which were further screened for L-asparaginase production.

### Screening of isolates for L-asparaginase production

The isolates obtained were primarily screened for L-asparaginase production using a rapid plate assay as described by Gulati et al.[21]. The isolates showing the L-asparaginase production were further screened using submerged fermentation process. The isolates were inoculated in glucose asparagine broth and L-asparaginase production was measured every 24 h using L-asparaginase assay. The isolate with highest amount of enzyme production was selected for further studies.

### L-Asparaginase assay

L-Asparaginase activity was measured by Nessler's reaction. The assay procedure is based on direct Nesslerization of ammonia. The assay was performed according to the procedure described by Alapati and Muvva [12], with certain modifications. Cell free extract was obtained by centrifuging the culture broth at 10,000 rpm for 15 min. This cell free extract (0.5 ml) was mixed with 0.8 ml of 50mM Tris-HCl buffer pH 8.6 and 0.2 ml of 40 mM L-asparagine. The reaction mixture was incubated for 30 min at 37°C, and was terminated by the addition of 15% trichloroacetic acid. Precipitated proteins were removed by centrifugation and the liberated ammonia was measured spectrophotometrically at 500 nm by nesslerization. Enzyme activity was determined with reference to ammonia liberated using ammonium sulphate standard graph.

### Identification of marine isolate

Different morphological, cultural and physiological characteristics of the isolates were studied for identification purpose and compared with the standard description of Bergey's Manual of Determinative Bacteriology.

### DNA extraction and 16S rDNA gene sequence analysis

The DNA was extracted using the method described by Ausubel *et al.*[22]. The 16S rDNA was amplified by polymerase chain reaction (PCR), using prokaryotic universal primers Bact8F (Forward primer): 5'-AGATTTGATCCTGGCTCAG-3' and Bact 1391R (Reverse primer): 5'-GACGGGCGGTGTGCA-3'. The PCR was carried out as per instruction manual provided by manufacturer (Applied Biosystems). The amplification conditions were as follows: Initial denaturation at 94°C for 2 min, 30 cycles: 94°C for 1 min, 55°C for 45 sec, 72°C for 2 min and final extension at 72°C for 20 min. The amplified samples were electrophoresed on 1% agarose gel in TAE buffer containing ethidium bromide (1 µg/ml). The sequence was determined by Geneombio Technologies. The sequence similarity searches were performed using the BLAST program available from the National Centre for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the organism was identified to generic level [23, 24] Using the CLUSTAL-X Multiple Sequence Alignment Program (Strasburg, France), the 16S rDNA sequences of the isolated strains were aligned with sequences of related organisms obtained from GenBank [25] A phylogenetic tree was constructed via the neighbour-joining method using the TreeView program [26].

### Optimization of L-asparaginase production

Influence of different carbon and nitrogen sources on the production of L-asparaginase was determined.

#### Nitrogen sources

Different nitrogen sources such as L-asparagine, yeast extract, beef extract, peptone, tryptone, alanine, hydroxyproline, arginine, and glutamine were added at a concentration of 0.1% (w/v) to the glucose asparagine broth. The optimal concentration of the best nitrogen source (0.02-0.15 % w/v) for L-asparaginase production was determined.

#### Carbon sources

To investigate the effect of different carbon sources on the L-asparaginase production by the strain, the glucose asparagine broth with optimal amount of superior nitrogen source was supplemented with different carbon sources such as glucose, galactose, sucrose, trehalose, and glycerol at the concentration of 1% (w/v). The best carbon source was checked

at different concentrations (0.5-2 % w/v) for L-asparaginase production by the strain.

### Enzyme solution preparation

For enzyme production, 24 hours old actively growing culture was added to 2.5 L of optimized medium composed of Galactose (2%);  $\text{KH}_2\text{PO}_4$  (0.05%); and asparagine (0.1%) and grown at ambient temperature (28°C – 30°C) on orbital shaker (100 rpm). After 48 h of incubation, the fermentation broth was collected, and centrifuged to obtain cell free crude enzyme extract.

### Partial Purification of L-Asparaginase

The purification of L-asparaginase was carried out by salting out method using ammonium sulphate and ion exchange chromatography by using DEAE cellulose column.

### Salting out by ammonium

A typical batch was carried out using cell free homogenate obtained from 2-3 litres of the medium. Solid ammonium sulphate was added to the homogenate to reach a saturation of 30%. The mixture was left overnight at 4°C and then centrifuged at 10,000 rpm for 20 min at 4°C. The precipitate obtained was dialysed and the supernatant was raised to 70% saturation with solid ammonium sulphate. After centrifugation, the supernatant was further raised to 90% ammonium sulphate saturation. The precipitate collected after each of the 30%, 70%, and 90% saturation steps were dissolved in minimum volume of 50mM Tris-HCl buffer (pH 8.6) and dialyzed overnight at 4°C against the same buffer.

All the four fractions, viz., crude enzyme and three fractions of ammonium sulphate precipitation were tested for enzyme activity and total protein content. The ammonium sulphate fraction showing the highest enzyme activity was further purified by ion exchange chromatography.

### Ion exchange chromatography

The ion exchange chromatography was carried out using DEAE cellulose column. The enzyme

fractions obtained at 70% saturation ammonium sulphate was further purified using DEAE-52 cellulose column (2.0 X 5.0 cm) chromatography. The protein sample was applied onto the DEAE-cellulose column, pre-equilibrated with 50mL of the 50mM Tris-HCl buffer at pH 8.6 and the protein was eluted using a linear gradient of 0.05-0.5M KCl in 50mM Tris-HCl buffer, pH 8.6 with a flow rate of 20ml/hr. Each fraction was analyzed for protein content and enzyme activity. The purity was calculated in terms of specific activity.

### Molecular weight determination

The mass of the native protein was determined by gel-electrophoresis. Slab gel electrophoresis was carried out using 10% gel in the presence of SDS and with or without  $\beta$ -mercaptoethanol according to the method of Laemmli [27] and stained with silver as described by Ausubel, et al. [28].

### Glutaminase activity

The partially purified enzyme obtained after ion exchange chromatography was checked for its glutaminase activity. The activity was determined by using the L-asparaginase assay, using 40 mM glutamine instead of 40 mM asparagine as substrate. The specific activity of the enzyme for glutamine was calculated and compared to that for asparagine.

## Results

### Isolation and screening of microbial source

A total of 11 marine isolates (MI) were obtained, of which only 7 isolates (MI 1- MI 5, MI 7 and MI 8) showed L-asparaginase production in primary screening. After secondary screening, marine isolate MI5 showed the highest enzyme production of  $75.73 \pm 0.82$  IU/ml after 48 hrs among all the isolates. Table 1, shows the enzyme produced by different isolates in secondary screening after 48 h time interval. Figure 1, shows positive and negative results of L-asparaginase production on M9 medium using rapid plate assay. MI 7 was not considered for secondary screening as it gave weakly positive result in primary screening.

**Table 1: L-Asparaginase activity of marine isolates after 48 h of incubation**

Isolate	L-Asparaginase activity (IU/ml)
MI 1	$43.60 \pm 0.44$
MI 2	$26.39 \pm 0.81$
MI 3	$33.03 \pm 0.84$
MI 4	$35.25 \pm 1.98$
MI 5	$75.73 \pm 0.82$
MI 8	$35.30 \pm 0.76$

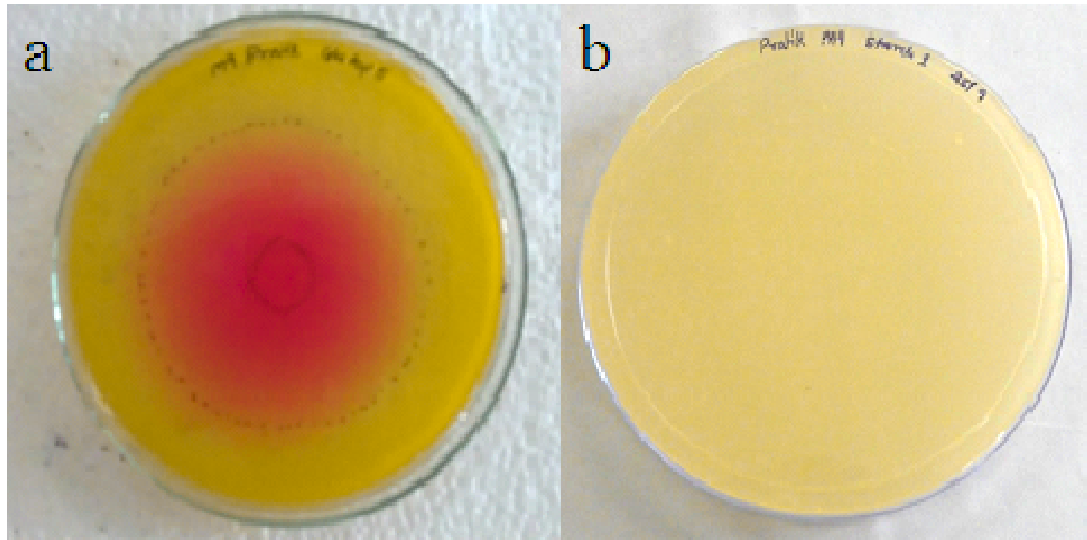


Figure 1: (a): Isolate showing positive result for L-asparaginase production on M9 medium; (b): Isolate showing negative result for L-asparaginase production on M9 medium

**Identification of microbial isolate**

The isolate MI 5 was identified on the basis of colony characteristics, biochemical tests, and 16S rDNA sequencing. The isolate was found to be aerobic, Gram-positive, motile, rod-shaped and endospore forming bacillus. The colonies when examined on solid media were white in color, circular, medium sized, glossy and flat in appearance. The bacillus was positive for both catalase and cytochrome oxidase test. Acid was produced without any gas from sugars like glucose, fructose and galactose. The isolate showed growth in nutrient broth with 10% NaCl.

**16S rDNA sequencing**

The absorbance ratio at 260 nm/ 280 nm of the extracted DNA was found to be 1.98 and the DNA concentration was 120.75 ng/μl. The PCR amplification of the 16S rDNA gene revealed efficient amplification and sharp band of around 1.3 kb was observed on 1% agarose. The 1.3 kb sequence obtained from the strain MI5 was aligned with all the presently available 16S rDNA sequences in GenBank Database. Based on the taxonomy report showed in the Figure 2, the strain MI5 was identified as *Bacillus pumilus*.

**Taxonomy Report**

Bacillus	58	hits	18
.. Bacillus safensis	36	hits	11
.. Bacillus pumilus	1	hits	1
.. Bacillus sp. SCMS (2010)	1	hits	0
.. Bacillus sp. 011105	1	hits	0
.. Bacillus sp. BBS1005 (2010)	1	hits	0
.. Bacillus sp. AU 866	1	hits	0
.. Bacillus sp. YX12	1	hits	0
.. Bacillus sp. HY11 (2010)	1	hits	0
.. Bacillus sp. SP-15	1	hits	0
.. Bacillus sp. SP1	1	hits	0
.. Bacillus sp. SP1-2	1	hits	0
.. Bacillus sp. S301Ydz-qs	1	hits	0
.. Bacillus sp. cp-h61	1	hits	0
.. Bacillus sp. HFB-h69	1	hits	0
.. Bacillus sp. HFB-10	1	hits	0
.. Bacillus sp. HFB-1b	1	hits	0

Figure 2: Taxonomy report of the isolate MI 5

**Optimization of L-Asparaginase production**

The effect of different nutritional parameters such as nitrogen sources and carbon sources on the production of L-asparaginase was determined.

**Nitrogen sources**

Different nitrogen sources like L-asparagine, yeast extract, beef extract, peptone, tryptone, alanine, hydroxyproline, arginine, and glutamine were amended

in glucose asparagine broth to determine their impact on L-asparagine production. Of all the nitrogen sources, L-asparagine showed highest enzyme production (Figure 3). Besides, the effect of different concentrations of L-asparagine (0.02-0.15%) on L-

asparaginase production was recorded (Figure 4). The optimal yield of L-asparaginase by the strain was achieved in the medium supplemented with 0.1% L-asparagine, whereas the enzyme production decreased with further increase in the L-asparagine concentration.

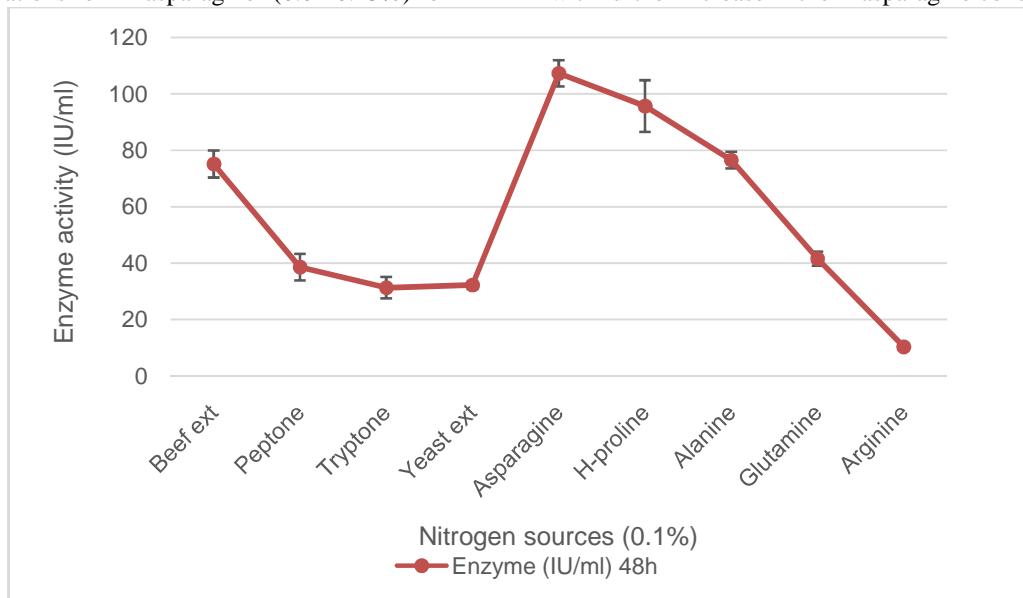


Figure 3: L-Asparaginase production by *Bacillus pumilus* grown in glucose asparagine broth amended with different nitrogen sources

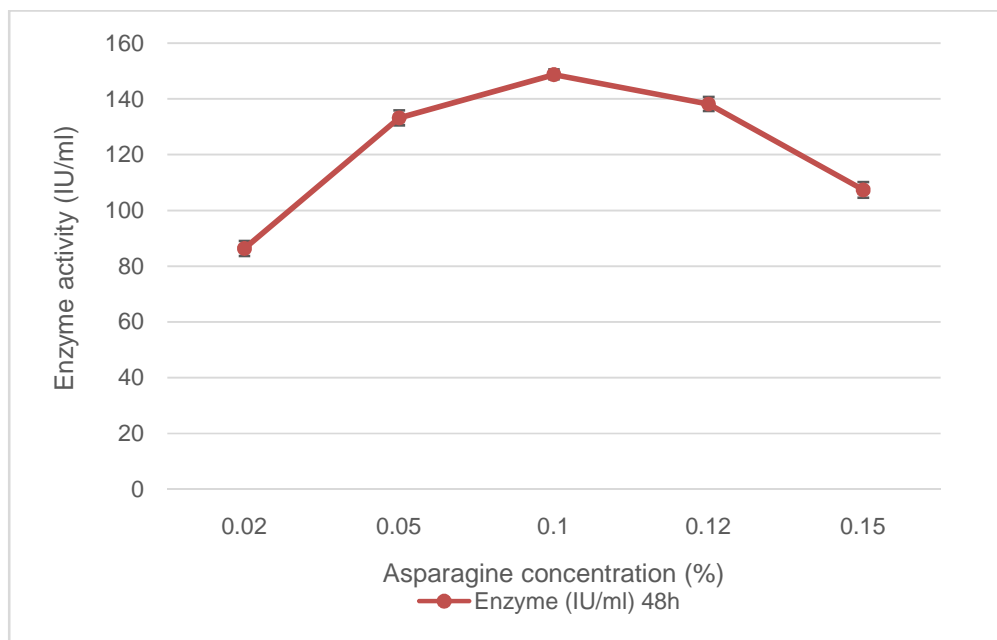
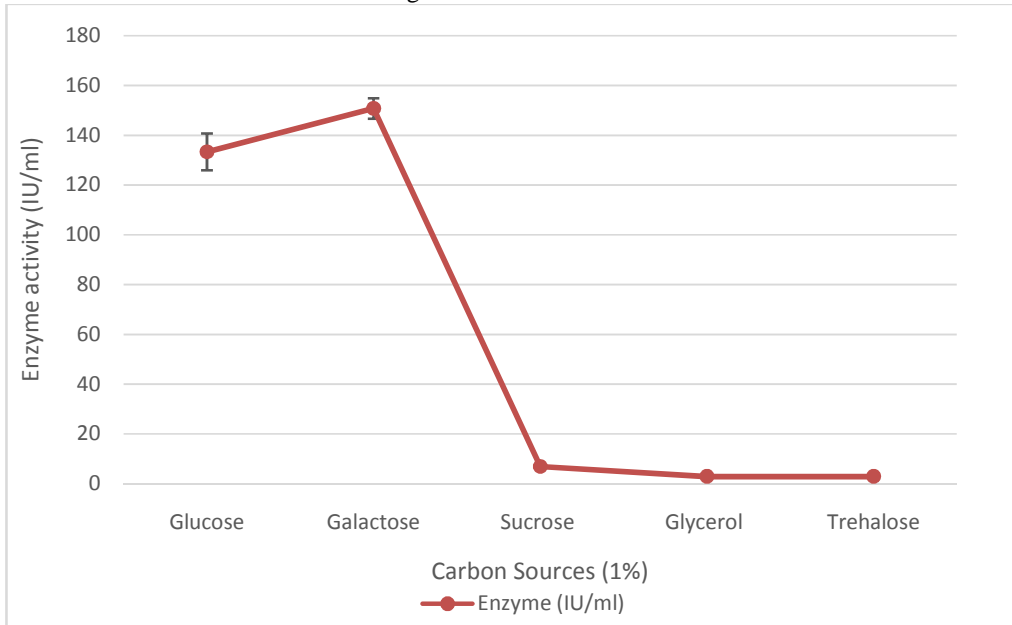


Figure 4: Impact of L-asparagine concentrations on L-asparaginase production by *Bacillus pumilus* grown in Glucose asparagine broth

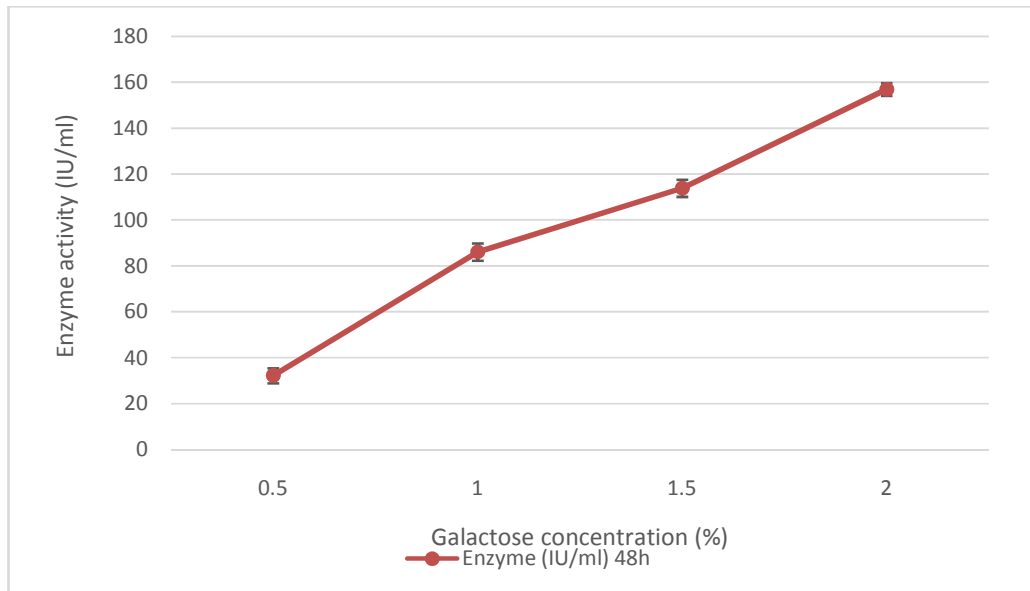
**Carbon sources**

The effect of carbon sources on the production of L-asparaginase by the strain was studied by incorporating different carbon sources to modified glucose

asparagine broth containing 0.1% L-asparagine. The enzyme production by the strain varied with different carbon sources tested (Figure 5). Among them, culture medium amended with 2% galactose favoured maximum enzyme production (Figure 6).



**Figure 5: L-Asparaginase production by *Bacillus pumilus* grown in modified glucose asparagine broth amended with different carbon sources**



**Figure 6: Impact of galactose concentrations on L-asparaginase production by *Bacillus pumilus* grown in modified Glucose asparagine broth**

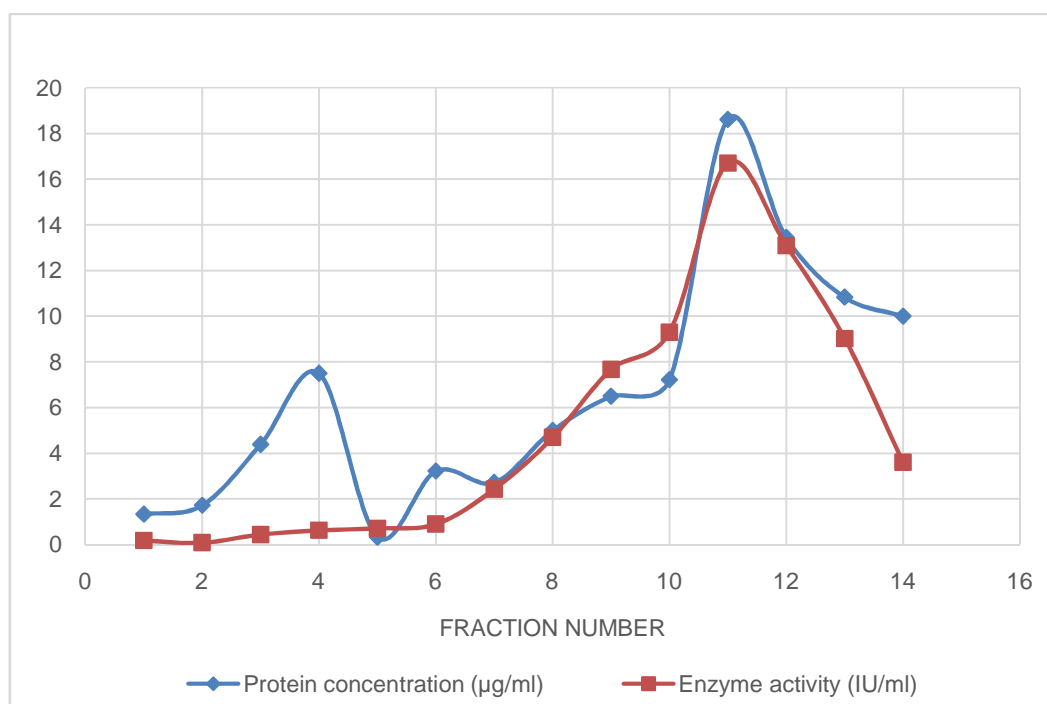
### Purification of L-Asparaginase

The enzyme L-asparaginase was purified from the strain *Bacillus pumilus* using ammonium sulphate fractionation and DEAE cellulose chromatography. The specific activity of the enzyme and the purity increased with every step of purification, whereas the

total protein, total activity and percent recovery decreased proportionally (Table 2). The enzyme precipitated out at 70% ammonium sulphate saturation was further purified using DEAE cellulose chromatography. Figure 7, shows the elution profile of the protein, where the enzyme was eluted at 0.4M KCl in Tris-HCl buffer (pH. 8.6).

**Table 2: L-Asparaginase purification summary**

Purification steps	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Recovery
Crude extract	258130	361	715	1.0	100
Ammonium sulphate precipitation	220	0.25	880	1.2	0.07
DEAE cellulose chromatography	167	0.16	1044	1.5	0.04



**Figure 7: DEAE cellulose chromatography of the dialyzed 70% ammonium sulphate precipitation fraction**

### Electrophoresis and Molecular Weight Determination

The L-asparaginase from *Bacillus pumilus* has a molecular mass of 136 kDa as determined by SDS PAGE (without  $\beta$ -mercaptoethanol) (Figure 8a); while the molecular mass of the enzyme subunit is 71 kDa as

observed in SDS-PAGE (with  $\beta$ -mercaptoethanol) (Figure 8b). The electrophoresis of the enzyme preparation from different steps of purification revealed that the resolved electrophoretic bands were progressively improved from the crude extract to the final step of purification.

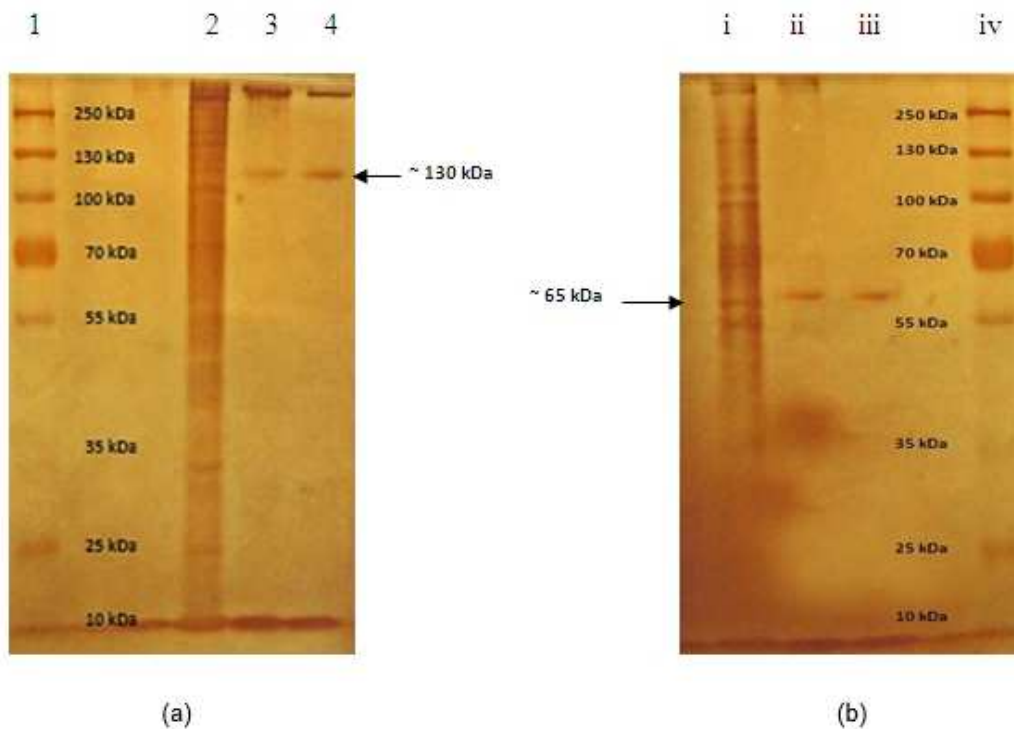


Figure 8: (a) SDS-PAGE (without  $\beta$ -mercaptoethanol) of the partially purified L-asparaginase from *Bacillus pumilus*, 1. Molecular weight marker proteins, 2. Crude preparation, 3. L-asparaginase after 70% ammonium sulphate precipitation, 4. L-asparaginase after DEAE column chromatography. (b) SDS-PAGE (with  $\beta$ -mercaptoethanol) of the partially purified L-asparaginase from *Bacillus pumilus*, i. Crude preparation, ii. L-asparaginase after 70% ammonium sulphate precipitation, iii. L-asparaginase after DEAE column chromatography, iv. Molecular weight marker proteins

#### Glutaminase activity

The specific activity of the purified enzyme for glutamine was observed to be 293.75 IU/mg and it was

3.5 times lesser as compared to the specific activity for asparaginase. The details are mentioned in Table 3.

Table 3: Glutaminase activity summary

Fraction	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)
DEAE cellulose chromatography	47.88	0.16	293.75

#### Discussion

L-asparaginase has been well documented to possess antilymphomic, anti-leukemic and antineoplastic activities. Organisms like *E.coli*, *Serratia marcescens*, *Erwinia carotovora*, *Pseudomonas acidovorans* and *P. geniculata* have been reported to produce L-asparaginase. The most important application of the

enzyme L-asparaginase is in the treatment of acute lymphoblastic leukemia, Hodgkin disease, chronic lymphocytic leukemia and melanosarcoma [16]. Hence, in the present study organism isolation from marine environment, having L-asparaginase activity was identified and optimized for maximum enzyme production. The isolate was identified as *Bacillus pumilus* and its presence in a marine environment falls



in line with reports by Ebrahiminezhad et al.[1]; Rohban et al.[29]; and Olajuyigbe and Ajele[30]. The classical method (one variable at a time) employed for optimization of nutritional parameters reported 0.1% asparagine and 2% galactose as potent substrate for enzyme production. This falls in line with reports that L-asparaginase production was enhanced by asparagine in *S. collins*, *S. karnatakensis*, *S. venezuelae* [12], *Actinomyces* [31], and *Bacillus cereus* [14].

Purification of *Bacillus pumilus* L-asparaginase was achieved by using 70% ammonium sulphate saturation and DEAE cellulose column, respectively. The enzyme was purified 1.5 times while its specific activity increased from 715 IU/mg to 1044 IU/mg for the crude extract and the final preparation respectively. This specific activity of the purified enzyme was around half of the specific activity of L-asparaginase from *Pseudomonas aeruginosa* 50071 [8] and *Corynebacterium glutamicum*[32], while it is significantly higher as compared to L-asparaginase from *Erwinia carotovora*[33], *Pseudomonas* 7A [34] and *Pseudomonas stutzeri*[35]. The final preparation was examined using SDS-PAGE (with and without  $\beta$ -mercaptoethanol), which revealed that its molecular mass was 136 kDa and that of its subunit was 71 kDa, respectively. This indicates that the enzyme is either active dimer or tetramer (dimer of intimate dimers). In this respect, the enzyme was higher than that obtained from *Corynebacterium glutamicum* (Mesas et al. 1990) with molecular weight of 80 kDa, while it matches the molecular mass of different native Asparaginases which ranges from 140- 150 kDa and also with *E. coli* L-Asparaginase which is 133-144 kDa [20]. Apart from this, the enzyme was also examined for its side glutaminase activity. The specific activity of the enzyme L-asparaginase for glutamine was 293.75 IU/ml which was 3.5 times lower than that for asparagine. High glutaminase activity (> 10%) was observed, as also seen in L-Asparaginase from *Acinetobacter glutaminasificans*, *Pseudomonas* 7A and *Erwinia chrysanthemi* strains [36].

The future prospects include enzyme kinetics, stability studies, amino acid sequencing and determination of the active sites will enhance the knowledge about its mechanism of action in treatment of ALL. The results are encouraging for further production and purification of L-asparaginase from *Bacillus pumilus*. Due to the applications of this enzyme in medical fields, its production from *Bacillus pumilus* could be attempted in pilot scale for its utility in pharmaceutical and food industry.

## Conclusion

In conclusion, the marine isolate *Bacillus pumilus* was identified as a promising candidate for L-asparaginase production and it showed significant enzyme production in nutrition medium supplemented with 0.1% L-asparagine and 2% galactose. The purified L-asparaginase had molecular weight identical to the enzyme extracted from *E. coli*. It is highly recommended that the future prospects of this enzyme should be explored for its utility in pharmaceutical and food industry.

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