World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Avoidable childhood death: Exploring the potential of marine bioactive products of halophilic bacteria in cancer treatment

Pallavi Rudrapati and Amrutha V. Audipudi*

Department of Microbiology, Acharya Nagarjuna University, Nagarjuna Naga, A.P. India

Received: 31-12-2014 / Revised: 15-01-2015 / Accepted: 24-01-2015

ABSTRACT

Enzymes are proteins that catalyze (*i.e.*, increase the rates of) chemical reactions. The manufacture of an enzyme for use as a drug is an important facet of today's pharmaceutical industry. Microbial L-Asparaginase (L-Asparaginase amido hydrolase) has been widely used as a therapeutic agent in the treatment of certain human cancers, mainly in acute lymphoblastic leukemia. Halophilic bacteria can be grown in minimal media with metabolic rates and growth yields comparable to those of mesophilic bacteria. L-asparaginase perform essential role in the treatment of acute lymphoblastic leukemia, lymphosarcoma and in many other clinical experiments relating to tumor therapy in combination with chemotherapy. Bacterial isolates were isolated from soil samples collected from different regions of the Nizampatnam mangrove, using nutrient agar medium by serial dilution method. The inoculated agar plates were incubated at 37˚C for 24-48 hours. It was found out that among the forty six isolates nine showed significant production of L-Asparaginase. One IU of L-Asparaginase is the amount of enzyme which liberates 1µmol of ammonia per minute per ml [µmole/ml/min]. From this work we conclude that more than 80% of the bacterial strains from marine soil sample had the ability to produce the enzyme L-Asparaginase. In the present study one of the potential *Pseudomonas* strain was selected and identified as *Bacillus cereus*AVP12 by 16s rRNA partial sequence. Effect of pH, temperature, carbon source, salt tolerance including various inducers and enhancers was studied for growth optimization and maximum production of enzyme.

Keywords: L- Asparaginase, Halophilic bacteria, Lymphoblastic leukemia, 16S rRNA partial sequence, *Bacillus cereus*, Solid state fermentation.

INTRODUCTION

Many enzymes have been used as drugs like for the treatment of especially Acute Lymphoblastic Leukemia (ALL) and Lymphosarcoma Cancer Cells [1]. Most of the normal tissue synthesizes L-asparagine in amounts for their metabolic needs but the Cancer or Cells (especially Malignant and Carcinoma Cell) require external source of L-asparaginase for their growth and multiplication [2]. In the presence of LA, the tumor cells deprived of an important growth factor and they may failure to survive. Thus this enzyme can be used as a chemotherapeutic agent for the treatment of ALL (mainly in children) as a potent antitumor or anti-leukematic drug [3]. L-asparaginase is used as a chemotherapeutic agent for acute lymphocytic leukemia and less frequently for acute myeloblastic leukemia, chronic lymphocytic leukemia, Hodgkin's disease, melanosarcoma and non-Hodgkin's lymphoma[4].Various bacteria such as, *E. coli* , *Erwinia aroideae*, *Proteus vulgaris*, *Streptomyces griseus*, *Vibrio succinogenes*, *Citrobacter freundi*, *Thermus aquaticus*, *Enterobacter aerogenes*, *Thermus thermophilus*, *Zymomonas mobilis*, *Pseudomonas aeruginosa* found to produce L-asparaginase[5,6,7,8,9,10]. The marine environment represents a relatively unexplored environment for microbial cultivation and subsequent drug discovery. Prokaryotes have been evolving over the past 3.5 billion years, which is presumed to have resulted in exceptionally high levels of genetic and phenotypic diversity[11].With the great diversity of bacteria and with genes adapted to a wide range of habitats (from deep sea trenches, to hydrothermal vents, to various marine invertebrates), a wide variety of unique secondary metabolites may be produced which can significantly increase the diversity of natural product screening libraries[12].

Corresponding Author Address:** Dr. A. Amrutha Valli, Assistant professor, Acharya Nagarjuna University, Guntur-522 210 Andhra Pradesh, India; **E-mail: audipudi_amrita@yahoo.com

Furthermore, by finding a microbial producer of bio medically important compounds, fermentation technologies can provide a continuous and sustainable source of supply to meet the demands required for human clinical trials. As prokaryotes from unique marine habitats have largely been unexplored with respect to their biomedical potential, this demonstrates that marine prokaryotes offer a wealth of diversity remaining to be exploited in clinical applications.

The microbial sources are very common for L-asparaginase, because they can be easily cultured and extraction purification of L-asparaginase from them is also convenient, facilitating for the Industrial scale production. The most commonly used microorganism to produce L-asparaginase are *Erwinia caratovora, Bacillus sp. Corynebacterium glutamicum, Pseudomonas stutzeri and E. coli*[6].L-asparginases has also been studied from marine bacteria[13] which are considered to be an important source of bioactive enzymes [14].Marine bacteria have halophilic in nature, can be used in industrially and pharmaceutically. We need new asparaginase with new immunological characteristics. Microorganisms inhabiting these environments are expected to have proteins with different features than proteins of non-saline environment organisms. They have halophilic enzymes with modified structure that creates tolerance of high salt concentration and low water activity. Therefore, Halophilic bacteria may contain L-asparaginase with novel immunological properties that can be used in hypersensitive patients.

Due to the hazards of chemotherapeutic drugs and its painful effects, L-asparaginase is emerging as safer source of anticancer enzymes [15].Genetic studies and enzyme purification studies has always been simpler with regards to bacteria hence bacteria are also preferred source for L-asparaginase production. Microbial Asparaginases have been particularly studied for their application as therapeutic agents in the treatment of certain types of human cancer [16].Study so far reports some of the fungal L-asparaginase is allergic and therefore there is a considerable need to find alternative bacterial L-asparaginase.

This treatment brought a major breakthrough in modern oncology, as it induces complete remission in over 90% of children within 4 weeks. With the development of its new functions, a great demand for L-asparaginase is expected in the coming years. Recent studies have indicated that the expression rate of L-asparaginase gene is very low or slow and their demands are ever increasing hence there is always short supply to Pharmaceutical Company. As production rate is low, the cost of enzyme is very high and is not available to many of the patients. The enzyme has received much attention, since it was reported as a potent anticancer agent against various types of tumor cell lines [17].

The aim of this work is to produce an immunologically and enzymologically compactable new type of L-asparaginase. This can be done by identifying new strain by means of screening from soil and optimization of various production parameters. Identification of low-cost natural substrate and optimization of other media components and process conditions are a significant step for cost efficient enzyme production.

ENZYMES IN MEDICINE

Since the mid-1950s there has been a considerable increase in both measurement of enzyme activities and the use of purified enzymes in clinical practice. In recent years, many enzymes have been isolated and purified, and this made it possible to use enzymes to determine the concentration of substrates and products of clinical importance. A further development, arising from the increased availability of purified enzymes, has been targeted to enzyme therapy. Commercial production of L-asparaginase appeared desirable only after Mashburn and Wriston showed that L-asparaginase from *E. coli* inhibits tumors in mice [18].

MATERIALS AND METHODS

Sample collection: Marine soil samples were aseptically collected from marine sediments of Nizampatnam, Guntur, A.P, India. In a sterile container for the isolation of L-Asparaginase producing organisms under laboratory conditions.

Pre-treatment of Soil Samples: The samples were dried at room temperature and processed by removing stones, leaves, roots and sand particles. Samples were then powdered by grinding soil in mortar and pestle and finally filtered through sieve.

Isolation of Bacteria: Isolation of bacteria were performed by the serial dilution technique[19]using nutrient agar medium (Peptone, 5.0 g; Beef-extract, 3.0 g; Sodium chloride, 5.0 g; agar-agar, 20.0 g per liter of distilled water). The sterilization of the media components were carried out in an autoclave at 121°C at 15 lbs. pressure for 30 minutes. Approximately 20 ml of media was poured into pre - sterilized petridishes and allowed to solidify. The sample (1g) was serially diluted up to 10^{-6} dilutions. A 0.1 ml of this dilution was aseptically spread over the surface of poured petridishes having nutrient agar medium. The plates were then incubated at 37°**C** for 48 hours. A colony appeared

with characteristics of bacterial morphology was isolated and purified using nutrient agar medium. After purification, all the isolated bacteria were maintained on nutrient agar slants and stored at 4°C for further use.

Screening of L-asparaginase activity on solid medium: All isolates were screened for L-asparaginase activity using modified - M9 medium [20] following tubed agar method with certain modification. The medium contained Na2HPO4.2H2O, 6.0 g; KH2PO4, 3.0 g; NaCl, 0.5 g; L-asparagine, 10.0 g; 1moll-1 MgSO4.7H2O, 2.0 ml; 0.1 M solution of CaCl2.2H2O, 1.0 ml; 20% glucose stock, 10.0 ml; agar 20.0 g. per liter of distilled water. The medium was supplemented with 0.005% phenol red dye (prepared in ethanol) and the pH was adjusted to 6.2 using 1N HCl. 9 ml of medium was poured in each tube and were sterilized. The tubes were inoculated with test organisms by using one loopful culture of bacteria and incubated at 37°C for 5 days. A set of tubes were also run as control without L-asparagine. The modified - M9 medium contains 1% L-asparagine as sole source of nitrogen. L-asparaginases hydrolyses L-asparagine into L-aspartic acid and ammonia. This can be easily detected by the change in the pH of the medium due to production of ammonia. The color change of the medium (from yellow to pink) indicates positive L-asparaginase production.

Production of L-asparaginase by Submerged Fermentation: Nine bacterial strains which showed excellent activity during primary screening were further tested for production of L-asparaginase in broth cultures. For this spore suspension of each test bacteria was prepared in modified - M9 broth. The flask having 50 ml broth were inoculated with spore suspension after autoclaving and incubated at 150 rpm for 72 hours at 37°C. Uninoculated medium was served as control. At the end of incubation, culture filtrates were obtained by centrifugation at 8000 rpm for 15 min. The supernatant was then used as crude extract for L-asparaginase activity and stored at 4°C for further use. The production of L-Asparaginase was studied at different inducers and enhancers like pH ranges, temperature, Salinity concentrations, Carbon sources, Amino acids, Nitrogen sources, Phosphate sources, Metal /Mineral salts

Production of L-asparaginase by Solid- state fermentation (SSF): Six different bioprocess residues were tried as substrates for L-asparaginase production. The substrates include coconut oil cake, coffee, Green tea, groundnut oil cake, sesame oil cake, Red gram. Solid-state fermentation was carried out in a 250 ml Erlenmeyer flask containing 5.0 g groundnut cake as a substrate, moistened with 10 ml of mineral salt solution containing (g/l): $Na_2HPO_4.2H_2O$, 6.0; $KH_2PO_4.7H_2O$, 3; 2ml of 1M $MgSO_4.7H_2O$; 1.0 ml of 0.1M CaCl2.2H2O; 1.0ml of 20% glucose stock; corresponding to 60% moisture level and at pH 6.0 then sterilized at 121^0C for 15min, cooled to desirable temperature, addition of 2.0 ml (2x106 cells/ml) inoculum and incubated for 48h at 37^0C. The enzyme was extracted at the end of the fermentation period by the addition of 90ml of 0.01 M phosphate buffer of pH 7.2 to the fermented medium, shaking for 15 min followed by centrifugation at 8,000 rpm for 20min. The cell free supernatant was used for the estimation of enzyme.

Assay of L-Asparaginase: Assay of enzyme was carried out [21].In this assay, the rate of hydrolysis of L-asparagine was determined by measuring the ammonia released using Nessler's reaction. A mixture of 0.5 ml of 0.04 M asparagine was taken in a test tube, to which 0.5 ml of 0.5 M buffer acetate buffer pH 5.4), 0.5 ml of enzyme and 0.5 ml of distilled water was added to make up the volume up to 2.0 ml and incubate the reaction mixture for 30 min. After the incubation period, the reaction was stopped by adding 0.5 ml of 1.5 M TCA (Trichloroacetic acid). 0.1 ml was taken from the above reaction mixture and added to 3.7 ml distilled water and to that 0.2 ml Nessler's reagent was added and incubated for 15 to 20 min. The OD was measured at 450 nm. The blank was run by adding enzyme preparation after the addition of TCA. One U of L-asparaginase is the amount of enzyme which liberates 1 µ mole of ammonia per minute per ml [µmole/ml/min].

Molecular Identification of the organism: Pure culture of AVP 12 bacterial isolate was grown until log phase achieved and genomic DNA was isolated essentially[22].The amplification of 16S rRNA gene was done by using universal bacterial primer 1492R (5´-TACGGYTACCTTGTTACGACTT-3´) and 27F (5´ AGAGTTTGATCMTGGCTC AG- 3´) as per the conditions[23].

DNA Isolation and Amplification Of 16S RNA Gene of *Bacillus cereus* AVP 12genus By Polymerase Chain Reaction (PCR): In polymerase chain reaction, the specific primers forward and reverse were used to amplify the genomic sequence of the open reading frame (ORF) of the gene. PCR condition were 94˚C for 2 min, and the 94˚C for 1 min, 60˚C for 1 min, 72˚C for 3 min, a total of 30 cycles, with the extension at 72˚C for 10 min[24].The PCR product was sequenced at Macrogen South Korea.

Phylogenetic Placement and Gen Bank Accession Number: The sequences obtained were compared with those from the GenBank using the BLAST program[25]and Phylogenetic trees reconstructions were obtained by the Neighbor joining method 1000 bootstrap replicates were performed to assess the statistical support for each branch in the tree [25,26].

Statistical analysis: Analysis of variance (ANOVA) and LSD test were carried out for the obtained results according to Steel and Torrie (1980) [27].

RESULTS AND DISCUSSION

Bacterial strains were isolated from soil samples collected from 10 ft depth of Nizampatnam marine soil sediment of Guntur district. The soil samples were serially diluted plated. The isolated strains were inoculated on the medium and pink color colonies were taken as L-asparaginase producing microorganisms. The organisms were isolated and were sub cultured for further works. Among these only the bacterial strain AVP12 was taken for maximum production of L-asparaginase activity was taken for molecular characterization.

Production of L-asparaginase by Submerged Fermentation: Production of L-asparaginase was found to be maximum at 37^0c (1.25IU/ml) and varies with incubation period at 37^0c indicating optimization of incubating period also exhibit a significant role (Figure 1). The significance of the incubation temperature in the development of sub merged fermentation process is such that it could determine the effects of inhibition, cell viability and death. However, the enzyme production reduced gradually with further increase in incubation temperature. This may be due to heat that accumulates in the medium, because of poor heat dissipation which could lead to a further drop in the oxygen level and thereby reducing the growth of the test organism. Sarqius et al., (2004) have reported 30°C is suitable for L-asparaginase production through submerged fermentation by using *A. terreus* and *A. tamari* [28]. K.G. Siddalingeshwara (2010) reported optimized temperature as 30^OC by *Emericella nidulans* **[29].** Yogendrasingh et al., (2012) observed the maximum activity at 30ºC by *Bacillus aryabhattai* strain ITBHU02 [30].

Enzyme production of AVP 12 showed variation at different pH. L-asparaginase production was found to be maximum (19.85 IU/ml) at pH 10 and observed to be gradually decreased beyond pH 12(Figure 2) Growth and metabolism along with enzyme production is governed by an important factor called pH. Different organisms have different pH optima and any modification in their pH optima could result in a decrease in their enzyme activity. Experiments were carried out to find the optimum pH in order to maintain the favourable conditions for increased L-asparaginase production. This was established by carrying out the fermentation by varying the pH from 5-14. G.Thirumurugan et al., 2011 reported an optimum asparaginase production at pH 8.0 by *Aspergillus terreus* [31].Selvakumar 2011 observed peak activity of asparaginase at pH 8.0 by *Streptomyces noursei* MTCC 10469[32].

Percentage of NaCl concentration also effect the L-asparaginase production of AVP 12.The production was inversely related with increasing concentration of NaCl and found to be maximum(21.32 IU/ml) at 0.5% concentration(Figure 3). Isolate AVP 12 showed maximum L-asparaginase production with Starch (145.32 IU/ml) (Figure 4). To determine the effect of carbon sources on L-asparaginase yield, different carbon sources were tested which include21 sugars. Each of them at a concentration of 0.5% w/v with other optimized conditions was supplemented to the production medium of AVP 12 and they have exerted a considerable effect on the biosynthesis of L-asparaginase. Baskar and Renganathan (2011) reported that glucose was found to be best carbon source for maximum L-asparaginase production using modified Czapek-dox media containing soya bean flour as substrate by *Aspergillus terrus* MTCC 1782[33].

AVP12 found to be produce maximum enzyme production in Yeast extract as principle nitrogen source (82.32 IU/ml) (Figure 6). The supplementation of 11nitrogen sources to the production medium had shown a profound impact on the production of L-asparaginase by AVP12.Gaffar and Shethna, (1977) observed the positive effect of supplementation of ammonium sulphate in the production of L-asparaginase [34]. Sreenivasulu et al., (2009) have reported ammonium sulphate exhibited maximum enzyme production by the isolated fungus VS-26[35].

Amino acid Tyrosine (79.91 IU/ml), Mineral source Aluminium sulphate (87.18 IU/ml) and Phosphate source Sodium dihydrogen phosphate (79.55 IU/ml) were observed to be potential inducers for L-asparaginase production (Figure 5,8 and 7). After optimization Starch, Yeast extract, Tyrosine, Aluminium sulphate and Sodium dihydrogen phosphate were selected as potential inducers and enhancers. An attempt was made to evaluate the extent of improvement in production of l-asparaginase in the modified formulated production media with necessary inducers and

enhancers. 5 folds of enhancement in L-asparaginase production observed with modified production medium indicating highly significant improvement so far observed.

Production of L-asparaginase by Solid- state fermentation (SSF): In present study an attempt was made for maximum production of l-asparaginase with 6 different solid substrates of different composition of carbon and energy sources. Out of the six substrates tested for their suitability to support SSF production of L-asparaginase, Red gram husk appeared to be the best substrate supporting maximum enzyme activity of 259.19 IU after 5 days of incubation. Groundnut oil cake supported 139.7 IU, Coconut oil cake supported 134.55 IU, Green tea supported 212.86 IU, Coffee 143.38 IU/ml, Sesame oil cake supported 137.5 IU activity while coffee supported 143.38 IU for L-asparaginase activity (Figure 9). As maximum activity was seen using Red gram husk therefore, Red gram husk might be the potential solid substrate for bioprocessing. All the substrates promoted enzyme production with Bacillus cereus AVP12.The maximum L-asparaginase activity of 259.19IU/ml was achieved in a medium containing red gram husk as the substrate followed by green tea and lowest activity of 134.55IU/ml was observed in case of coconut oil cake. Abha Mishra et al., 2006 reported production of L-asparaginase from *Aspergillus Niger* using agricultural substrates like bran of Cajanus Cajan, Phaseolus mungo and Glycine max [36]. Hymavathi et al., 2009 reported asparaginase production by isolated *Bacillus circulans* MTCC 8752 under solid state fermentation using different agricultural materials like red gram husk, Bengal gram husk, coconut, and groundnut cake [37].

Molecular Identification of the organism: The bacterial strain AVP 12 was classified to be *Bacillus sp.* A 1466 bp PCR product of gene was amplified from the genomic DNA of AVP 12 .A sequence similarity showed that the 16srDNA gene sequence of AVP 12 had 99% similarity to the 16srDNA of *Bacillus cereus* strain and *Bacillus* species PPB2(AC:HM771657).The sequence was blast in NCBI and for analysis.

Phylogenetic Placement and Gen Bank Accession Numbers: Based on phylogenetic analysis revealed that AVP 12 was closely related to *Bacillus cereus* (Figure 10) and sequence was deposited in NCBI as *Bacillus cereus* AVP 12 with accession number KF527826.

The selected AVP12 bacterial strain with anticancer properties was found to be with L-asparaginase activity, an essential tumor controlling enzyme. Our results supported the hypothesis that the marine bacterial strains with medicinal properties.

CONCLUSION

Increasing global warming, malnutrition, and various environmental insults continue to increase the incidences of cancer. According to the American Cancer Society, the global burden is expected to grow as 27 million new cancer cases and 17.5 million cancer deaths simply due to the growth and aging of the population by 2050. Natural derivatives play an important role to prevent the cancer incidences as synthetic drug formulations cause various harmful side effects to human beings. Marine floras are potential source of anticancer compounds, but they are least explored .Of the anticancer compounds extracted so far, the marine algae contribute 65.63%, the mangroves 28.12% and the bacteria 6.25%.Owing to a diverse chemical ecology, the marine organisms especially marine flora have a great promise for providing potent, cheaper and safer anticancer drugs, which deserve an extensive investigation. Mangroves from the southwest coast of India were studied for the first time for bioactivity. From the preliminary screening, we have identified mangrove soil with pronounced biological activities. Nature has supplied several active anticancer agents which have significantly improved the management of many types of human cancers. The challenge of identifying new anticancer agents in the oceans has been taken up by a group of scientists who have formed a worldwide collaboration to investigate the organisms found on These living organisms represent a rich reservoir of genetic and metabolic diversity, which is ready to be exploited and which will certainly make anticancer drug discovery even more challenging in the next few years.

Conflict of interest: The authors have declared no conflict of interest.

Compliance with ethics requirements: This article does not contain any studies with human or animal subjects.

Acknowledgement: Authors are grateful to University Grants Commission–Special Assistance Propgramme-Basic Science Research New Delhi India for sanctioning Junior Research Fellowship to Pallavi Rudrapati, Department of Microbiology Acharya Nagarjuna University Guntur Andhra Pradesh INDIA.

Production of L-asparaginase by Sub-merged fermentation

Figure 1

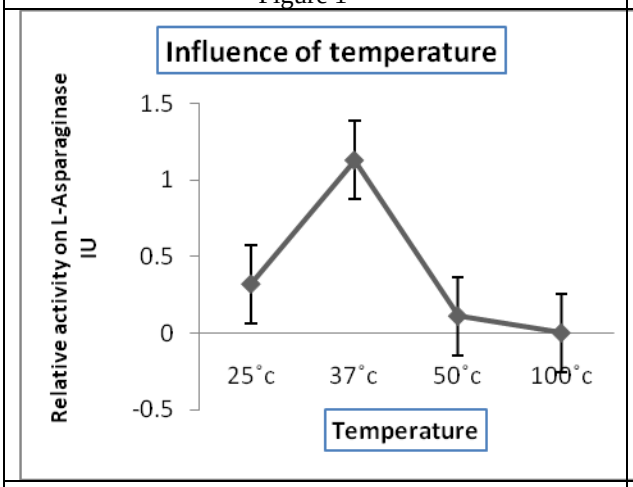


Figure 2

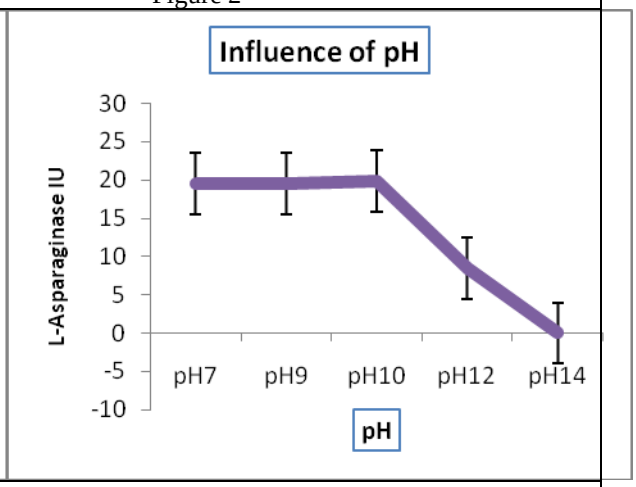


Figure 3

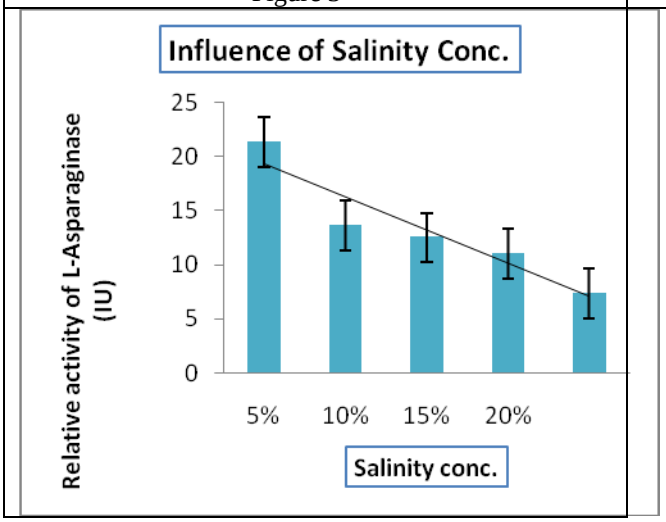


Figure 4:Influence of Carbon sources on L-Asparaginase enzyme

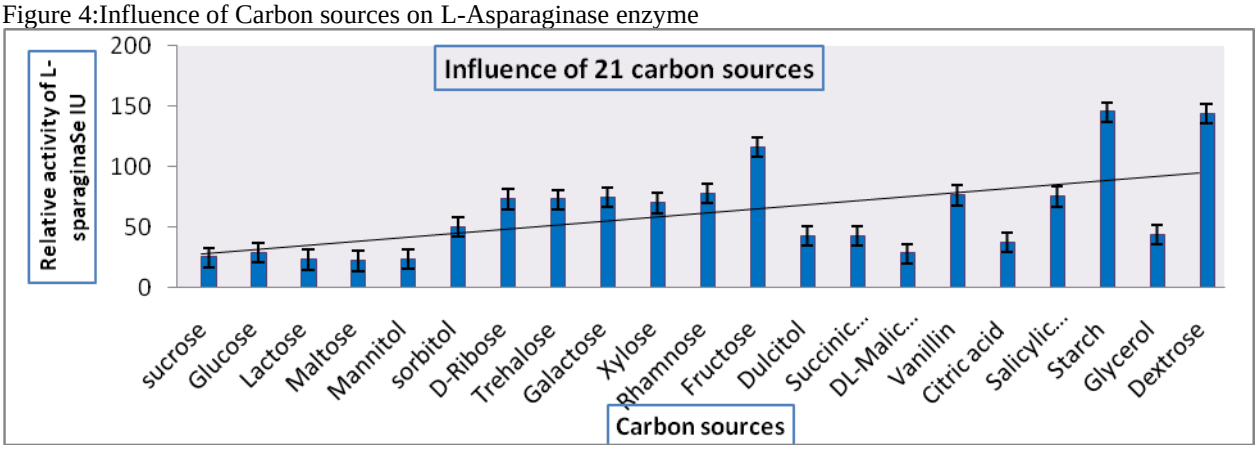


Figure 5:Influence of Amino acids on L-Asparaginase enzyme

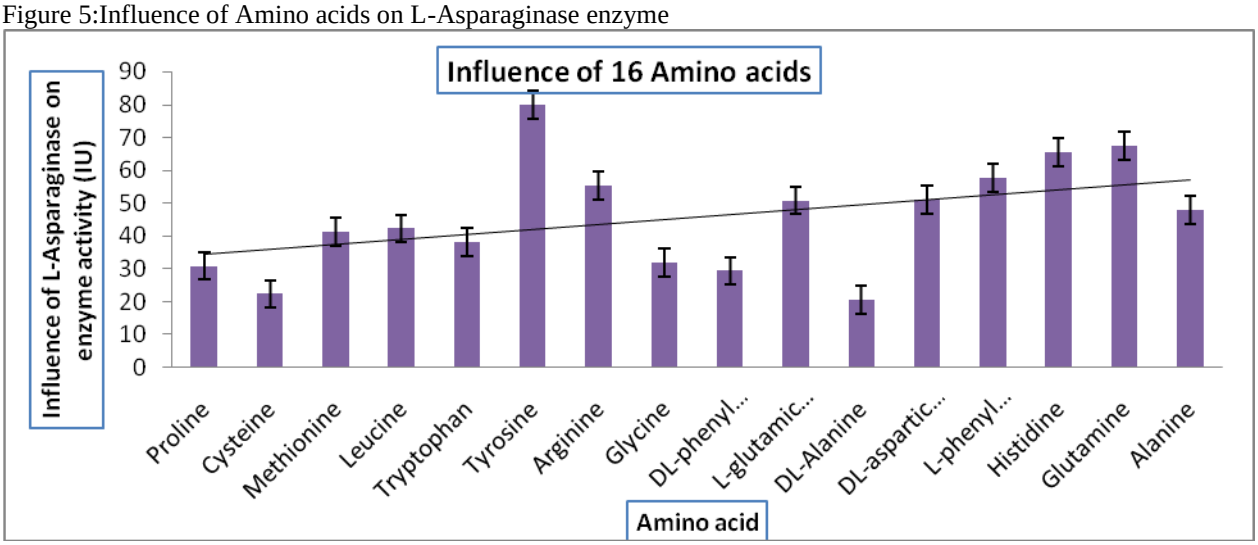


Figure 6: Influence of Nitrogen sources on L-Asparaginase enzyme

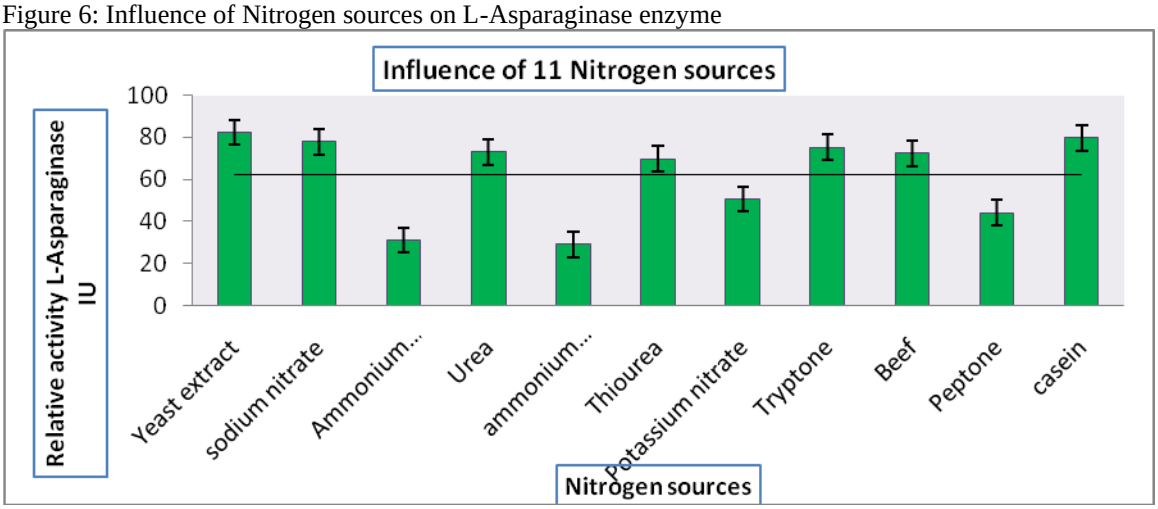


Figure 7:Influence of Phosphate sources on L-Asparaginase enzyme

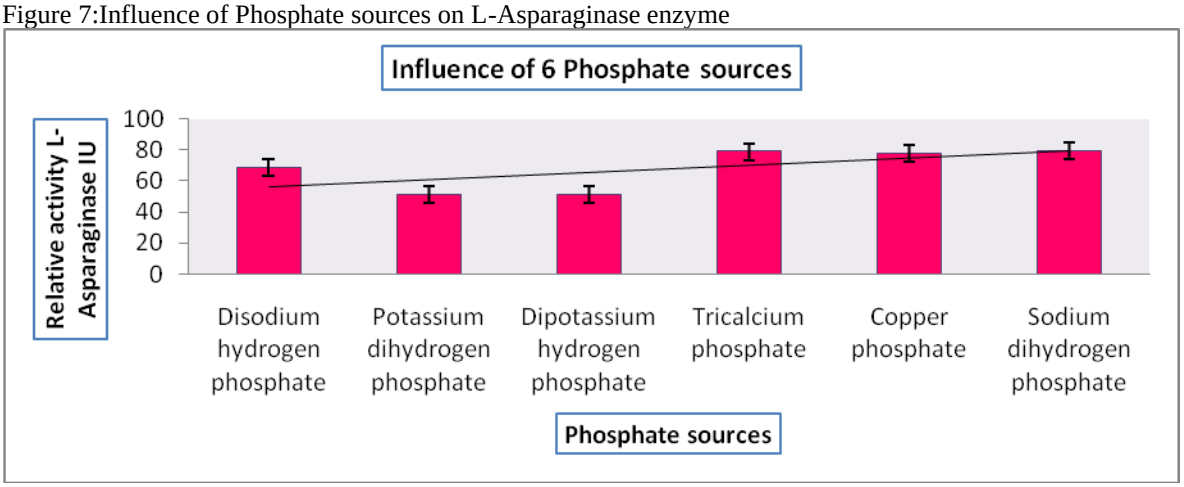


Figure 8: Influence of Mineral sources/Metal salts on L-Asparaginase enzyme

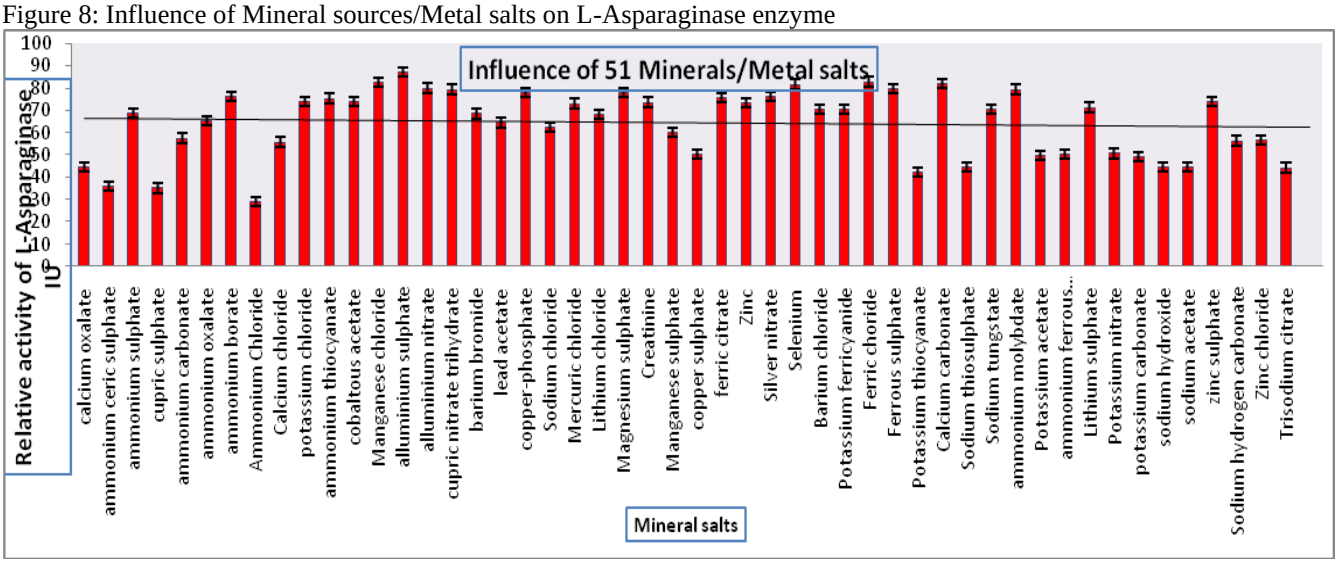


Figure 9: Production of L-asparaginase by Solid- state fermentation

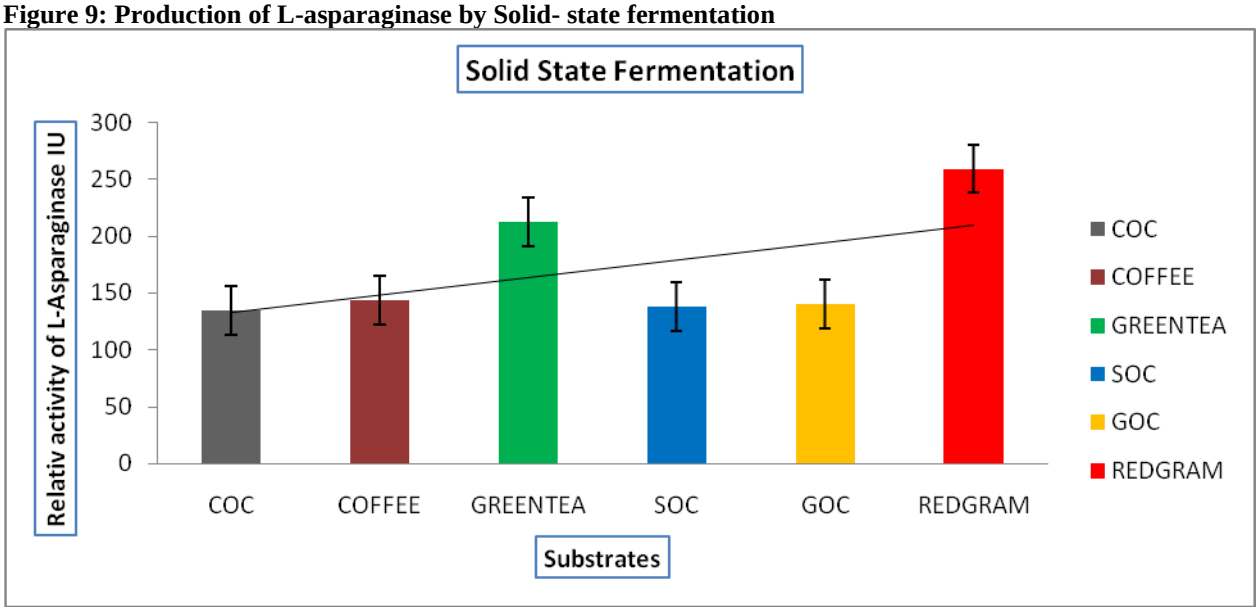
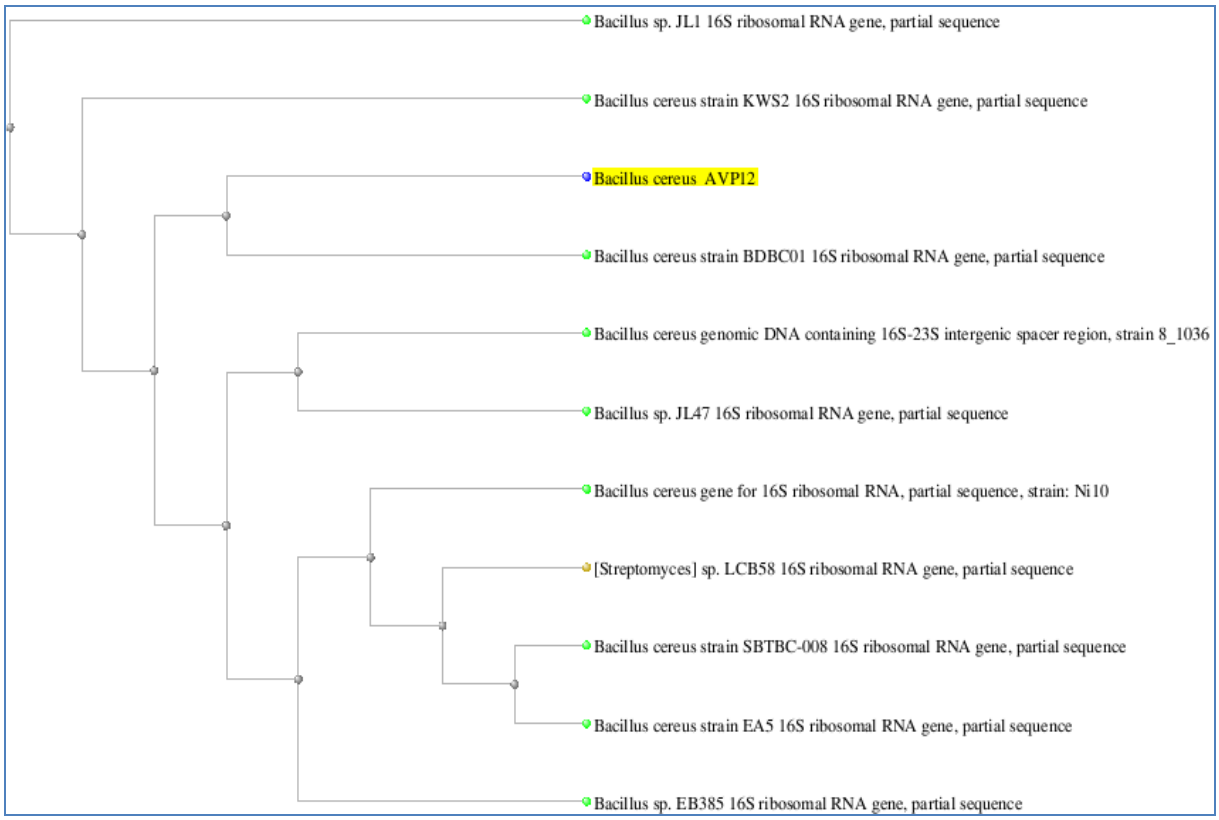


Figure 10: Phylogenetic tree showing the placement of the *Bacillus cereus* AVP 12 Marine soil isolate.



REFERENCES

1. Head DR and Behm FG. Acute lymphoblastic leukemia and the lymphoblastic lymphomas of childhood. Semin Diagn Pathol.1995; 12(4):325-34.
2. Broome JD. L-asparaginase EC-II from Escherichia coli. Some substrate specificity characteristics. Biochemistry 8.1963; 3766-3772.
3. Nachman JB et al. Augmented post-induction therapy for children with high-risk acute lymphoblastic leukemia and a slow response to initial therapy. N Engl J Med.1998; 338: 1663- 1671.
4. Baskar G and RenganathanS. Indian Journal of Science and Technology.2009;**2, (**5): 45-48.
5. Pritsa A.A et al. Anticancer Drugs.2001; 12: 137-142.
6. Howard C andSchwartzJ.H.J.Bacteriol.1968; 96: 2043-2048.
7. Peterson RE and Ciegler A. Appl. Microbiol.1969; 18: 64- 67.
8. TosaT et al.Appl. Microbiol.1971; 22: 387-392.
9. Dejong. Appl. Microbiol.1972; 23:1163- 1164.
10. PinheiroIO*et al*.Biomaterial and Diagnostic, 2001; **6**: 243-244.
11. Sogin ML et al.Microbial Diversity in the Deep Sea and the Underexplored rare Biosphere- Proceedings of the National Academy of Sciences 2006; 103.32:12115-2120.
12. Harvey AL et al. Strategies for discovering drugs from previously unexplored natural products. Drug Dis. Today 5,2000; 294–300
13. Benny PJ and Kurup GM. L-asparaginase activity in bacteria from estuarine sediments and mollusks. Indian Journal of Marine Sci.1991; 20:36-39.
14. Williams ST and Vickers JC.The ecology of antibiotic production, Microbiol. Ecol.1986; 12:43–52.
15. KambleKD and Khade PJ. Studies on antineoplastic enzyme producing bacteria from soil, Int J Pharm Biomed Res. 2012; 3(2): 94-99
16. Gallagher MP et al. L-asparaginase a drug for treatment of acute lymphoblastic leukemia. Essays Biochem. Vol. 24, 1989; pp:1-40.
17. Tan Y et al. Polyethylene glycol conjugation of recombinant methioninase for cancer therapy. Protein ExprPurif 1998; 12: 45-52.
18. Mashburn LT and Wriston JC .Tumor inhibitory effect of L-asparaginase from *Escherichia coli*. Arch BiochemBiophys.1964; 105: 450-452.
19. Aneja KR. Experiments in Microbiology Plant Pathology and Biotechnology, Fourth Edition, New Age International (P) Ltd.Publishers, New Delhi. 2003; pp 320.
20. Gulati R et al. A rapid plate assay for screening L-asparaginase producing microorganisms. LettApplMicrobiol 1997; 24: 23-26.
21. Mashburn LT et al. Tumor Inhibitory Effect of L-asparaginase from Escherichia coli. Archives of Biochemistry and Biophysics, 1964; 105:450-452.
22. Bazzicalupo M and Fani R. Humana Press Inc. Totowa NJ. 1995; 112-124.

23. Pandey P and Kang SC. CurrSci. 2005; 89(1):177-180.
24. Volosiouk T *et al*. Direct DNA extraction for PCR- Mediated assay of soil organisms. Appl. Environ microbial.1995; 61: 3972-3976.
25. Astchul SF.J MolBiol.1990; 215:403-410.
26. Tamura K. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution.2007; 24: 1596-1599.
27. Steel R.G.B and Torrie J.H.Principles and Procedures of Statistics, second ed. Mc-Graw-Hill. Inc.1980; p. 633.
28. Sarquis MI et al.ManInstOswaldo Cruz.2004; 99(5):489
29. Siddalingeshwara KG and LingappaK. An International Journal of Pharmaceutical Sciences.2010; 1(1):103.
30. Thirumurugan G et al. Effect of Inducers and Physical Parameters on the Production of L-Asparaginase Using *AspergillusTerreus*. J Bioprocess Biotechniq.2011; 1: 110 doi:10.4172/2155-9821.1000110.
31. SelvakumarDharmaraj. Study of L-asparaginase production by *Streptomyces noursei* MTCC 10469, isolated from marine sponge *Callyspongiadiffusa*. Iranian Journal of Biotechnology.2011; Vol. 9, No. 2: 102-108.
32. Baskar G and Renganathan S. Design of experiments and artificial neural network linked genetic algorithm for modelling and optimization of L-asparaginase production by *Aspergillusterreus* MTCC 1782. Biotechnology and Bioprocess Engineering. 2011; 16: 50-58.
33. Gaffar SA and Shethna YI. Purification and Some Biological Properties of Asparaginase from Azotobactervinelandii. Appl Environ Microbiol.1977; 33(3): 508-514.
34. Sreenivasulu Vet al.Optimization of process parameters for the production of Lasparaginase from an isolated fungus. Research J. Pharmacognosy and Phytochemistry.2009;1(1):30-34.
35. Abha Mishra. Production of L-Asparaginase, an anticancer agent, from *Aspergillusniger* using agricultural waste in solid state fermentation. Applied Biochemistry and Biotechnology.2006; 135.
36. Hymavathi M et al. Enhancement of L-Asparaginase production by isolated *Bacillus circulans*(MTCC 8574) using response surface methodology. ApplBiochemBiotechnol.2009; 159(1):191-198.