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, Bhavya, Amit Rana, Shrish, Shivani Dutt, Abhishek, Diksha Bisht, Ashutosh Singh,Vibhu Mishra
rashmiwardhan56@gmail.com
Shivaji College, University of Delhi, New Delhi

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.

Key Words: Anti-tumor enzyme, Apoptosis, L-Asparaginase, L-Glutaminase, PCR, Sequencing, Thermal stability, Tumor cells.

INTRODUCTION

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-Asparagine is a non-essential amino acid used by normal and cancer cells for their proliferation (1,2).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 acidslike Arginine

and Asparagineand cannot synthesize sufficient endogenous L-Asparagine due to very low levels of L-Asparagine synthetase present in tumor cells. Therefore, these cells are dependent on serum levels of Asparagine for their proliferation and survival.One more attributed reason to this is the inability of tumor cells to increase L-asparagine synthetase activity after L-Asparaginase administration. So the tumor cells use Asparagine from the diet (blood serum) as well as what they make themselves (which is limited) to satisfy their large L-Asparagine demand(3).Thus, administration of L-Asparaginase deprives dependent tumor cells of their extracellular source of L-Asparagine 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. 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 carcinomas(4, 5)but limitation to biomedical use of this enzyme is its short life for the processes of production, thermal instability and dual substrate specificity with L-Glutamine and rapid plasma clearance**.**Asparagine and Glutamine differ structurally with only one methyl group, and hence L-Asparaginases will have dual substrate specificity and thus results in decreasing concentrations of both amino acids in the body. Due to the L-Glutaminase side activity of L Asparaginase, patients may suffer from various side effects such as leucopenia, immuno-suppression, acute pancreatitis, hyperglycemia and neurological seizures. Various microorganism hadshownvarious degree of toxicity related to L-Glutaminase side effectand have different level of L- Asparaginase enzyme activity, that's why these microbes are of great use for production and modification of the enzyme for clinical use without these side effects(6).The aim of this present study is to isolate and identify microorganisms from sewage water showing high L -Asparaginase activity and lowest L- Glutaminase activity for modification for increasing thermal stability and enzyme activity,so this can be used for clinical purpose to the maximum. Identification is done by16SrRNA sequencing. Quantitative assay for L-Asparaginase and L-Glutaminase activity are done after primary and secondary qualitative enzyme activity screening of selected microorganisms for selection of microorganisms.

METHODOLOGY

Luria Bertoni, MacConkey and XLD differential media were purchased from Hi media Laboratory. L-Asparagine, L- Glutamine,Tris -HCl buffer and Nesseler's reagent were purchased from Merck,Germany. Other chemical were purchased from CDH of analytical grade.

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(7) was used. 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 (8) purified and preserved on master plates and on LB slant for further study.

Screening of L- Asparaginase enzyme activity

For screening for L -Asparaginase enzyme activity, Modified Czapek Dox's medium(11)was prepared for production of enzymeand plate assay was done with phenol red as indicator of ammonia production .Czapek Dox's media contain [1 X M9 stock of $6gL^{-1}$ Na_2HPO_4;3g L^{-}

[1]KH_2PO_4;0.5gL^{-1} NaCl;5g^{L-1} ,L Asparagine and 5gL^{-1}]. M9 stock is autoclaved separately. In this media sterilized 2ml of 1M $MgSO_4$, 1ml of $CaCl_2.2H_2O$,10ml of 20% Glucose stock and 20g of Agar were added (pH 7 ± 1). A2.5% stock of Phenol red dye was prepared in ethanol, 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 5mmcontainingmodified Czapek Dox'smedia as mentioned above.The filterate was allowed to diffuse for 24hr at 37_0C. The color of plates were observed to see L- asparaginase activity(12).

Quantitative assay for L- Asparaginase and L- Glutaminase enzyme activities:The enzyme activity was assayed by nesselerization (13,14).The culture were inoculated in 500ml Erlenmeyer flask containing 150ml of Czapek Dox's media. The flasks were incubated at 37^0C,125rev min $^{-1}$ for 24 hrs in controlled environment incubator shaker. Uninoculated media serves as control. After 24hrs the cultures were centrifuged at 6500 rpm for 10 min at 4^0C.The cells were harvested. The cells were suspended in 0.1M Tris -HCl buffer,pH 8.6 with 20% glycerol and PMSF protease inhibitor cocktail (10mM stock) from sigma was added to final concentration of2mM. 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 were collected and stored at -20^0C in deep freezer for further assay. For enzyme activity,0.5 ml of Tris HCl buffer pH 8.6,0.5 ml of 0.04 M 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 reaction, centrifuged at 10,000rpm for 10 min. 0.1ml supernatant was taken into fresh tube and volume was made up to 2.7 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.

Protein assay

Protein was estimated by Bradford and Stoscheck method (15,16).1mg BSA per ml was used for standard. The linearity was found from 5µg to 50 µg of protein. Absorbance was taken at 595nm.

Identification of isolated culture**:** Identification of isolated culture was done by PCR using Universal primer for 16SrRNA. Genomic DNA was isolated using Insta gene ^{TM} Matrix Genomic DNA isolation kit. Using universal primer27FAGAGTTTGATCMTGGCTCAG and 1492R TACGGYTACCTTGTTACGACTT ,16S rRNA 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 (9, 10,). Sequences were submitted to Gen bank and microorganisms were identified.

RESULTS

A. Primary and secondary Screening for L- Asparaginase enzyme

In the present study10colonies isolated from sewage water of Shivaji College, had shown dark red zone in screening analysis on Modified Czapek Dox's modified agar plates.These colonies had shown Pink or red zone in different intensities.Pink or red color is because of L-Asparaginase enzyme activity. LAsparaginase hydrolyze Asparagine to Aspartic acid and release ammonia, that increases pH of media.Phenol redchanges color from yellow to pink or red because of alkaline pH by ammonia.(Figure-I).

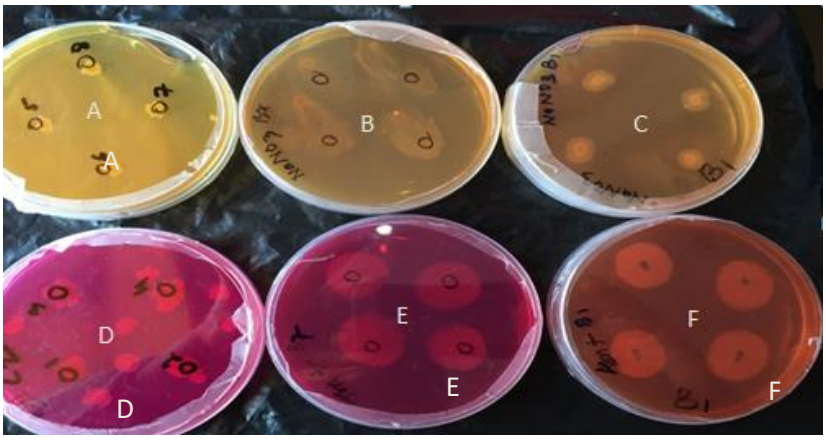

Figure-I Isolated Micro organism showing L- Asparaginase activity. A, B and C, Control with $NaNO_3$ as nitrogen source. D,E and F with L- Asparagine in media . Red zone indicates positive result for L- Asparaginase enzyme presence.

Figure – II A. Control B. Extracellular Enzyme L- Asparaginase activity

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

16srRNA sequencing analysis confirmed five organisms from these isolated colonies,so data is given for identified five colonies only.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.(TableI , Figure-III).

Table- I Screening of micro organism 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 (Growth of microbes) ,with L Asparagine	Red zone diameter(cm) media, L-Asparaginase presence, with L Asparagine
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.	*Enterobactera erogenes*	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.	*Alcaligene faecalis*	2.100±0.041	1.159±0.008	4.250±0.000

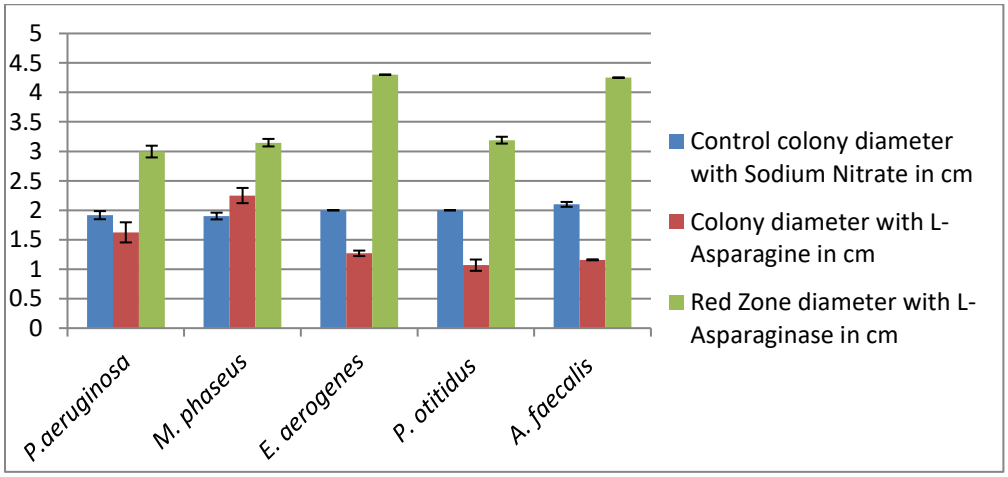

Figure- III Graph showing screening of micro organism for L Asparaginase enzyme presence (Red zone diameter (cm) and cell growth ring diameter(cm) along with control.

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

Enzyme activity for L- Asparaginase 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 micro organisms were also calculated .The data was statistically

analysed 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±0.03in *Myroid phaeus* to *pseudomonas aeruginosa*(0.522±0.040).*Enterobacter aerogenes* had shown lowest L-Glutaminase activityamong five that is 0.101±0.006.L- Glutaminase activity was low in all the micro organisms in comparison to L- Asparaginase.(Table II, Figure-IV).

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

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

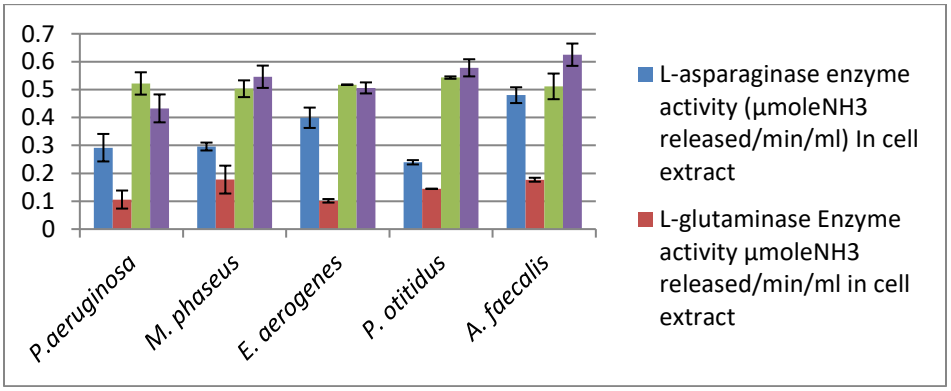

Figure -IV Quantitative assay for L- Asparaginase and L-Glutaminase enzyme activity in selected Microorganisms.

C. Identification of Microorganisms

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

S.No.	Gen Bank accession No	Name of microorganism
1.	KU682196	*Myroids phaeus str* 14 Y15
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*

DISCUSSION

In present study all selected five microorganism *Pseudomonas aeruginosa, Pseudomonas otitidus, Enterobacter aerogenes, Myroid phaseus* and *Alcaligenes faecalis* had shown high L-Asparaginase enzyme activity in Czapek Dox'S media and two microbes *Alcaligenes faecalis and Pseudomonas aeruginosa,* had extracellular L- Asparaginase activity. *Alcaligenes faecalis* and *Pseudomonasaeruginosa* are reported earlier also for high enzyme activity and showing two type of L- Asparaginase I and II(19,20). These two microorganism will be more useful for further characterization because of the presence of class I and classII L-Asparaginase in them.Bacterial sources proved to be an abundant source of L-Asparaginase as they are easy to manipulate. The two organisms *Alcaligenes faecalis* and *Pseudomonas* are showing two types of L- Asparaginase activity so these two may be used for further study and their activity can be compared with other organisms like *E.coli* and *Ervinia chrysanthami* ,which are presently being used for lymphoma treatment and other endophytic bacteria showing high L Asparaginase activity(4,21,22,and 23) but L-Asparaginase enzyme gene of *pseudomonas aeruginosa* has already being tried for cloning and expression (21)so *Alcaligenes faecalis* may be new organism to explore for its enzyme modifications and comparisons for future .

CONCLUSIONS

In the present study five Microorganisms are identified for having high L Asparaginase and Low L Glutaminase activity and one of them *Alcaligenes faecalis* will be used for enzyme purification, characterization and modification.

ACKNOWLEDGEMENTS

This work is financially supported by University of Delhi under Innovation Project Scheme. We are also extremely thankful to our mentor Professor R.P. Singh, Department of Biotechnology, IIT, Roorkee, Utrakhand for his guidance.

REFERENCES

1. Campbell, H.A.Mashburn L.T.Boyse E.A. Old L.J (1967). Two L-Asparaginases from *Escherichia coli*B. Their separation, purification, and antitumor activity. *Biochemistry*, Vol 6: pp (721–30).
2. David, E. A.(2004). Structure and Function of Arginases .*J. Nutr*. Vol. 134(10): pp(2760S-2764S)
3. Rajan,S .Shehla ,P. Ardeshir, K.Cederbaum. S. and Chaudhuri, G.(2000).Arginase Activity in Human Breast Cancer Cell Lines: *N* ωHydroxylargininеSelectively Inhibits Cell Proliferation andInduces Apoptosis in MDAMB468Cells. *Cancer Res,* Vol.60:pp (3305)
4. Gökbuget, N., Hoelzer ,D., (2006). Treatment of adult acute lymphoblastic leukemia. Hematology.*Am SocHematolEducProgram*Vol1: pp (33–41)
5. Verma, N.Kumar, K. Kaur ,G. Anand, S. Lasparaginase: A promising chemotherapeutic agent (2007). *Crit Rev Biotechnol* Vol. 27: pp(5–62).
6. Zen, X.Li1,Y. Fan, J. Zhao, H. Xian,Z.Sun,Y.Wang,Z.Wang,S. Zhang, Gand Ju.D. (2013). Recombinant human Arginase induced caspase dependent apoptosis and autophagy in non hoddgkin,s lymphoma cells. *Cell Death and Disease*. Vol 4: pp (e840) doi:10.1038/cddis.2013.35913.
7. Waksman, S.A.Reilly,H.C. (1945).Agar streak method for assaying antibiotics substances.*Analytical chemistry*.Vol 17(9):pp(556-558).
8. Holt,J.G.Krieg, Noel, R. Sneath Peter , H.A.Stanley James ,T.Williams ,S. T. Bergey'S manual of Determinative Bacteriology. 9th edition, Baltimore, USA, Waverly press, INC.
9. Edward, U.Rogall.T, Blocker ,H. Emde, M. Bollger E.C(1989). Isolation and direct complete nucleotide determination of entire genes:characterization of a gene encoding for 16srRNA;*Nucleic acid research* 17: (7843-7853).
10. Edger R c: MUSCLE: multiple sequence alignment with high accuracy and high through put(2004). *Nucleic acid research*32(5).: (1792-1797).
11. GulatiR, Saxena R.K and Gupta R.(1997). A rapid plate assay for screening L-asparaginase producing micro organism.*LettApplMicrobial*24:(23-26).
12. Jain, R.Z aidt K.V. Verma Y. Saxena P. (2012).L–Asparaginase :A promising enzyme for treatment of acute Lymphoblastic leukemia,*People's Journal of scientific Research*Vol 5(1):pp(29-35)
13. Imada ,A,Igarasi,S,Nakahama,K. and Isono ,M. (1973) .Asparaginase and glutaminase activities of microorganism ;*Journal of General Microbiology*; 76:(86-89).
14. Meshburn, L.T. Wriston,J.C.(1964),Tumor inhibitory effect of L-asparaginase from E.coli, *Archives of Biochemistry and Biophysics*Vol105:pp(450-452).
15. Bradford,M.M.(1976). Rapid and sensitive method for the quantitation of microgram quantitiesof protein utilizing the principle of protein dye binding ,*Anal Biochem*.Vol 72: pp(248-254).
16. Stoscheck ,C.M,(1990).Quantitation of protein, *Methods in Enzymology*Vol 182: pp(50-69).
17. Warwll,R.P. Jr Arlin, Z.A.Gee ,T.S, Chou,T.C.Roberts, J.Young, C.W.(1982),Clinical evaluation of Succinylated Actinobacter Glutaminase, Asperginase in adults leukemia;*Cancer Treat Rep* Vol 66(7):pp(1479-1485).
18. Kafkewitz,D.Bendich,A.(1983).Enzyme induced Asperginaseand glutamine depletion and immune system function;*Am J clinical Nutr*;Vol 37(6):pp(1025-1035).
19. Hiroko Sakato, and Kenji soda, (1970).Asparaginase of Alcaligenes faecalis,*Bull.inst.Chem.Res,Kyoto univ*48(6): (283-292).
20. Komathi, S. Rajalakshmi, G. Savitha,S.and Balaji, S. (2013).Isolation ,production and partial purification of L Asparaginase from Pseudomonas aeruginosa by solid state fermentation,*Scholar Academic Journal Of Pharmacy* Vol2(2): pp(55-59)

21. Kishore, V. Nishita,K.P. and Manonmani,H.K. (2015). Cloning, Expression and characterization of L A sparaginase from pseudomonas fluorescence for large scale production in *E.coli* BL21,3*Biotech*Vol 5:pp(975-981). DOI10.1007/s13205-015-0300-y
22. Rudrapati,Pallavi. Audipudi, Amrutha ,V.(2015).Characterization and bioprocessing of oncolytic enzyme L –Asparaginase isolated from marine Bacillus AV14.*International Journal of Pharm.Sci.Rev.Res.*Vol 30(2):pp(195-201)
23. Renuka ,D .Joshi,Nikhilesh, S Kulkarni, (2016). Optimization studies on L-Asparaginase production from endophytic bacteria .*International Journal of Applied Science* .Vol 2(3):pp(624-629)