University of Delhi
Innovation Project 2015-2016
Final report
SHC 312

"L-Asparaginase, an Antitumor Agent: Production, Characterization & Molecular Approaches"

Investigators from Shivaji college

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University of Delhi

Certificate of Originality

This is to certify that the research work carried out and the final report submitted by the Project Investigators and the students of Innovation Project having Project code **SHC 312** and title "**L-Asparaginase, an Antitumor Agent: Production, Characterization & Molecular Approaches"** of Shivaji College is original. Any plagiarism/academic dishonesty reported at any stage will be our responsibility.

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Utilization Certificate

Innovation Project 2015-16
SHC – 312

Project Title L-Asparaginase, An Anti Tumor Agent : Production, Characterization And Molecular Approaches

Audited Financial Statement under Innovation Project scheme

College: Shivaji College

Project Investigators: Dr. Rashmi Wardhan, Dr. Prabhavathi

Grant Sanctioned Rs	Rs. 7,00,000/- (Rupees Seven Lacs Only)		
	Grant Received	**Grant Utilized**	**Unspent Grant**
Equipments/Consumables	4,25,000/-	5,42,933/-	(1,17,933)*
Travel	55,000/-	NIL	55,000/-
Stipend	1,20,000/-	95,000/-	25,000/-
Honorarium	25,000/-	25,000/-	NIL
Stationery	20,000/-	2,930/-	17,070/-
Contingency	55,000/-	8,911/-	46,089/-
Total	**7,00,000/-**	**6,74,774/-**	**25,226/-**
Total amount utilized	Rs. 6,74,774/- (Rupees Six Lacs Seventy Four Thousand Seven Hundred Seventy Four Only)		
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Certified that out of **Rs. 7,00,000/- (Rupees Seven Lacs Only)** sanctioned to Innovation Project Code **SHC-312, Rs. 6,74,774/- (Rupees Six Lacs Seventy Four Thousand Seven Hundred Seventy Four Only)** has been utilized during the period of the project. The remaining amount **Rs. 25,226/- (Rupees Twenty Five Thousand Two Hundred Twenty Six Only**) and is being returned back to the University.

Note : Over expenditure under the head "Equipment/ Consumables has been met from balance in Travel, Stationery and Contingency with prior approval from the Innovation Des

(Dr. Rashmi Wardhan) **(Dr. Prabhavathi)**

Principal

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To Whomsoever It May Concern

Nov 01, 2016

This is to say that the innovation project, entitled ***"L-asparaginase, an antitumor agent: Production, Characterization & Molecular Approaches"*** **SHC 312** has been taken up by **Dr. Rashmi Wardhan, Dr V. Prabhavathi, Shivaji College, University of Delhi** as Principal Investigators along with their team of students. I have interacted and discussed the results with Principal Investigators and students working for this project. The work done in the project is satisfactory.

R. P. Singh

Abstract

L-Asparaginase (EC 3.5.1.1) belongs to a group of homologous amidohydrolases family, which catalyses the hydrolysis of the amino acid L-Asparagine to L-Aspartate and ammonia. L-Asparaginase enzyme is used to treat cancers like melanoma, lung cancer, renal cell carcinomas, acute Lymphoblastic Leukemia (ALL) and hepatocellular carcinomas. The reason for using L-Asparaginase enzyme for treatment is that the tumor cells have an unusually high requirement for amino acids like Arginine and Asparagine. The enzyme, Asparagine synthetase in healthy cells converts aspartate to asparagine by using ATP as energy source but tumor cells cannot synthesize sufficient endogenous L-Asparagine due to very low levels of L-Asparagine synthetase and therefore are dependent on serum levels of Asparagine for their proliferation and survival. Administration of L-Asparaginase to tumor cells deprive them from L Asparagines sources and lead to apoptosis. However, healthy cells escape unaffected as they are capable of synthesizing Asparagine de novo with the aid of the enzyme L-Asparagine synthetase.

Currently *Escherichia coli* and *Ervinia chrysanthami* L Asparaginase are being used to treat acute lymphoblastic leukemia but the limitation to biomedical application of this enzyme is its short life and instability for the processes of production and treatment and side effect of L- glutaminase activity in some cases.

In the present study we isolated colonies by serial dilution on differential media, screened them for L- Asparaginase enzyme presence in M9 media with 0.009% phenol red and 10% asparagine and then enzyme activity and specific activities for L- Asparaginase and L- Glutaminase was assayed in isolated colonies.

The best 10 samples showing high L- Asparaginase activity and low glutaminase activity were sent for 16SrRNA sequencing to Yaazh Xenomics, Madurai for identification. The sequence BLAST was done from NCBI and five microorganisms were identified after submitting these sequences to Gen bank. The data was analyzed statistically to select microorganism for having high L- Asparaginase activity and low L- Glutaminase activity for study. L Asparaginase Enzyme activity and specific activity from *Alcaligene faecalis* and *E.coli* HB101 are compared and had

shown similar level of activity because *Ervinia* and *E.coli* L Asparaginase is approved by FDA,US to use as clinical enzyme for treatment of leukemia.

Alcaligene faecalis L Asparaginase enzyme in this present study is confirmed to be of periplasmic type II. This organism has only one type of L Asparaginase enzyme gene, not two types. L Asparaginase enzyme isolated from various sources had limitation of its use because of high Km, temperature sensitivity and difficult time consuming purification procedure. The periplasmic fraction in present study has shown high specific activity, very low Km and easily purified from the protocol standardized for this study. L Asparaginase enzyme type II of *Alcaligene faecalis* is inducible by L Asparagine presence in the media. *Alcaligene faecalis* L Asparaginase has low Km in the range of 0.6 µM to 1.0 µM, which is important for the use of enzyme as medicine and has shown highest enzyme activity at pH 6.5, with optimum temperature 37^0C. L Asparaginase gene is amplified and inserted into pGEMT vector. purified, digested with RE XhoI and NdeI and then inserted into pET 22b vector. DH5a *E.coli* strain cells were transformed with pET22b plasmid having Alcaligene faecalis L Asparaginase gene. Now we are trying to express in Rossetta /BL21for further study.

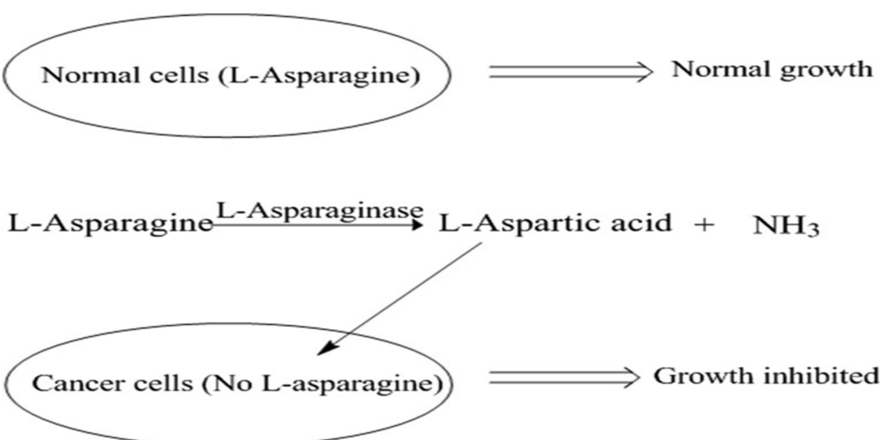
Introduction

L-Asparaginases (EC 3.5.1.1) belong to group of homologous amidohydrolase family, which catalyses the hydrolysis of L-asparagine into L-aspartate and ammonia and to lesser extent the hydrolysis of L-glutamine to L-glutamate (1,2,3)

Schematic illustration of the reaction mechanism of L Asparaginase. The proposed intermediate is formed through nucleophilic attack by the enzyme. Red arrows indicate nucleophilic attack.

L-Asparagine is a non-essential amino acid used by normal and cancer cells for their proliferation. The enzyme, human Asparagine synthetase converts Aspartate to Asparagine by using ATP as energy source. Tumor cells have an unusually high requirement for the amino acids like Arginine and Asparagine and cannot synthesize sufficient endogenous L-Asparagine due to very low levels of L-Asparagine synthetase present in tumor cell, so these Neoplastic or cancer cells depend on circulating sources. The L-Asparaginase administration deprives dependent tumor cells of their extracellular source of L-Asparagine and lead to apoptosis. For this reason, the commonest therapeutic practice is to inject the enzyme intravenously in order to decrease the blood concentration of l-asparagine, selectively affecting the neoplastic cells (4,5). However, healthy cells escape unaffected as they are capable of synthesizing Asparagine de novo with the aid of the enzyme L-Asparagine synthetase. L-Asparaginase enzyme has gained attention because of its potential in causing apoptosis in cancers like melanoma, lung cancer, renal cell carcinomas, Acute Lymphoblastic Leukemia (ALL) and hepatocellular carcinoma (6). Bacterial L Asparaginases

from *Escherichia coli* and *Erwinia chrysanthemi* have been extensively used as drugs for the treatment of acute lymphoblastic leukemia.



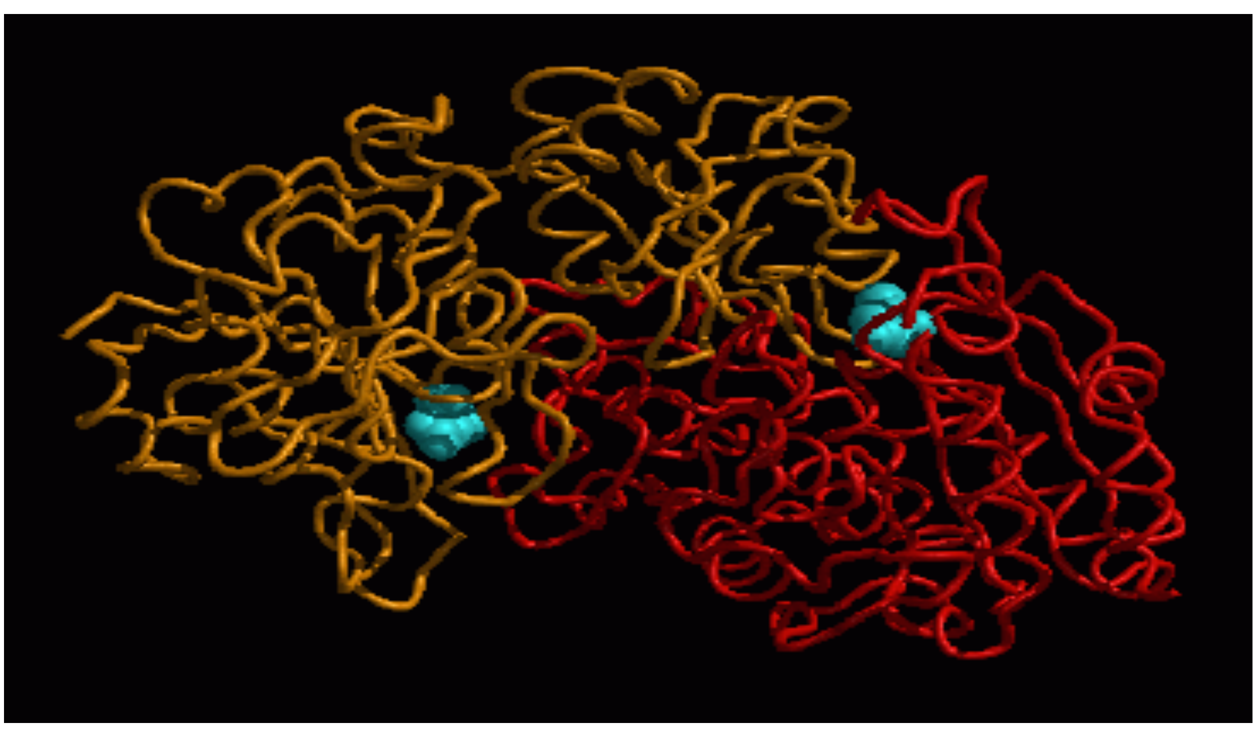
Schematic diagram of L-Asparaginase inhibiting growth of tumor cells

All Asparaginases are hometetramers with molecular mass in the range of 140 -150 kDa with a highly conserved overall fold(7,8,9 &10).Two types of bacterial L-Asparaginase have been identified and named type I and type II (11).Type I L Asparaginases are expressed in the cytoplasm and characterized by enzymatic activity for both L-Asparagine and L-Glutamine. Type II L Asparaginases are expressed under anaerobic conditions in the periplasmic space of the bacterial membranes with high specific activity against L-Asparagine. Each monomer consists of about 330 amino acid residues that form 14 β strands and 8 α helices, arranged into two easily identifiable domains, the larger N terminal domain and the smaller C terminal domain, connected by a linker consisting of ~20 residues. There are three gene sequences available for L-Asparaginase (L-Asparaginase, L-Asparaginase I and L-Asparaginase II) of *E. carotovora* subsp. atroseptica SCRI 1043 in NCBI gene bank (12). Type II L Asparaginases display antitumor activity and are used as chemotherapeutics in bacteria (13,14) . The L-Asparaginase enzyme is not very stable in blood plasma, which limits its use in treatment of leukemia. These enzymes along with their antitumor activity also have some side effects. The Glutaminase activity is basis of side effects such as serious **liver disorders, acute pancreatitis, thromboembolism, hyperglycemia,** acute **pancreatitis**, and **immunosuppression** (15). Although many microorganisms such as *Aerobacter, Bacillus*, *Erwinia*, *Pseudomonas*, *Serratia*, *Xanthomona*s, *Photobacterium* (16) , *Streptomyces*

(17) , *Proteus* (18), *Vibrio* and *Aspergillus* (19) all have a potential for L Asparaginase production. The purified enzyme from *E. coli* and *Erwinia sp*. has been reported as anti-tumor and anti-leukemia agent. *Erwinia* L-Asparaginase is reported to show less allergic responses compared to the *E. coli* L-Asparaginase but had a shorter half-life than E. coli Asparaginase (20). L-Asparaginase from bacterial origin can cause hypersensitivity in the long term used, leading to allergic reactions and anaphylaxis. The toxicity is partially attributable to the L glutaminase activity of these enzyme (21).

However, the L-Asparaginase produced by *E. coli* and *Erwinia* contains L Glutaminase activity albeit at low level that limits this enzyme usage, as administration of this enzyme causes side effects like hyper-triglyceridemia in acute lymphoblastic leukemia with 11q23 abnormality, hepatotoxicity, neurotoxicity and impairments in blood coagulation (22,23).

The crystal structure of L Asparaginase from *E.coli* and *Ervinia chrysanthemi* have been known active as tetra homodimer. The tetramer consists of a pair of dimers, each with an extensive dimer interface: it can be described as a dimer of "intimate dimers". Each "intimate dimer" contains two active sites, and each active site contains some residues from both monomers in the intimate dimer. The crystal structure of *E .coli* type I (EcA) contains four molecules of L-Aspartate. one bound into each active site. Two threonine residues - T12 and T89 in EcA - which are conserved throughout the L-Asparaginase family, and are known to be essential for activity,lie close to the bound aspartate. These threonine residues are both able to act as primary nucleophiles. They are both necessary for activity (24).



"Intimate dimer" of *E.coli* type I L Asparaginase (EcA) , showing L-aspartate (cyan, van der Waals representation) bound in each of the equivalent active sites.

L-Asparaginase is an injectable drug used for the treatment of tumors. The sensitivity of the application of this enzyme requires a high degree of purity. For purification secretory production of recombinant proteins in *E. coli* has been particularly useful for production of pharmaceutical Proteins (25,26). The periplasmic enzyme is preferred for protein expression because of significantly fewer proteins, particularly proteases, compared to the cytoplasm, having network of redox enzymes for the formation and isomerization of disulfide bonds and of N-linked glycosylation. The Periplasmic proteins are often easier to isolate, less prone to crowding-induced aggregation and/or proteolytic degradation, and more efficiently folded compared with their cytoplasmic counterparts (27). L-Asparaginse type I is a low affinity cytoplasmic enzyme and expressed constitutively while L-Asparaginase type II, a high affinity periplasmic enzyme is under complex co-transcriptional regulation by both Fnr and Crp (28).

The human genome encodes at least three enzymes capable of hydrolyzing Asparagine. The first, belonging to the bacterial-type family, is lysophospholipase due to its ability to hydrolyze lyso-phospholipids in addition to Asparagine (29). The other human enzyme is lysosomal aspartyl-glucosaminidase (AGA), a plant-type enzyme,which remove carbohydrate groups linked to

Asparagine and third is the human L-Asparaginase (30). Human L Asparaginase is not being used as anticancer medicine because of its high Km (31). L Aspraginase enzyme also has potential application in food industries for the reduction of acrylamide in starchy products (32,33). L-Asparaginase is also used for the development of L-Asparagine biosensor in detection of leukemia in a blood sample (34). *Withenia Somnifera* ,a plant was found to have high specific activity of enzyme to use as bio-sensor for cancer cells (35).Plant-type Asparaginases share a high degree of amino acid sequence similarity (60-70%) with the human aspartyl glucosaminidase (AGA) family (EC 3.5.1.26) as both enzyme groups belonging to the N-terminal nucleophile (Ntn) hydrolase protein superfamily(36).

Objectives

1. Isolation of bacteria from sewage water
2. Screening of various microorganisms for L Asparaginase and L Glutaminase enzyme activity
3. Identification of bacteria by 16srRNA gene amplification and sequencing of PCR product.
4. Comparison of microorganism for high L Asparaginase and low L glutaminase activity
5. Purification of enzyme from *Alcaligene faecalis,* selected organism for L Asparaginase enzyme study
6. Comparison of *Alcaligene faecalis* L Asparaginase enzyme activity and specific activity with *E.coli* HB101 in cell extract.
7. Study of kinetic parameter of L Asparaginase from *Alcaligene faecalis*
8. Bio-informatic studies to know gene sequence and for cloning strategy

Methodology

Chemicals and reagents: Luria Bertoni, MacConkey and XLD differential media were purchased from Hi Media Laboratory. L-Asparagine, L- Glutamine, Tris -HCl buffer and Nesseler reagent were purchased from Merck, Germany. Chemicals and reagents for cloning was purchased from Promega. The other chemical were purchased locally from CDH of analytical grade and from Merck, Germany. *E.coli* HB101strain was procured from Dr Rekha Kansal ,Principal scientist, National research center on plant Biotechnology, IARI, Pusa campus , New Delhi.

Isolation of Bacteria: Sewage water sample were collected from Shivaji College in sterilized bottle for isolation of bacteria producing L- Asparaginase enzyme. In order to isolate bacteria serial dilution method of Waksman and Reilly was used (37).

Different dilution from 10^{-2} to 10^{-5} was streaked on LB medium, XLD and MacConkey agar. Colonies were isolated on the basis of morphological characters. The isolated colonies were preserved on master plates and on LB slant for further study (38).

Screening of L- Asparaginase enzyme activity: For screening for L -Asparaginase enzyme activity (39), A Modified Czapek Dox's medium was prepared for production of enzyme and plate assay was done with phenol red as indicator of ammonia production. Czapek Dox's media contain 1 X of M9 stock [10 X of M9 stock of 60gL-1 Na2HPO4 ; 30g L-1KH2PO4 ;5gL-1 NaCl], L Asparagine 10 gL-1]. M9 stock is autoclaved separately. In this media sterilized 2ml of 1M MgSO4, 1ml of CaCl2.2H2O, 10ml of 20% Glucose stock and 20g of Agar were added (pH 7.2 ± 1) (40).

A 2.5% stock of Phenol red dye was prepared in ethanol and pH was adjusted to 7.0 with 1M NaOH. The dye was added to production media to final concentration 0.009%. The plates were prepared. Control plated were also prepared with modified Czapek Dox's media having $NaNO_3$ as source of nitrogen and without substrate L- Asparagine. Plates were inoculated with isolated culture and kept for 24hr at 37^0C.The zone and colony diameter and red zone diameter were observed.

Agar well diffusion technique: 8µl of cell free culture was poured into agar plates with well diameter of 5mm containing modified Czapek Dox's media as mentioned above. The filterate was allowed to diffuse for 24hr at 37^0C. The color of plates was observed to see L- Asparaginase activity (41)

Quantitative assay for L Asparaginase and L-Glutaminase activity:

Quantitative assay for L- Asparaginase and L- Glutaminase enzyme activities was assayed by nesselerization (42,43,44).

Nessler's reagent is alkaline solution of potassium tetra iodomercurate (II). In the presence of ammonium ions with Nessler's reagent, it forms a precipitate of mercury (II) amido-iodide. The color of the precipitate varies from yellow to brown, depending on the quantity of ammonium ions.

$NH_4^+ + 2[HgI_4]^{2-} + 4OH^- \longrightarrow HgO.\ Hg(NH_2I\ (s) + 7I^- + 3H_2O$
(Yellow or brown)

The culture was inoculated in 500ml Erlenmeyer flask containing 150ml of Czapek Dox's media. The flasks were incubated at 37^0C, 125rev min -1 for 24 hours in controlled environment incubator shaker (khera instruments). Uninoculated media serves as control. After 24hrs the cultures were centrifuged at 6500 rpm for 10 min at 4^0C.The cells were harvested and suspended in 0.1M Tris -HCl buffer, pH 8.6 with 20% glycerol and PMSF protease inhibitor cocktail (10mM stock) from sigma and was added to final concentration of 2mM. The Cells were sonicated in MISONIX-Ultra Sonicator, energy712 J, Amplitude 45,30sec pulse on and off for 5 min on ice. After sonication extracts were centrifuged at 10,000 rpm for 30 min at 4 ^{0}C to remove debris and unbroken cells. The supernatant was collected and stored at -20^0C in deep freezer for further assay. For enzyme activity, 0.5 ml of 50mMTris HCl buffer pH 8.6, 0.5 ml of 0.05M L-Asparagine/ L - Glutamine substrate and 0.5ml enzyme extract from various microorganisms were added and kept at 37^0C for 30 minutes and then 500µl of 10% TCA was added to terminate the reaction, centrifuged at 14,000rpm for 10 min. 0.1ml supernatant was taken into fresh tube and volume was made up to 1.2 ml with D/W. 0.2ml Nesseler's reagent was added and Kept for 20 min at 37^0C to develop color. Absorbance was taken at 450nm. One international unit of enzyme activity is defined as amount of enzyme required to form 1µmole of ammonia released in unit time. Standard curve with 1mM Ammonium sulphate was prepared (45).

Protein assay: The protein assay was done by Bradford and by absorbance at 280nm (46,47).1 mg BSA per ml was used for standard. The linearity was found from 5µg to 50 µg of protein by brad ford method. The absorbance was taken at 595nm. Another protein standard curve was prepared with 1mg BSA per ml at 280nm. Absorption at 280nm by proteins depends on the Tyrosine and Tryptophan content (and to a very small extent on the amount of Phenylalanine and disulfide bonds).

Purification of DNA: Bacterial Genomic DNA was isolated using the Insta GeneTM Matrix Genomic DNA isolation kit – As per the kit instruction. An isolated bacterial colony was picked and suspend in 1ml of sterile water in a microfuge tube. This was Centrifuged for 1 minute at 10,000–12,000 rpm to remove the supernatant. Then 200 µl of Insta Gene matrix was added to the

pellet and incubated at 56 °C for 15 minutes. The sample was vortexed at high speed for 10 seconds and placed the tube in a 100 °C in heat block or boiling water bath for 8 minutes. Finally, the content was vortexed at high speed for 10 seconds and Spin at 10,000–12,000 rpm for 2 minutes. 20µl of the supernatant was used per 50 µl PCR reaction.

Identification of isolated culture: Identification of isolated culture was done by PCR using Universal primer for 16SrRNA that is 27F AGAGTTTGATCMTGGCTCAG and 1492R TACGGYTACCTTGTTACGACTT. 16S r RNA gene fragment was amplified by MJ Research Petlier Thermal cycler. The PCR product were purified by using Montage PCR clean up kit (Millipore) and their single pass sequencing was performed using 785F GGATTAGATACCCTGGTA and 907 R CCGTCAATTCMTTTRAGTTT sequences by Yaazh Xenomics. Sequence data was aligned and analyzed for identification by Yaazh Xenomics, Madurai. The program MUSCLE 3.7 is used for multiple alignments of sequences. These sequences were aligned by using BLAST of NCBI and then submitted to Gen bank for microorganism identification (48,49). The sequences were approved by Gen bank and on that basis of sequence submission, accession number was provided by Gen bank.after publication .

Isolation of periplasmic enzyme: The procedure for periplasmic enzyme purification was standardized with the help and protocol of Ms. Vaishali Verma from Prof V.K Chaudhary laboratory, Department of Biochemistry, South campus , University of Delhi in this project. Three fractions were collected;1. Sucrose fraction,2. periplasmic fraction and 3. Spheroplast. Enzyme activity and specific activity were assayed in all fraction. The procedure for periplasmic enzyme purification is as follows: 15 ml of induced culture of Alcaligene faecalis (Absorbance at 600nm =1per ml) in 50 ml falcon tube was taken and centrifuged at 5000 rpm for 10 minutes at 4°C.Supernatant was discarded and pellet was re suspended in 30 ml 10 mM Tris-HCl, pH 8.0 (ice-cold), and centrifuged at 5000 rpm for 10 minute at 4°C.Again supernatant was discarded and pellet was re suspended in 1 ml10 mM Tris-HCl, pH 8.0. After this cells were transferred to 2 ml microfuge tube and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded carefully (as pellet is loose) and pellet was re suspended in 1 ml sucrose solution and kept at room temperature (30 mM Tris-HCl pH 8.0 + 1mM EDTA + 20 % Sucrose), and mixed by inversion for 5 min at room temperature. Suspension was centrifuged at 10,000g for 10 min at 4°C. The

supernatant was collected in fresh microfuge tube labeled as "Sucrose wash". The pellet was re suspend in 1 ml 5 mM MgCl2 (ice-cold) and tube was kept on ice for 10 minutes with mixing by inversion every 1-2 min.The suspension was centrifuged again at 10,000g for 10 minutes at 4°C and supernatant was collected in fresh microfuge tube labeled as "Periplasm". The pellet again was re suspended in 1 ml of 40 mM Tris-HCl pH 8.0 solution containing 1 mM EDTA (called as "spheroplast"). In this protocol three fractions were collected and named as 1. Sucrose fraction, 2. Periplasmic fraction and 3. Spheroplast

SDS PAGE:The purity of L-Asparaginase was assayed by SDS-PAGE (12% gels) stained with Coomassie Brilliant Blue R250 by the Laemmli method (50) with sensitivity range 0.2~0.5μg/band.

Study of Kinetic parameters: Enzyme activity was assayed by taking one parameter variable and rest others constant as per protocol mentioned above. Enzyme activity was assayed by nesslerization at different pH, temperature and at different substrate concentration. These all are discussed in results.

Observations and Results

A. **Primary and secondary Screening for L- Asparaginase enzyme**: In the present study 10 colonies isolated from sewage water of Shivaji College, had shown dark red zone in screening analysis on modified Czapek Dox's agar plates. These colonies had shown Pink or red zone in different intensities. Pink or red color is because of L- Asparaginase enzyme activity. L Asparaginase hydrolyze Asparagine to Aspartic acid and release ammonia, that increases pH of media. Phenol red changes color from yellow to pink or red because of alkaline pH by ammonia. (Figure-I).

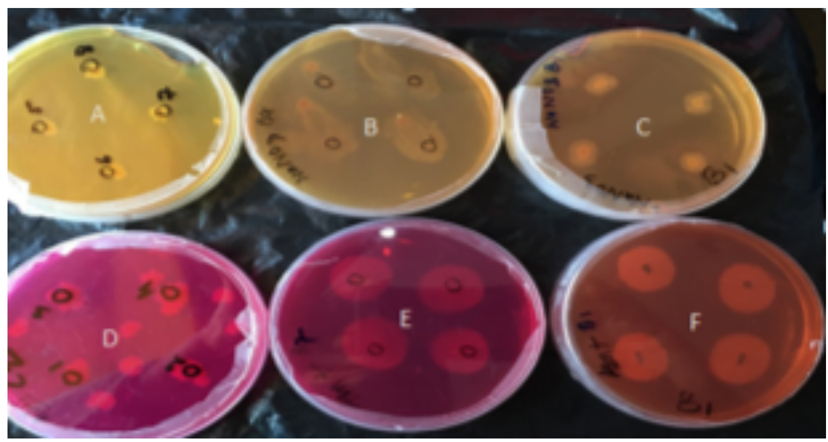


Figure-I : Isolated Microorganism showing L- Asparaginase activity. A, B and C, Control with NaNO3 as nitrogen source. D, E and F with L- Asparagine in media . Red zone indicates positive result for L- Asparaginase enzyme presence.

There are two types of L- Asparaginase enzyme, intracellular type I and extracellular type II. For extracellular enzyme cell free extract was loaded in well on Czapek Dox's modified agar media and incubated for 24hr. Only two microorganisms were found to have extracellular enzyme in this study. Later on these microorganisms were identified as *Alcaligenes faecalis* and *pseudomonas aeruginosa* (Figure-II A, B).

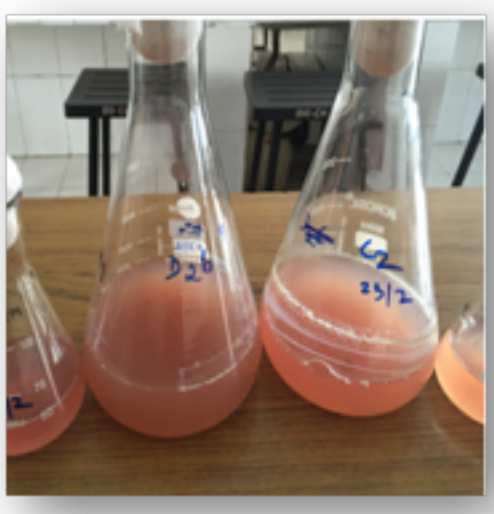


Figure II : A. Control (left) , B. Extracellular Enzyme L- Asparaginase activity (Right).

16srRNA sequencing analysis confirmed five organisms from these isolated colonies, so data is given for identified five colonies. The diameter of red zone ring because of L-Asparaginase activity and diameter of microorganism growth ring was observed. The data was statistically analyzed and graph shows red zone diameter, indicator of L-Asparaginase presence and cell growth diameter, indicator of organism growth and control where $NaNO_3$ was added as nitrogen source. (Table I, Figure-III)

Table- I: Screening of microorganism for L Asparaginase activity (Red zone diameter, cell growth ring diameter, and control).

S.No.	Name of Microorganism	Colony diameter(cm) Control(media with NaNO3)	Colony diameter(cm) media with L Asparagine	Red zone diameter(cm) media with L Asparagine L Asparaginase presence
1	*Pseudomonas aeruginosa*	1.917±0.068	1.625± 0.170	2.995±0.099
2	*Myroids phaeus*	1.902±0.057	2.250±0.129	3.146±0.063
3	*Enterobacter aerogenes*	2.00±0.00	1.269±0.046	4.300±0.000
4	*Pseudomonas otitidus*	2.00±0.00	1.068±0.095	3.189±0.057
5	*Alcaligenes faecalis*	2.100±0.041	1.159±0.008	4.250±0.000

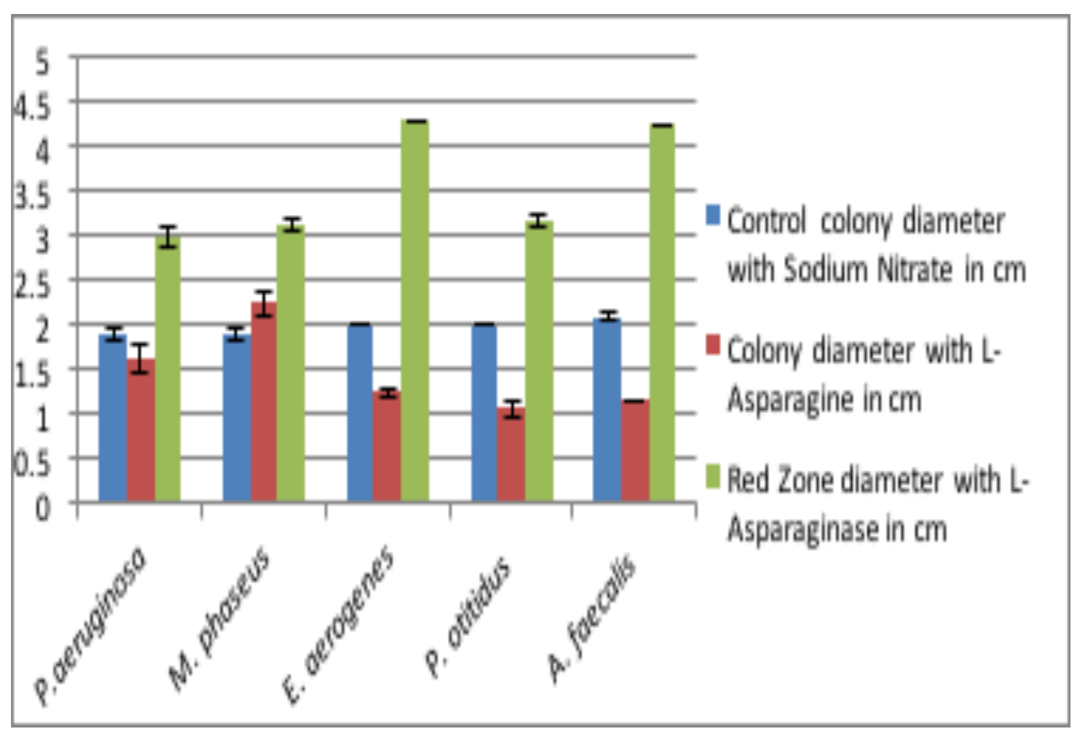


Figure-III: Graph showing screening of microorganism for L Asparaginase enzyme presence (Red zone diameter (cm) and cell growth ring diameter(cm) along with control

B. Quantitative assay for L Asparaginase and L Glutaminase enzyme activity:

Enzyme activity for L- Aparaginase and L- Glutaminase were assayed from various organisms, which were found to be positive in screening. Protein and Specific activity of L Asparaginase from these microorganisms were also calculated.The data was statistically analyzed and compared . One unit of enzyme activity is taken as 1µmole ammonia produced /min/ml enzyme. Maximum L- Asparaginase enzyme activity was observed in microorganism later identified as *Alcaligenes faecalis* (0.480±0.0282). The specific activity of these five organisms were very similar,ranging from 0.503±0.03 in *Myriad phaeus* to *pseudomonas aeruginosa* (0.522±0.040). *Enterobacter aerogenes* had shown lowest L-Glutaminase activity among five that is 0.101±0.006. L- Glutaminase activity was low in all the microorganisms in comparison to L- Asparaginase. (Table II, Figure-IV)

Table II – Quantitative assay for L- Asparaginase and L- Glutaminase enzyme activity in selected microorganism.

SNo.	Name of Microorganism	L- Asparaginase activity µmoleNH3 /min/ml	L- Glutaminase Activity µmoleNH3 /min/ml	Specific activity of L Asparaginase enzyme activity/mg	Protein mg/ml
1	*Pseudomonas aeruginosa*	0.291±0.0492	0.106±0.0323	0.522±0.040	0.432±0.05
2	*Myroids phaeus*	0.296±0.014	0.176±0.05	0.503±0.03	0.546±0.04
3	*Enterobacter aerogenes*	0.399±0.036	0.101±0.006	0.517±0.001	0.506±0.019
4	*Pseudomonas otitidus*	0.239±0.007	0.144±0.007	0.543±0.004	0.578±0.030
5	*Alcaligenes faecalis*	0.480±0.0282	0.177±0.007	0.511±0.0459	0.625±0.04

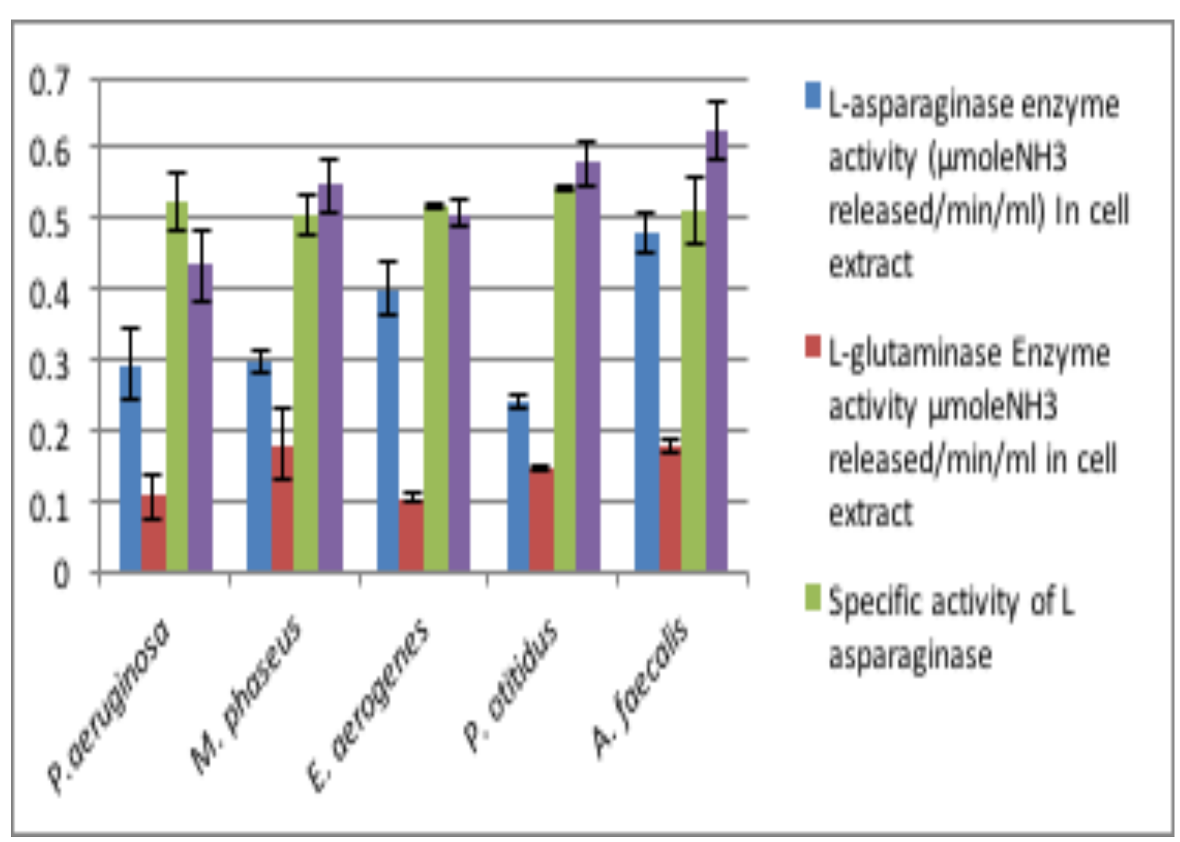
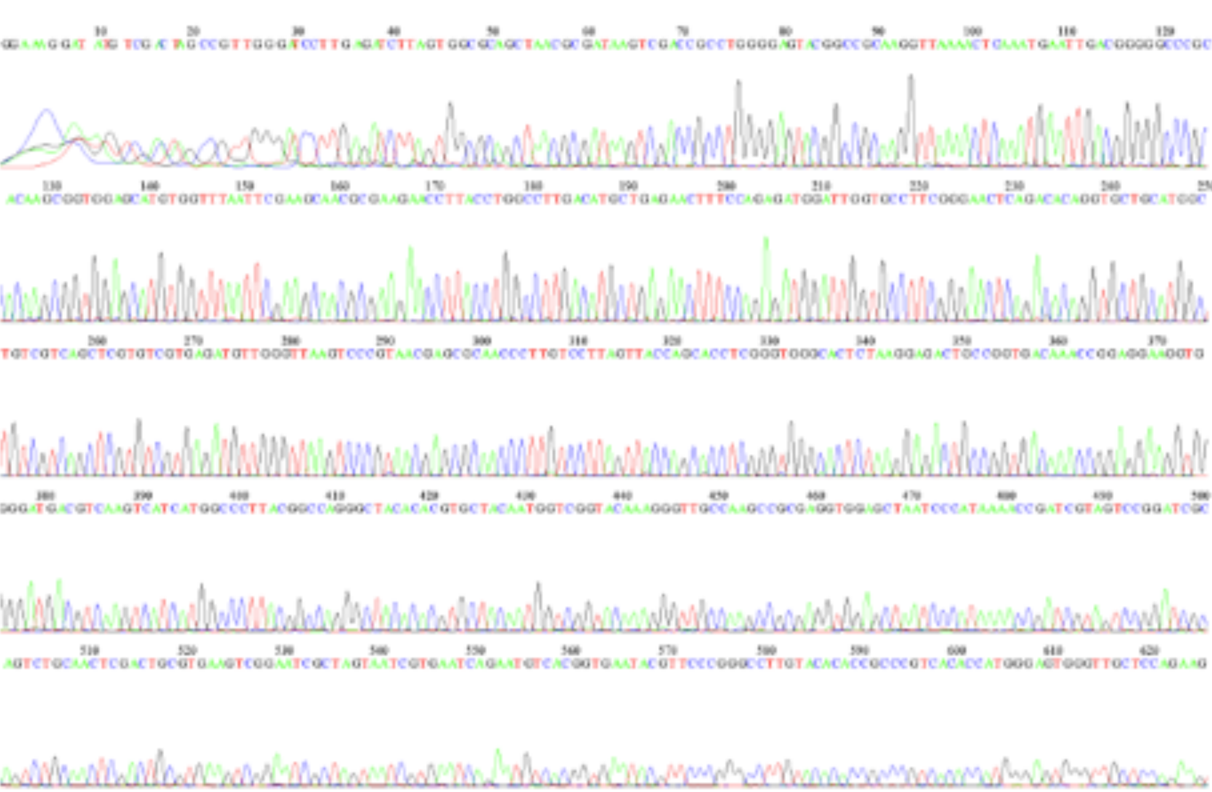


Figure -IV Quantitative assay for L- Asparaginase and L-Glutaminase enzyme activity in selected Microorganisms.

C. **Identification of Microorganism**: 10 samples were sent to YAAZH Xenomics ,Madurai for 16SrRNA sequencing .These sequences were submitted to Gen bank , only 8 colonies sequences were accepted and accession number were provided. Five organisms identified by Gene bank from these sequence submission are *Pseudomonas aeruginosa*, *Pseudomonas otitidus*, *Enterobacter aerogenes*, *Myroid phaseus* and *Alcaligenes faecalis* (TableIII).

Table III: Identification of microorganism by 16SrRNA gene sequencing

SNo.	Gen Bank Accession No	Name of microorganism
1	KU682196	*Myroids phaeus*
2	KU682197	*Myroids phaeus*
3	KU682198	*Pseudomonas aeruginosa*
4	KU682199	*Pseudomonas aeruginosa*
5	KU682200	*Enterobacter aerogenes*
6	KU682201	*Pseudomonas otitidus*
7	KU682202	*Pseudomonas aeruginosa*
8	KU682203	*Alcaligenes faecalis*



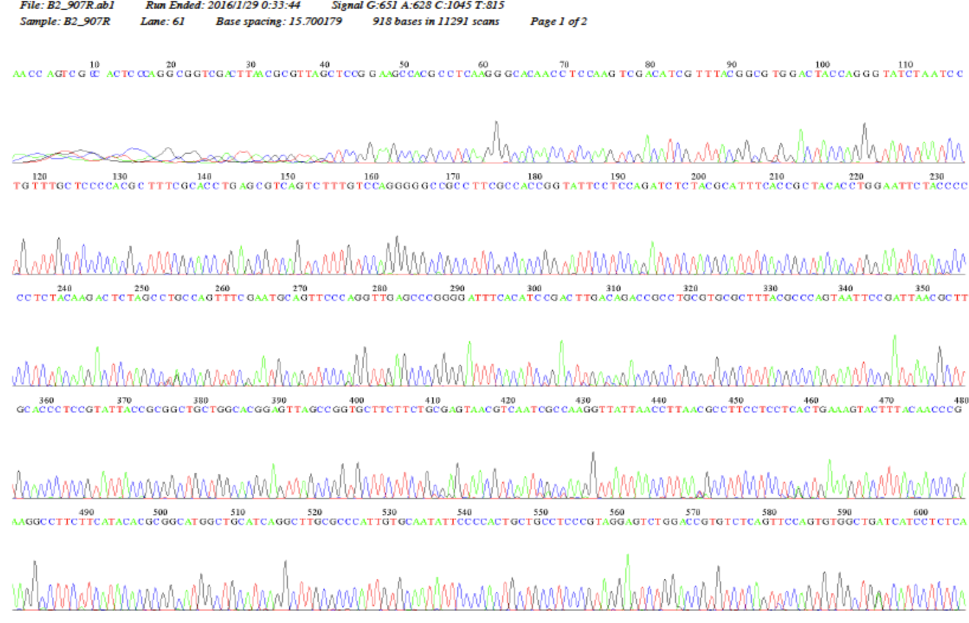


Figure -V Chromatogram of DNA sequencing

```
>gb|JX847782.1| Alcaligenes sp. cf3-A 16S ribosomal RNA gene, partial
sequence
Length=1379

 Score =  544 bits (294),  Expect = 2e-150
 Identities = 517/621 (83%), Gaps = 30/621 (5%)
 Strand=Plus/Plus

Query  10    ATGTC-ACTGGCTGTTGGGGCCGTTATGTCTTAGTA-C-CAAGCTAACGCGTGAAGTTGA
66
             ||||| ||| |||||||||||||||| | ||||||| | | |||||||||||||||||||
Sbjct  765   ATGTCAACTAGCTGTTGGGGCCGTTAGGCCTTAGTAGCGC-AGCTAACGCGTGAAGTTGA
823

Query  67    CCGCCTGGGGAGTACGGTCGCAAGGTTAAAACTCAAAGGAATTGACGGGGACCCGCACAA
126
             |||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  824   CCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAA  883

Query  127   GCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCCACCCTTGACATG  186
             ||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  884   GCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATG  943

Query  187   TCTAG-AATCTTGAAGAAATTTATTAGTGCCCGCAAGGGAACTGGAACACAGGTGCTGCA  245
             ||| | || |  ||||| ||||    |||| |||||| |||| |||||||||||||||||
Sbjct  944   TCTGGAAAGC-CGAAGAGATTTGGCCGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCA  1002

Query  246   TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT  305
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1003  TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT  1062

Query  306   TGTCATTAGTTGCTACATTCGATTGA-TCTAATGAAACTGACTGTGACTGACCA-ACCGA  363
             ||||||||||||||||    ||   | |||||||| |||| | |||||  |||  |  ||
Sbjct  1063  TGTCATTAGTTGCTACGCAAGAGC-ACTCTAATGAGACTGCCGGTGACAAACCGGAG-GA  1120

Query  364   AGGAGGGGATGAATGACA-GTCCTCCCGCCCCTTATTCATAGGGCTTC-CTACATCCTAC  421
             ||| ||||||||  | || ||||||  | |||||||   ||||||||| | || || |||
Sbjct  1121  AGGTGGGGATGAC-GTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCAC-ACGTCATAC  1178

Query  422   AAAGAACGGCACATACGGACGCCTACCCACCAGGGGGAGCTAATCTCTCAAACCCGATCG  481
             || |  ||| ||| | || |||| |||| | ||||||||| ||||||  |||||||||||
Sbjct  1179  AATGGTCGGGACAGAGGGTCGCCAACCCGCGAGGGGGAGCCAATCTCAGAAACCCGATCG  1238

Query  482   TACTCAGGATGG-A-TCGCTGCCAGTC-ACTTGAC-TGAA-TGCAGAATCACTCGTTATC  536
             || || |||| | | ||  ||| | || ||| | | |||| | | ||||| || || |||
Sbjct  1239  TAGTCCGGATCGCAGTC--TGCAACTCGACT-G-CGTGAAGT-CGGAATCGCTAGTAATC  1293

Query  537   ACGGATGA-AACTGCCTG-GGTGAATGAA-TTCCCGGGTCTTGCATGCACCGACCGTCAC  593
              ||||| | || || | | ||||||| |  ||||||||||||| |  ||||| |||||||
Sbjct  1294  GCGGATCAGAA-TGTC-GCGGTGAAT-ACGTTCCCGGGTCTTGTACACACCGCCCGTCAC  1350

Query  594   TCCAAGGAAGTGAGTTTCACC  614
              ||| || |||| ||||||||
Sbjct  1351  ACCATGGGAGTGGGTTTCACC  1371
```

Figure VI: 16s ribosomal RNA gene of *Alcaligene faecalis*

D. *Alcaligene faecalis* and *E. coli* HB101 strain L Asparaginase enzyme activity comparison:

In this present study, enzyme activity and specific enzyme activity of L Asparaginase enzyme from *E.coli* and *Alcaligene faecalis* has been compared in cell extract after ultra-sonication because *E.coli* and *Ervinia crysthanthami* L Asparaginase is approved by food and drug administration for cancer treatment either in native form or pegylated form

(covalent conjugation of polyethylene glycol to enzyme) by USA. This was found that enzyme activity and specific activity were comparable to each other. *E. coli* has shown enzyme activity 3.52µmole /min/ml and specific activity 2.45µmole /min/mg protein .*Alcaligene faecalis* had 3.13 µmole /min/ml enzyme activity and 2.93µmole /min/mg protein. The values are very closely comparable. Table IV Figure VII.

Table IV: comparison of enzyme activity and specific activity

S.no	Activity	*E.Coli* HB101 strain	*Alcaligene faecalis*
1	Enzyme activity µmole/min/ml	3.52	3.13
2.	Specific activity µmole/min/mg	2.45	2.93

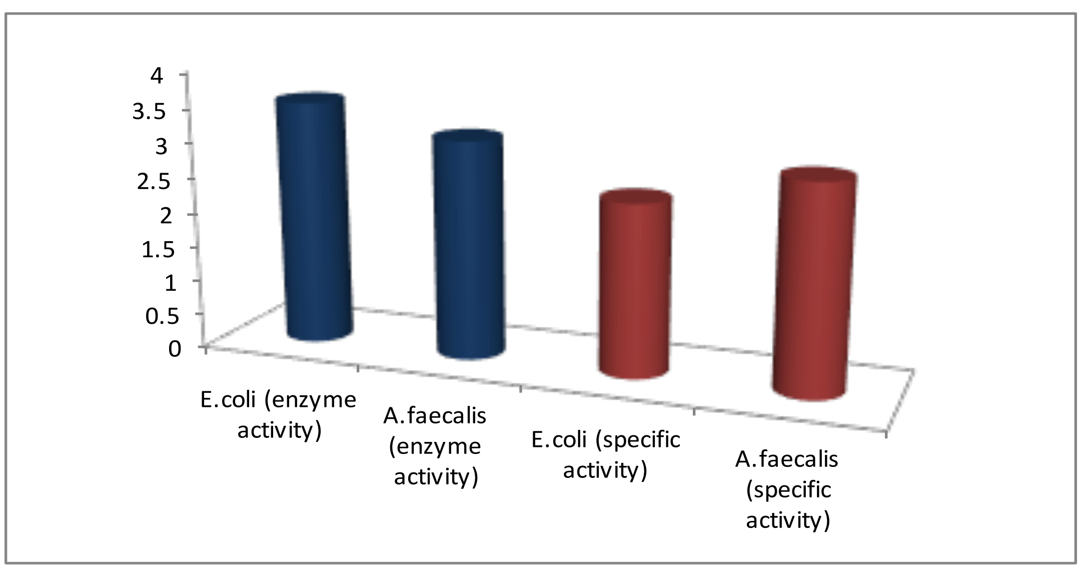


Figure VII: Comparison of L Asparaginase enzyme activity and Enzyme specific activity from *E. coli* and *Alcaligene faecalis*.

Alcaligene faecalis was reported to show highest specific activity among *pseudomonas* , *Achromobacter* and *proteus species* (Hiroko and Kenzi,1970), so *Alcaligene faecalis* was

selected for characterization of enzyme and grown on M 9 medium as per protocol mentioned in methods(51).figure VIII

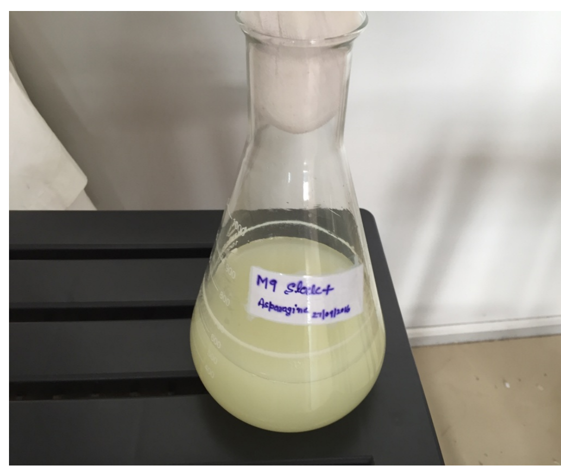


Figure- VIII: Growth of *Alcaligenes faecalis* in M-9 medium

Alcaligene faecalis was grown on for 72 hrs in M 9 medium to give maximum Periplasmic Type II enzyme L Asparaginase production as per protocol mentioned in methods (52).

E. *Alcaligene faecalis* Periplasmic L Asparaginase enzyme fractions comparison for specific activity:

Sucrose fraction was showing high turbidity and no protein was found in SDS PAGE from this fraction.The Periplasmic fraction had shown very high specific activity 2.84µmole/mg/min in comparison to Spheroplast (0.04µmole/mg/min) . Table V

TableV: Comparison of enzyme activity of periplasmic fraction and spheroplast fraction

S.No.	Fraction	Enzyme Activity (µmole/min/ml)	Specific Activity(µmole/mg/min)
1.	Periplasmic	2.14	2.84
2	Spheroplast	6.50	0.04

F. Periplasmic fraction had shown only one band in SDS PAGE while many diffuse bands were visible in spheroplast fraction (Figure IX).

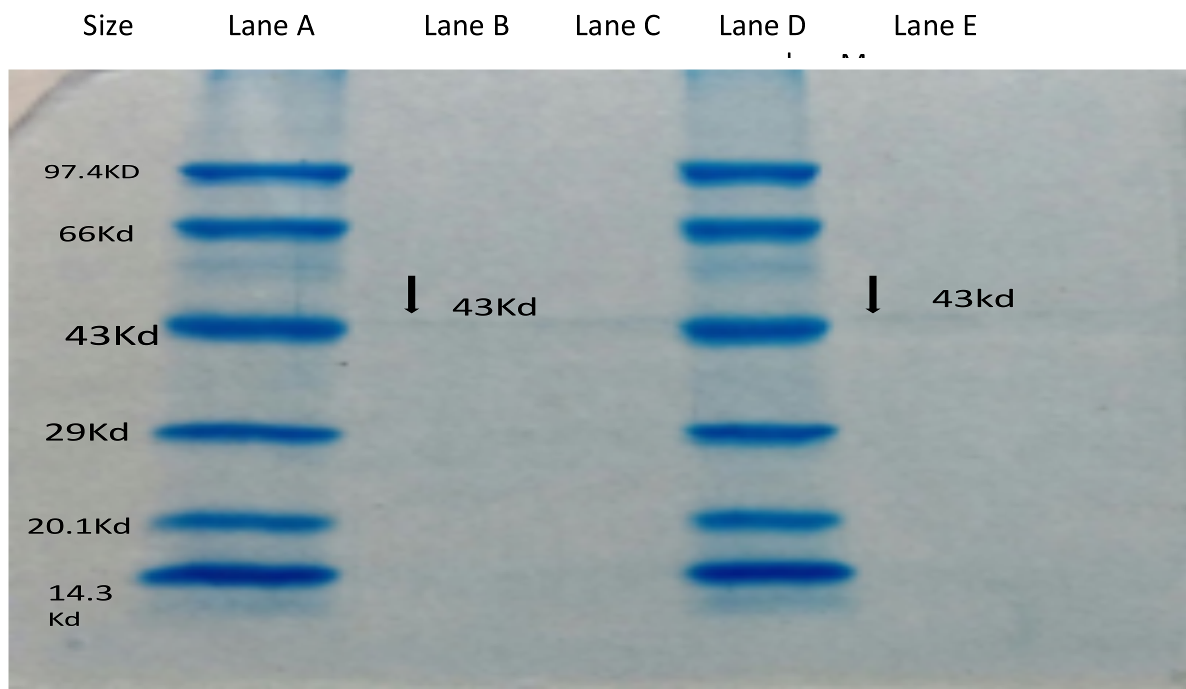


Figure IX: SDS PAGE for Periplamic Fraction showing monomer of 43 kDa. Lane A and D with protein markers, Lane B, C and E with periplasmic extract.

The Periplasmic extract has shown only one band of 43Kd, this may be because of L Asparagine presence in media induced more production of type II L Asparaginase enzyme.

L-Asparaginase type II, a high affinity periplasmic enzyme is under complex co-transcriptional regulation by both Fnr and Crp (28). SDS gel is stained with Coomassie blue (G-250), which has Sensitivity 0.2~0.5μg/band protein The other proteins might be in very low concentration which can be detected by silver staining having sensitivity of 2ng/band staining. The periplasmic enzyme is preferred for protein expression because of significantly fewer proteins, particularly proteases, compared to the cytoplasm, having network of redox enzymes for the formation and isomerization of disulfide bonds and of N-linked glycosylation. The Periplasmic proteins are often easier to isolate, less prone to crowding-induced aggregation and/or proteolytic degradation, and more efficiently folded compared with their cytoplasmic counterparts (27). Though the band is light but matching with 43kDa marker protein which is approximately closer to monomer of L Asparaginase . spheroplast has shown many diffusible band but low specific activity ,so periplasmic fraction is considered more than 90% pure protein and kinetic parameters were studied with this fraction.Two types of L Asparaginase enzyme were reported by Hiroko sakato and Kenji soda in alcaligene faecalis (50).To confirm this we searched from NCBI nucleotide sequence and came to know that ***Alcaligene faecalis*** **has only one gene for type II periplasmic L Asparaginase enzyme. Cytosolic fraction was loosing activity after two days and had low specific activity in comparison to periplasmic fraction that had stability till 10-15 days with high specific activity , Figure :IX**

L asparaginase CDS of *Alcaligenes faecalis*

AATCTGTATCAGGCCGTGCAAGTGGCCTGCTCGCCCTTGGCCAACGACCAAGGTGTGCTGGTGCTGCTGAATGATCAAATCCACA
GCGCCCGCTTTCTGAGCAAGCAGCACACCACCCAGACCAGCGCCTTTGGTTCACCCGATGCAGGCCCGCTGGGCCTGGTATCAG
GAGGCCAGCCGCGCTTCATGATGCGTACACTGCTGCCCCACACTCACAGCAGCCTGTTCGATGTGCGCTCTTTGCACAGCCTGCC
TAAAGTGCAGATTTTTTATGATCACCCCGATACTTTGGCCGAACTGTACCGCCACGCCGTACAAAGCGGTGTGCGAGGCATTGTG
GTGGCGGCGACTGGCAACGGCAGCCTGACCCCCGGCGCCTTGGAAGGTGTCAGCCGGGCACACCGACTGGGCGTAGTGTGTG
TGCGGGCCTCTCGCATTCATGCAGGACCTGTGACAGACAGTGCTTATGACCAGGATCACCACACCATTGCCGCGCATTACTTGCC
TGCTCAAAAGGCCCGAATTTTATTAATGCTGTGTCTGGCTGCTCAGCTGGAGCACGATGAGATAGAGGCGGCTTTCCGCGACTAT

Protein

MPTRPKLALVGTGGTIAATTQSNLGLTDYDITQGVETLLQAVPGITELADLECHQIFNVD
SRAMGSLMLLELSHKLNALLARPDINGVVITHGTDTLEESAFFLHLTLKTDKPVVMVAAM
RPASALSADGPLNLYQAVQVACSPLANDQGVLVLLNDQIHSARFLSKQHTTQTSAFGSPD
AGPLGLVSGGQPRFMMRTLLPHTHSSLFDVRSLHSLPKVQIFYDHPDTLAELYRHAVQSG
VRGIVVAATGNGSLTPGALEGVSRAHRLGVVCVRASRIHAGPVTDSAYDQDHHTIAAHYL
PAQKARILLMLCLAAQLEHDEIEAAFRDY

Number of amino acids: 329
Molecular weight: 35278.37
Theoretical pI: 6.16

Figure X: CDS of L Asparaginase enzyme of *Alcaligene faecalis* and its protein sequence T04157

This enzyme has 329 amino acids and 990 nucleotides. The protein is homotetramer or ‘dimer of dimer’ having catalytic site on interface between two dimers of dimer.

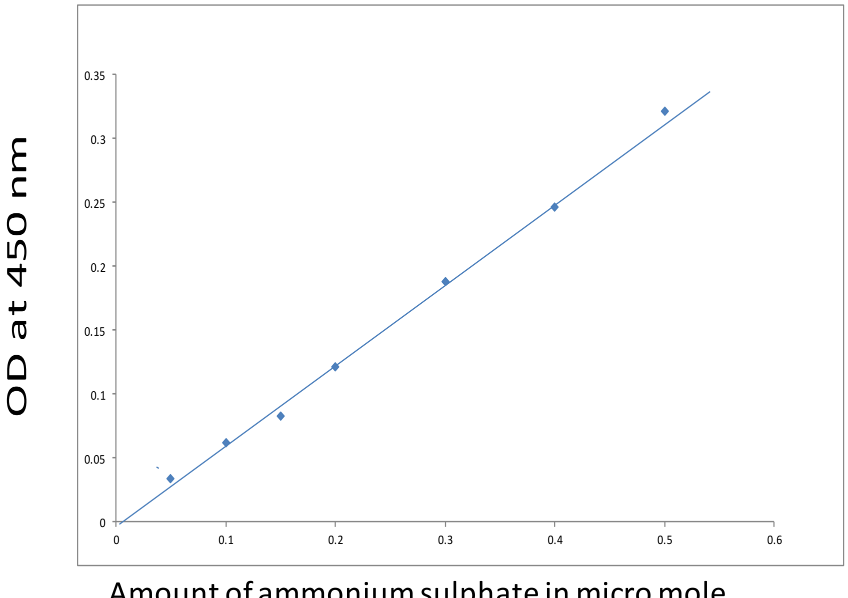


Figure XI: standard curve of Ammonium Sulphate (1mM)

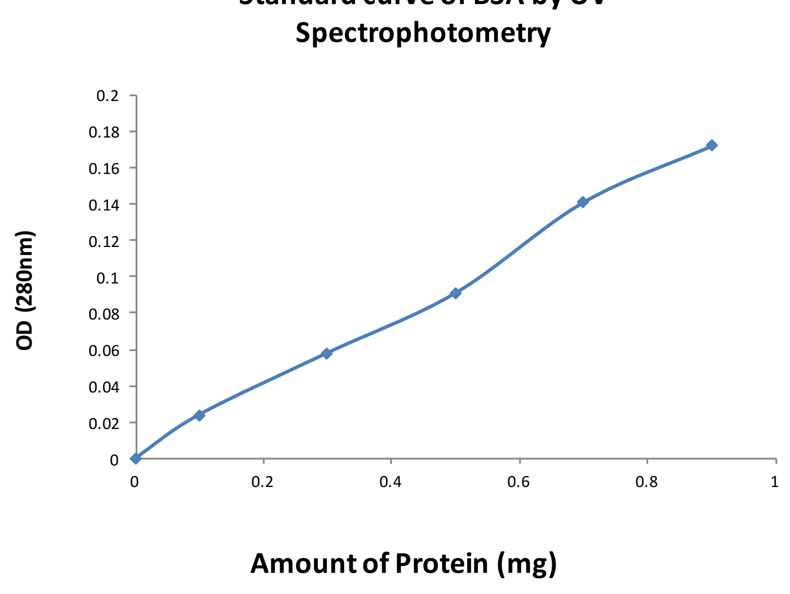


Figure- XII: Protein standard curve of BSA at 280nm

H. Kinetic parameter study of periplasmic L Asparaginase:

i.Effect of pH on periplasmic L Asparaginase enzyme:

In this study enzyme has shown two pH at which enzyme had high enzyme activity , that is pH 6.5 and 8.5. At pH 6.5 maximum activity was observed and second highest point of pH was 8.5. The *Alcaligenes faecalis* has only one gene to code for periplasmic enzyme then two pH for activity may be because of an adaptation of enzyme to environment, which needs to be verified further. Table VI and Figure XIII

Table VI: Effect of pH on Enzyme activity (micromole /ml/min)

SN	pH	Enzyme activity µmole/min/ml
1	4	1.67
2	4.5	1.46
3	5	1.75
4	5.5	2.07
5	6	2.51
6	6.5	2.68
7	7	2.6
8	7.5	2.45
9	8.0	2.09
10	8.5	2.28
11	9	2.21

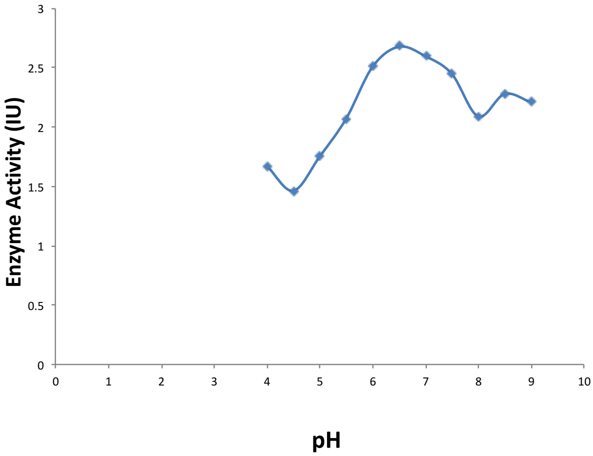


Figure XIII: Graph between pH and enzyme activity of L Asparaginase periplasmic enzyme

ii. Effect of temperature on enzyme activity:

Enzyme has highest enzyme activity at 37^{0}C (2.35 µmole /min /ml) but did have some activity till 55^{0}C(1.44 µmole /min /ml).**Table VII and figure XIV**

Table VII: Effect of temperature on Enzyme activity (micromole/minute/ml)

SN	Temperature in ^{0}C	Enzyme activity µmole/min/ml
1	0	0
2	15	0.815
3	25	2.15
4	37	2.35
5	45	0.97
6	55	0.167
7	65	0.005

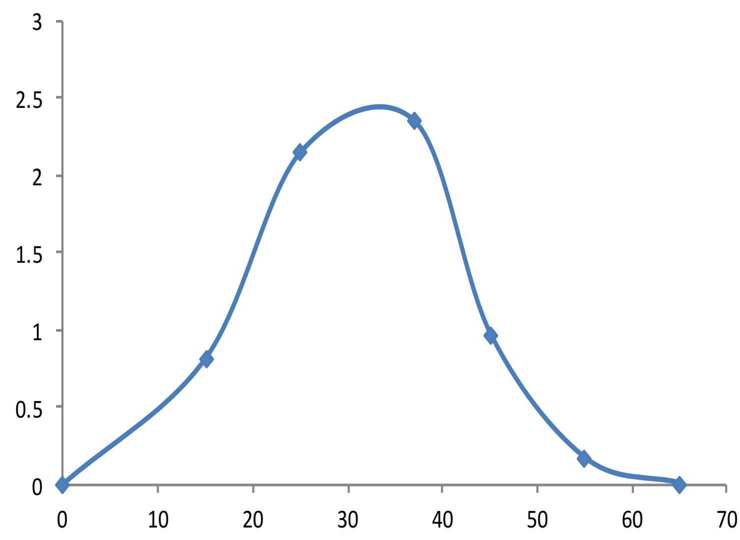


Figure XIV: Graph between pH and enzyme activity of L Asparaginase enzyme

ii. Effect of substrate concentration on L Asparaginase enzyme activity:
The line weaver burk plot between 1/S and 1/V, Table VIII and figure XV

Table VIII: Substrate concentration VS enzyme activity

S.No.	[S] µM	V , µmole /min /ml	1/s μM^{-1}	1/V µmole-1 x min x ml
1.	1.25	1.115	0.8	0.896
2.	2.5	1.313	0.4	0.761
3.	6.25	1.364	0.16	0.746
4.	12.5	1.595	0.08	0.702
5.	13.8	1.499	0.072	0.69

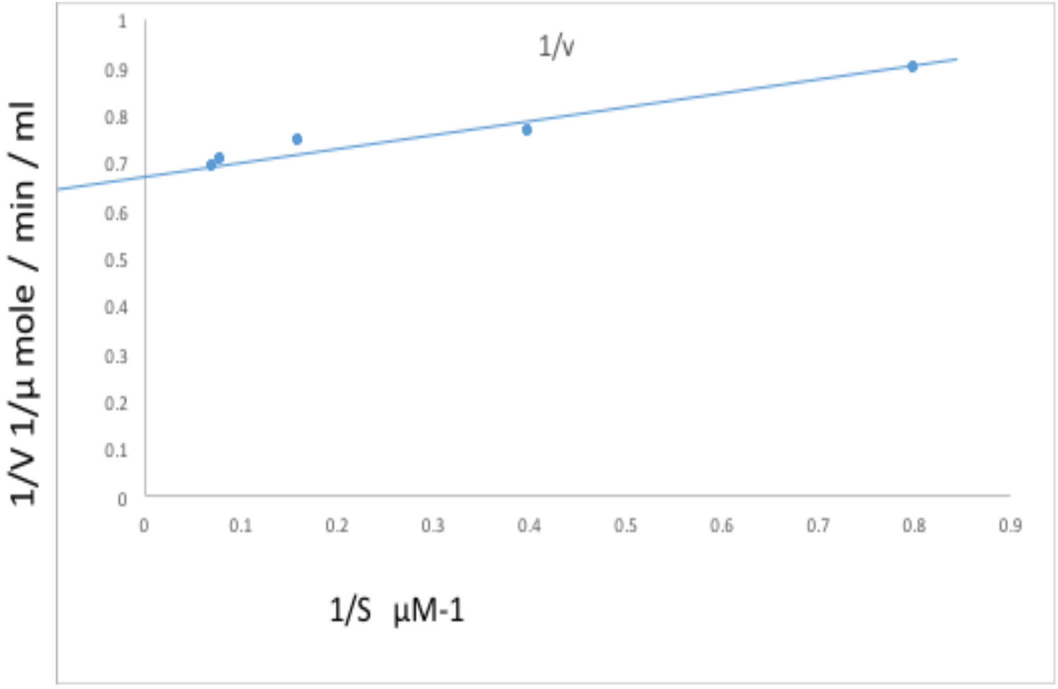


Figure XV: Lineweaver Burke Plot (1/V vs 1/S)

Effect of substrate concentration on enzyme activity: The Km of periplasmic enzyme was found to be in the range of 0.6 micro molar to 1micro molar . This is better to have low Km of the enzyme in micromolar range for its use as medicinal enzyme (54) as serum Asparagine is about ~50 µM concentration in serum (55). Human L Asparaginase type III has high Km that why not good for use.

I. Cloning

Cloning strategy

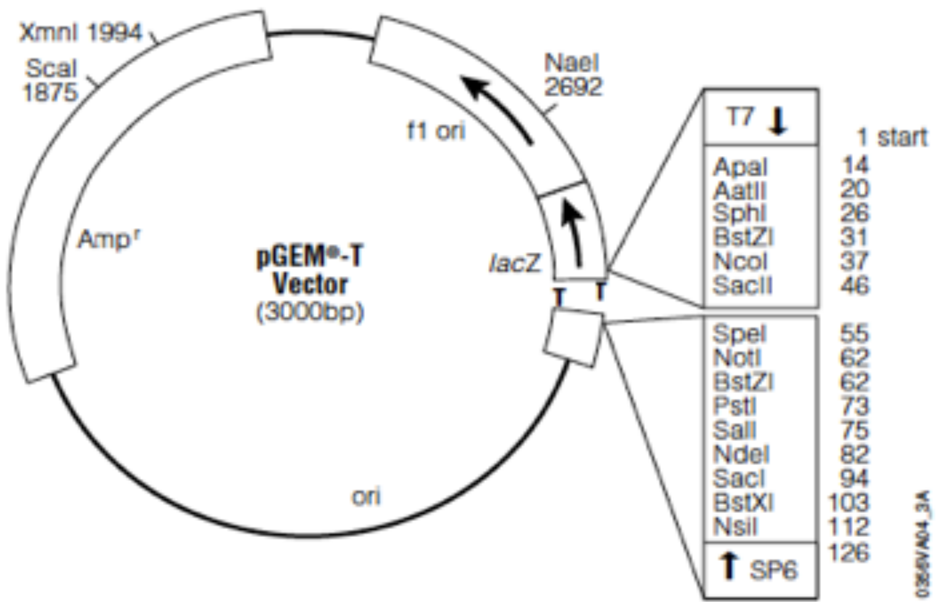
1. Isolation of genomic DNA
2. Amplification of L Asparaginase by using forward and reverse primer and with taq polymerase of high fidelity
3. Purify PCR product and insert into pGEMT
4. Digest with Xho I and NdeI restriction endonuclease
5. Ligate and amplify
6. Digest with Restricted endonuclease and isolate plasmid on agarose

electrophoresis.

7. Clone into pET22b

8. Transformation in to DH5a *E.coli* strain

9. Isolate plasmid from DH5a and transfer into BL21 / Rossetta expression vector for protein expression.



FigureXVI : pGEM®-T Vector Map and Sequence Reference Points

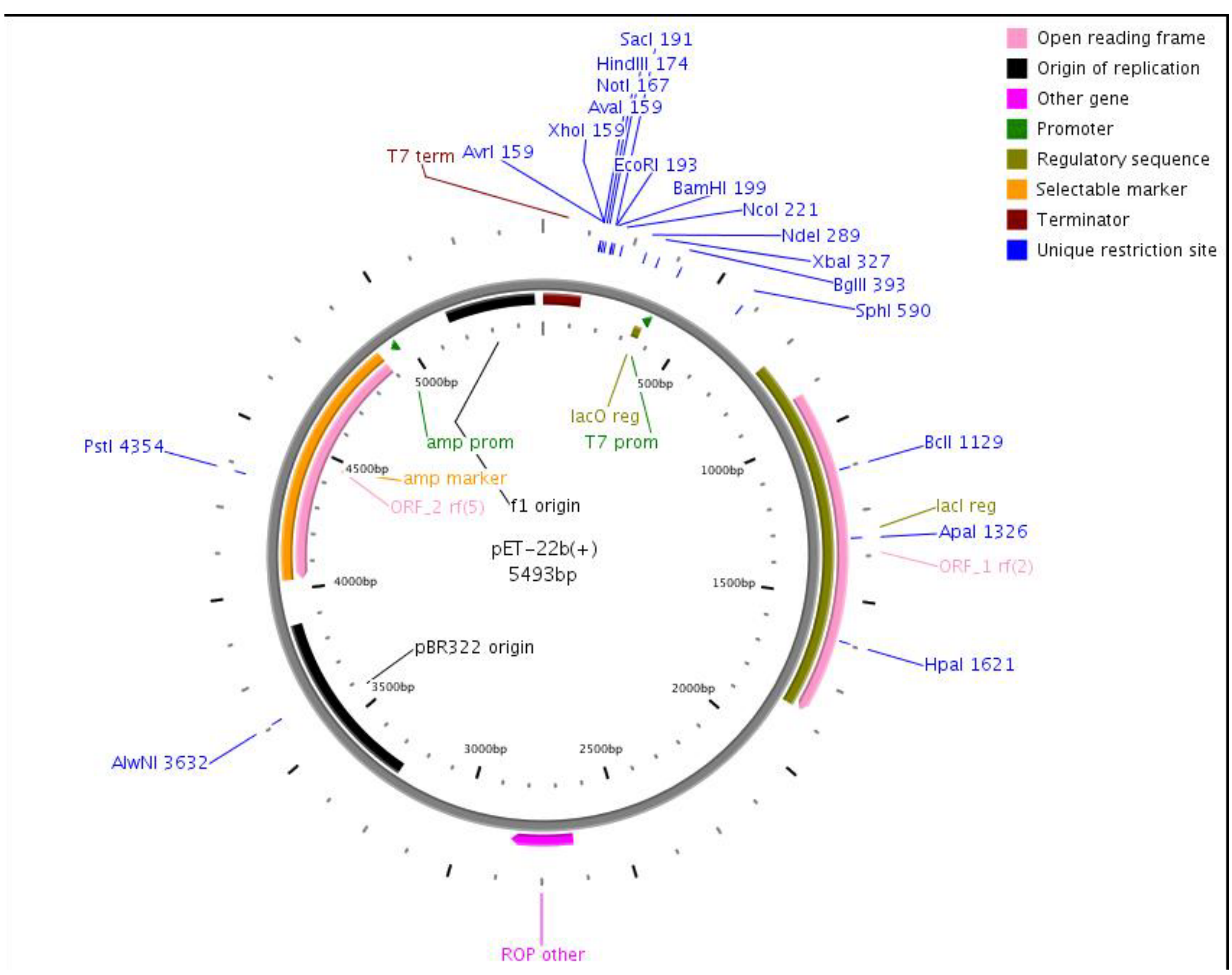


Figure XVII: The pET-22b(+) vector

The pET-22b(+) vector carries an N-terminal pelB signal sequence for potential periplasmic localization, plus optional C-terminal His•Tag® sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3).

J. Cloning Experiments

Experiment A: Genomic Isolation from *Alcaligene faecalis*: Genomic isolation is done as procedure given in promega DNA isolation kit and DNA is rehydrated in 100µl Rehydration buffer.

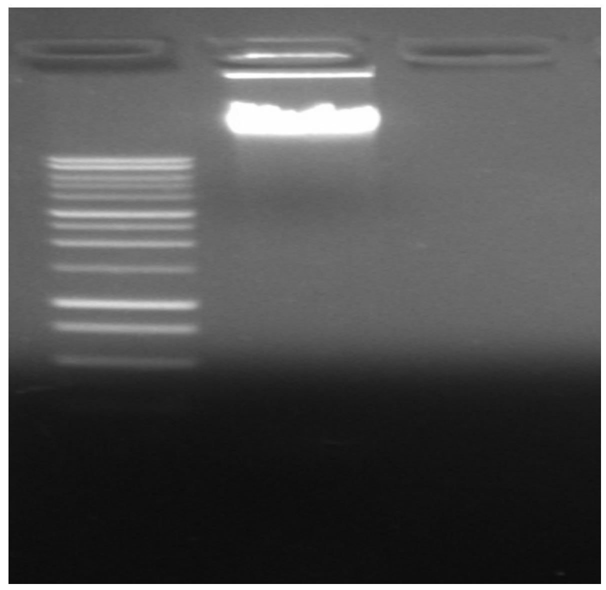


Figure XVII: Genomic DNA isolation from *Alcaligenes feacalis*

Experiment B:

PCR to amplify Asparaginase (1Kb) using genomic DNA as template. The primer for L Asparaginase;

Alcaligene faecalis primer are:

Forward primer : 5' AAAA CATATG CCG ACA CGC CCC AAA CTG GCC 3'and
Reverse primer : 5' AAAA CTCGAG ATA GTC GCG GAA AGC CGC CTC TAT CTC3'

E.Coli HB101 Primers are:
Forward Primer: 5' AAAA CATATG GAGTTTTTCAAAAAGACGGCACTTG 3' and
Reverse Primer: 5' AAAA CTCGAG GTACTGATTGAAGATCTGCTGGATC 3'.

Table IX: The reaction mixture composition for PCR is as following table.

NEB10X PCR buffer	**5µl**
2mM dNTPs	**4µl**
AF1 Forward primer Of *A.Faecalis*	**3µl**
AF2 Reverse primer of *A. faecalis*	**3µl**
Template	**1µl**
Taq pol	**1µl**
Mq	**33µl**
Total Volume	**50µl**

Table X: PCR conditions for 30 cycles

94C	94C	58C	68C	68C	16C
5min	30sec	30sec	1min	5min	Hold

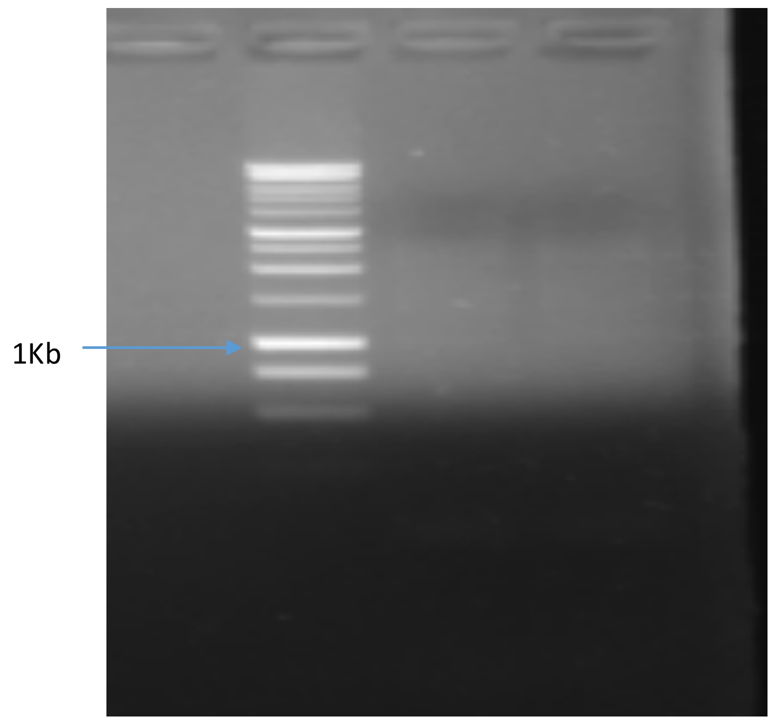
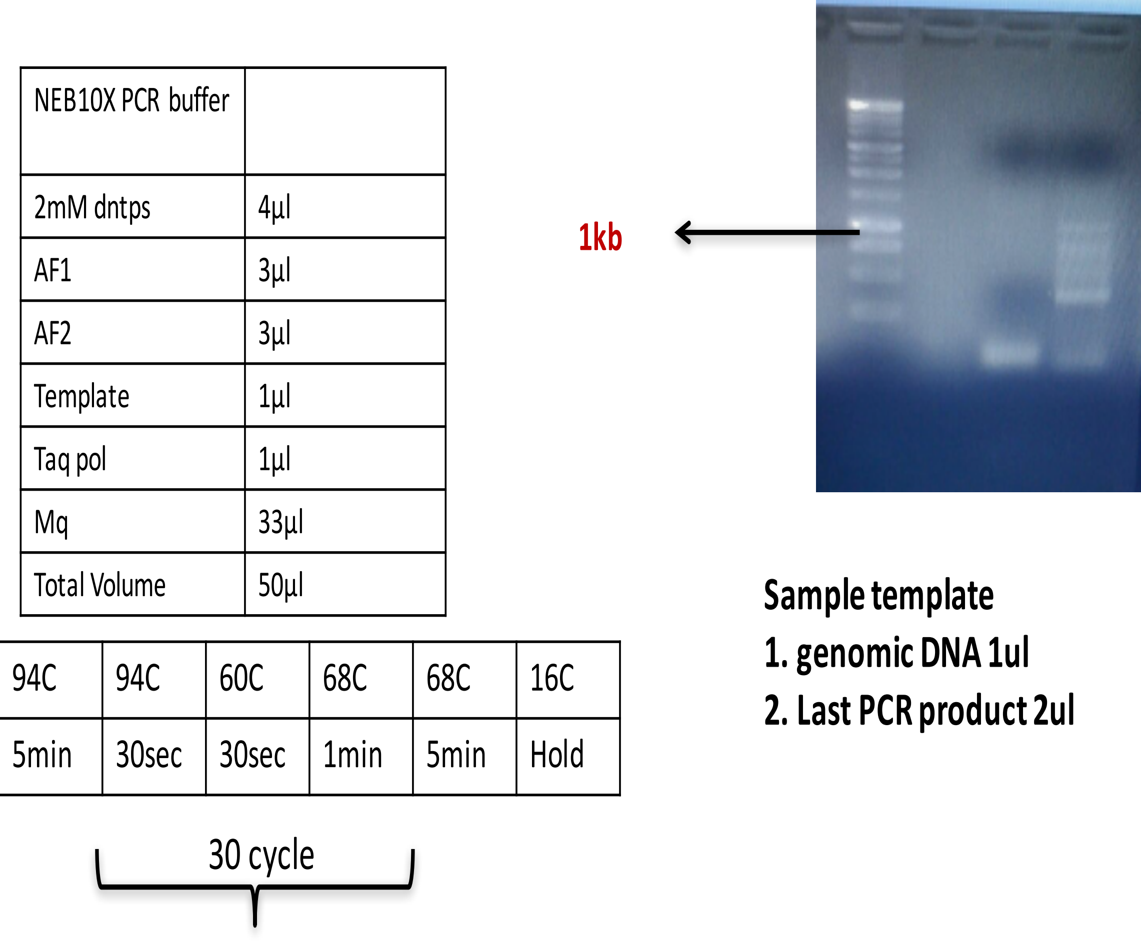


Figure XIX : Result , A very faint band near 1Kb

Experiment: B

PCR to amplify Asparaginase (1Kb) using genomic DNA as template.



NEB10X PCR buffer	
2mM dntps	4µl
AF1	3µl
AF2	3µl
Template	1µl
Taq pol	1µl
Mq	33µl
Total Volume	50µl

94C	94C	60C	68C	68C	16C
5min	30sec	30sec	1min	5min	Hold

30 cycle

Sample template

1. genomic DNA 1ul

2. Last PCR product 2ul

PCR conditions:

Table XI and Figure XX: L Asparaginase PCR amplification

Experiment: C

Table XI: PCR to amplify Asparaginase (1Kb) from *Alcaligene faecalis* using genomic DNA as template. Reaction mixture for assay is as follows:

NEB10X PCR buffer	5µl
2mM dNTPs	4µl
Forward Primer for *E.coli*	3µl
Reverse primer for E.coli	3µl
Template	1µl
Taq pol	1µl
Mili Q water	33µl
Total Volume	50µl

Table XII: Conditions for 33 PCR cycle.

94C	94C	55C	68C	68C	16C
7min	40sec	30sec	1min	5min	Hold

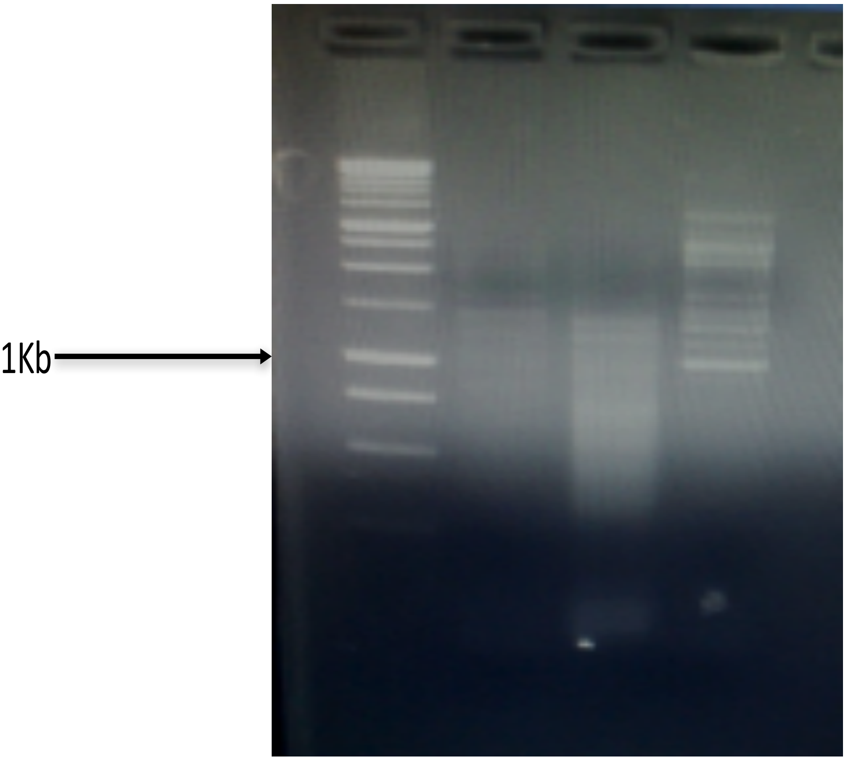


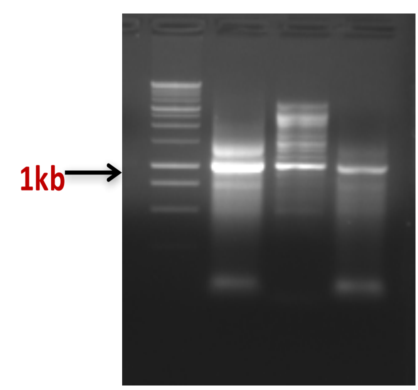
Figure XXI: PCR to amplify Asparaginase (1Kb) using genomic DNA as template.

1. PCR product from expt. A , 2. PCR product from expt. B, 3. Genomic DNA, and Sample 3 has a positive band.

Experiment: D

PCR to amplify Asparaginase (1Kb) using genomic DNA as template. (D)

NEB10X PCR buffer	5µl
2mM dntps	4µl
AF1	3µl
AF2	3µl
Template	1µl
Taq pol	1µl
Mq	33µl
Total Volume	50µl



94C	94C	53C	68C	68C	16C
8min	40sec	30sec	1min	5min	Hold

35 cycle

Figure :
.Sample. 1 PCR product from Expt C-3
2. Genomic DNA.,
3. PCR product of A

Result: all samples are positive, sample no -1 is subjected to gel purification and and TA ligation

Table XII and figure XXII:PCR of Products from various experiments

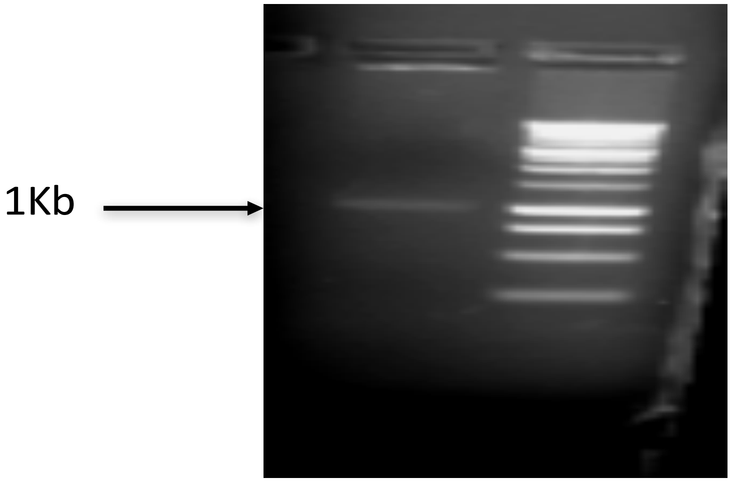


Figure .Gel Purified insert before ligation in pGEMT

Figure XXIII: Agarose electrophoresis of Purified insert

Experiment E: Ligation of insert

Table XIII : ligation reaction Mixture composition

Component	Volume
2X pGEMT ligation buffer	5µl
Insert	3µl
Vector	1µl
Ligase	1µl
Total reaction volume	10µl

O/N 4^0C ligation.

Experiment: F

Plasmid isolation and Restriction Digestion analysis : To confirm presence of insert in pGEMT vector.

Restriction Digestion Reaction: 37°C 6hrs

10X res dig buffer: 2.5µl
Plasmid : 10µl
NdeI : 1µl
XhoI : 1µl
Mq : 10.5µl
Total rxn Volume : 25µl

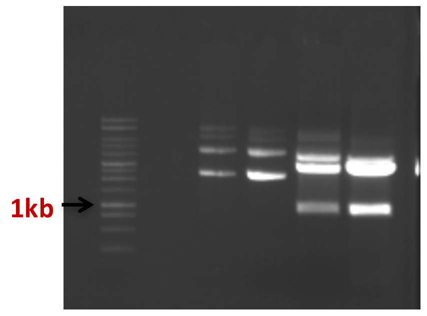


Fig:-7.Restriction Digestion analysis
Asparaginase (AF)pGEMT DH5α 1 and 2

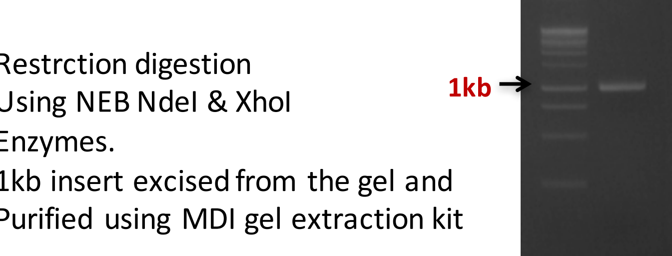


Fig:-8.Gel Purified1kb insert
Before ligation in pET22b+

Figure XXIV: Plasmid isolation and restriction digestion analysis

Experiment: G, Cloning of Asparaginase in pET22b+

TableXIV : Rosetta/BL21 & DH5a transformation using ligation mix.

Ligation Reaction

10X NEB ligation buffer	2µl
Insert	3µl
Vector	2µl
T4 DNA ligase	1µl
MQ	12µl
Total reaction volume	20µl.

O/N 16°C ligation.

We have completed transformation in DH5a. We are now working to express our gene of interest into an expression host BL21 for further study.L-Asparaginase is an injectable drug used for the treatment of tumors. The sensitivity of the application of this enzyme requires high degree of purity, so the simple isolation and purification procedure will be an advantage for developing L Asparaginase as drug. This is better to have low Km of the enzyme in micromolar range to work (54) as serum Asparagine is about ~50 µM concentration in serum (55). The Periplasmic extract has shown only one band of 43Kd, this may be because of L asparagine presence in media induced more production of type II enzyme in this organism. SDS gel is stained with Coomassie blue (G-250) which has Sensitivity of 2ng/band staining. The other proteins are in very very low concentration which can be stained by silver staining having sensitivity of 0.2~0.5µg/band.L Asparaginase enzyme in this present study has shown very low Km.

Innovations shown by Project SHC 312:

1. This was confirmed by bioinformatics work and with experiments that *Alcaligene faecalis* has only one type of L Asparaginase enzyme, that is type II periplasmic and not of two type of L Asparaginase as reported previously.
2. A Periplasmic enzyme purification procedure is standarized in this present study, which is highly reliable and stable procedure and had shown one single band of L Asparaginase protein in SDS PAGE when visualized with Coomassie blue.
3. Type II periplasmic L Asparaginase enzyme is characterized and had shown very Low Km which is very good for its use as an anticancer agent.
4. L Asparaginase enzyme gene from *Alcaligene faecalis* has been cloned in to pET22b and DH5aand finally into BL21/Rossetta in this project for modification and stability.

Conclusion and Future direction:

1. At present we are trying to express L Asparaginase gene in to BL21/Rossetta expression vector that might be modified to be suitable for clinical purpose.
2. Purification procedure may be further modified for better result and cost effective production

3. kinetic parameters of enzyme: Modified enzyme may be tested on different type of cancer cells for its effect**.**

Acknowledgement

This gives me immense pleasure to acknowledge support and encouragement by prof. R.P. Singh as mentor in this project. There are many people needs to be mentioned here for their unconditional support and guidance. Ms. Vaishali Verma from Prof.V.K Chaudhary laboratory is acknowledged for developing and standardizing protocol for Enzyme purification. Prof. Chaudhary, department of Biochemistry has always supported this project with his experience and guidance. Dr. Saurabh Raghuvanshi from plant Molecular Biology is our mentor to solve all bioinformatics problems and design cloning for the project. Many thanks to Prof. Aloo nag, Department of Biochemistry for helping us in ultrasonication and providing us pET28a. Thanks to Prof. Suman Kundu and his students for helping us for in gel picture and doing native gel Electrophoresis.

This innovation project work was financially supported by University of Delhi. we are very thankful to the coordinator of innovation desk for their positive and immediate response and support.We are thankful to technical staff, department of Biochemistry, account department and administration for supporting this work.

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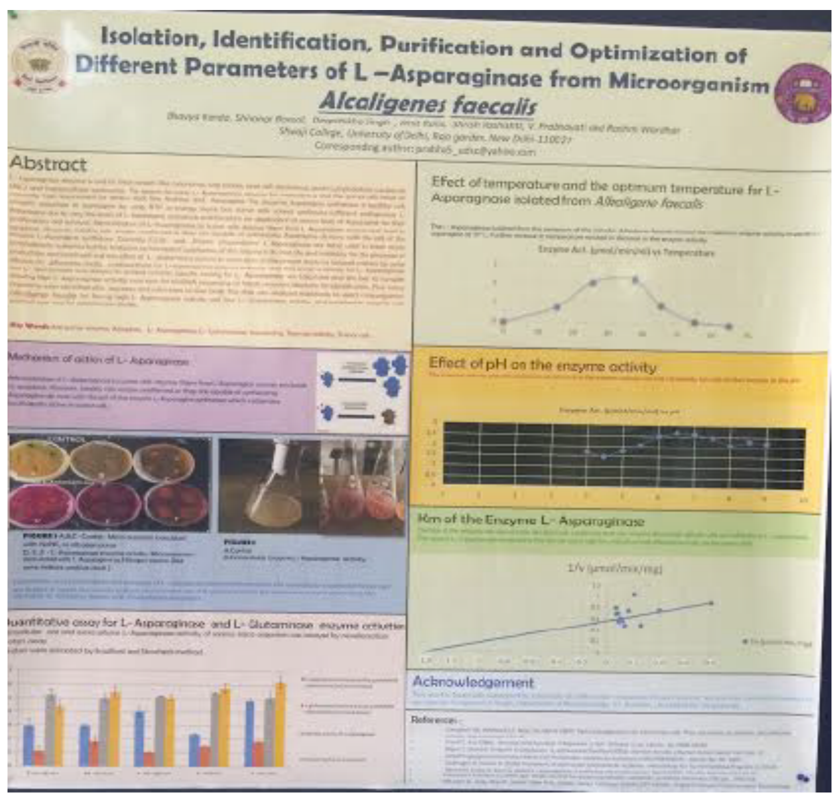
Publications and Poster presentations

1. Rashmi Wardhan , V.Prabhavathi , Shivangi Bansal, Deepshikha , Bhavya , Amit Rana, Shrish, Shivani Dutt, Abhishek , Diksha Bisht, Ashutosh Singh , Vibhu Mishra, (2016) Isolation and Identification of microorganisms with high activity of L –Asparaginase : Anticancer enzyme , **DU Journal of Undergraduate Research and Innovation** Volume Issue 1 pp 171-179, 2016 , Research Innovation ISSN-2395-2334 .

2. **Poster presentation** : Rashmi wardhan , V.Prabhavathi , Shivangi Bansal, Deepshikha , Bhavya , Amit Rana, Shrish,Shivani Dutt, Abhishek , Diksha Bisht, Ashutosh Singh ,Vibhu Mishra, **(2016**) Isolation ,identification ,purification and optimization of different parameters of L Asparaginase from microorganism *Alcaligene faecalis*. Poster presented in **Innovation conclave 2016**, Nurturing research at UG level, organized by **ANDC Delhi University** on 25th -26th oct. 2016.



Poster Presentation at ANDC, **This poster got first prize in Biological Sciences.**

3. Poster presentation: Rashmi Wardhan ,V.Prabhavathi , Shivangi Bansal, Deepshikha ,Bhavya , Amit Rana, Shrish,Shivani Dutt, Abhishek , Diksha Bisht, Ashutosh Singh ,Vibhu Mishra **(2015),**Screening isolation and confirmation of L Asparaginase from microorganism in sewage water. poster presented in **National symposium on trends in research and innovation in life sciences at undergraduate level** on **30th march 2015, at DDU College**, University of Delhi.

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DU Journal of Undergraduate Research and Innovation Volume 2, Issue 1 pp 171-179, 2016

Isolation and Identification of Microorganisms with High Activity of L–Asparaginase: Anti-Cancer enzyme

Rashmi Wardhan*, V. Prabhavathi, Shivangi Bansal, Deepshikha Singh,
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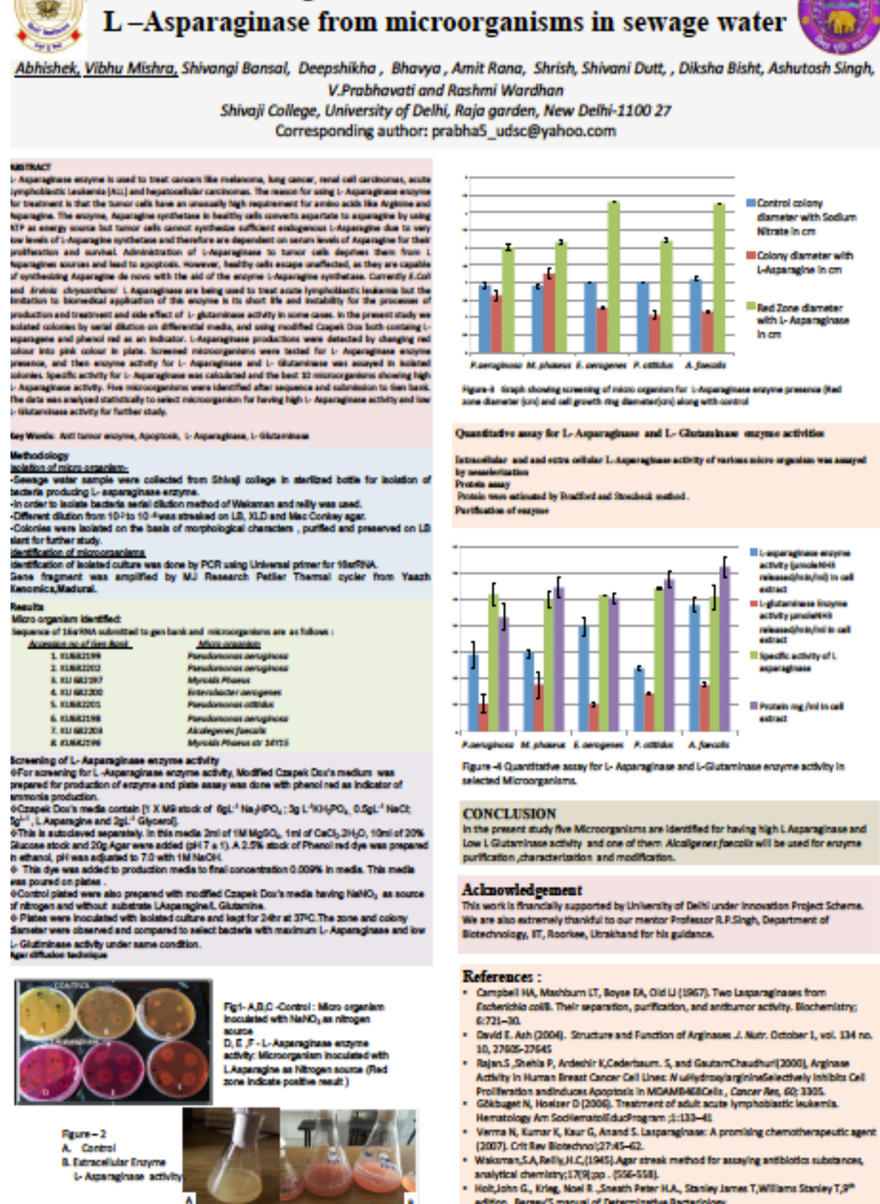
ABSTRACT
L-Asparaginase (EC 3.5.1.1) belongs to a group of homologous amidohydrolases family, which catalyses the hydrolysis of the amino acid L-Asparagine to L-Aspartate and ammonia**.**L- Asparaginase enzyme is used to treat cancers like melanoma, lung cancer, renal cell carcinomas, acute Lymphoblastic Leukemia (ALL) and hepatocellular carcinomas. The reason for using L- Asparaginase enzyme for treatment is that the tumor cells have an unusually high requirement for aminoacids like Arginine and Asparagine. The enzyme, Asparagine synthetase in healthy cells converts aspartate to asparagine by using ATP as energy source but tumor cells cannot synthesize sufficient endogenous L-Asparagine due to very low levels of L-Asparagine synthetase and therefore are dependent on serum levels of Asparagine for their proliferation and survival. Administration of L-Asparaginase to tumor cells deprive them from L Asparagines sources and lead to apoptosis. However, healthy cells escape unaffected as they are capable of synthesizing Asparagine de novo with the aid of the enzyme L-Asparagine synthetase. Currently *E.Coli*and *Ervinia chrysanthami* L Asparaginase are being used to treat acute lymphoblastic leukemia but the limitation to biomedical application of this enzyme is its short life and instability for the processes of production and treatment and side effect ofL- glutaminase activity in some cases. In the present study we isolated colonies by serial dilution on differential media, screened them for L- Asparaginase enzyme presence, and then enzyme activity for L- Asparaginase and L- Glutaminase was assayed in isolated colonies. Specific activity for L- Asparaginase was calculated and the best 10 samples showing high L- Asparaginase activity were sent for 16SrRNA sequencing to Yaazh xenomics,Madurai for identification.Five micro-organisms were identified after sequence and submission to Gen bank. The data was analyzed statistically to select micro-organism for having high L- Asparaginase activity and low L- Glutaminase activity for further study.



Poster presentation at DDUC, University of Delhi

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Dr. Arijit Chowdhuri
Assistant Professor

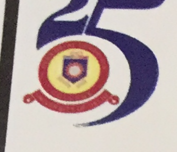
Dated : 28th October 2016

TO WHOMSOEVER IT MAY CONCERN

This is to certify that Poster presentation with title "Isolation, Identification, Purification and Optimization of different parameters of L Asparaginase from microrganism Alcaligene faecalis has been awarded first prize under the Life Sciences category at the Innovation Conclave - 2016: Nurturing Research at the UG level held from 25 – 26 October 2016 at Acharya Narendra Dev College (University of Delhi).

The details of the authors of aforementioned presentation (LS - 20) listed on page 61 of the abstract book are as follows:
Amit Rana, Shivangi Bansal, Deepshikha, Bhavya, Shrish, V. Prabhavathi*, Rashmi Wardhan# - Shivaji College, University of Delhi, Raja garden, New Delhi – 110027

(Arijit Chowdhuri)
Convener
Innovation Conclave – 2016



NATIONAL SYMPOSIUM

on

"Trends in Research and Innovations in Life Sciences at Undergraduate level"

organized by

Department of Zoology
Deen Dayal Upadhyaya College (University of Delhi)
Karampura, New Delhi-110015

CERTIFICATE

Dr. /Mr. /Ms. Prabhavathi ....from ...... Shivaji College ....

has participated/presented poster entitled ......Screening, Isolation and Conformation of L-Asparaginase .... Sewage Water ...in the symposium.

Dr. S.K. GARG
(Patron)

30th March, 2016

Dr. ANITA GULATI
(Convenor)

Pictures related to the project.

Students are interacting with staff and volunteers about patients

Visit to Rajiv Gandhi Cancer Institute & Research Centre

Doctors are briefing to students

Students working in lab

Utilization Certificate

Innovation Project 2015-16
SHC – 312

Project Title L-Asparaginase, An Anti Tumor Agent : Production, Characterization And Molecular Approaches

Audited Financial Statement under Innovation Project scheme

College: Shivaji College

Project Investigators: Dr. Rashmi Wardhan, Dr. Prabhavathi

Grant Sanctioned Rs	Rs. 7,00,000/- (Rupees Seven Lacs Only)		
	Grant Received	**Grant Utilized**	**Unspent Grant**
Equipments/Consumables	4,25,000/-	5,42,933/-	(1,17,933)*
Travel	55,000/-	NIL	55,000/-
Stipend	1,20,000/-	95,000/-	25,000/-
Honorarium	25,000/-	25,000/-	NIL
Stationery	20,000/-	2,930/-	17,070/-
Contingency	55,000/-	8,911/-	46,089/-
Total	**7,00,000/-**	**6,74,774/-**	**25,226/-**
Total amount utilized	Rs. 6,74,774/- (Rupees Six Lacs Seventy Four Thousand Seven Hundred Seventy Four Only)		
Amount remaining Rs. (In figures and words)	Rs. 25,226/- (Rupees Twenty Five Thousand Two Hundred Twenty Six Only)		

Certified that out of **Rs. 7,00,000/- (Rupees Seven Lacs Only)** sanctioned to Innovation Project Code **SHC-312, Rs. 6,74,774/- (Rupees Six Lacs Seventy Four Thousand Seven Hundred Seventy Four Only)** has been utilized during the period of the project. The remaining amount **Rs. 25,226/- (Rupees Twenty Five Thousand Two Hundred Twenty Six Only**) and is being returned back to the University.

Note : Over expenditure under the head "Equipment/ Consumables has been met from unspent balance in Travel, Stationery and Contingency with prior approval from the Innovation Desk.

(Dr. Rashmi Wardhan) **(Dr. Prabhavathi)**

Principal

University of Delhi

RC/2015/9435 31 August, 2015

The Principal,
Shivaji College
Ring Road, Raja Garden,
New Delhi-27

Subject: - **Innovation Projects 2015-16**

Dear Principal,

The University of Delhi is pleased to announce the third round of the undergraduate research initiative in colleges, Innovation Projects 2015-16. You will be glad to know that the following project submitted by your college has been selected for award

Project Code: SHC 312
Project Title: L-Asparaginase, An Anti Tumor Agent: Production, Characterization And Molecular Approaches

The distribution of grant under different budget heads as below:

Sr. No.	Budget Head	Amount
1.	Equipment/Consumables	Rs 3,25,000/-
2.	Stipends	Rs. 1,20,000/- (1000x10x12)
3.	Travel	Rs 55,000/-
4.	Honorarium	Rs 25,000/-
5.	Stationery/Printing	Rs 20,000/
6.	Contingency	Rs 55,000/-
	Total	Rs 600,000/-
Rs 6 lakhs (Rupees six lakhs only)		
Amount to be released in first phase by Finance Branch- Rs 450,000/		

Budget head No. 1 and half of the remaining grant will be released as the first instalment. The second and final instalment will be released after submission of half-yearly report (by 15 February 2016), satisfactory review and recommendation of release of the second instalment.
Please refer to the detailed guidelines for implementation of the project. Any queries may be addressed to- innovationprojects1516@gmail.com.

With best wishes,

Yours sincerely,

Prof. Malashri Lal