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L–Asparaginase an Antitumor Agent: Identification from different Microorganisms

V. Prabhavathi[1] and Rashmi Wardhan[2]

Shivaji College, University of Delhi, Raja garden, New Delhi-1100 27
E-mail: [1]prabha5_udsc@yahoo.com

Abstract—*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 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 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, 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 microorganism for having high L- Asparaginase activity and low L- Glutaminase activity. Based on enzyme activity these arganisms are used for further studies.*

Keywords*: Anti tumor enzyme, Apoptosis, L- Asparaginase, L-Glutaminase, Sequencing, Thermal stability, Tumor cells.*

1. 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- 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 *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 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, 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.

2. METHODOLOGY

Identification of microorganisms

Identification of isolated culture was done by PCR using Universal primer for 16srRNA.Gene fragment was amplified by MJ Research Petlier Thermal cycler from **Yaazh Xenomics,Madurai.**

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 (1) 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 (2) purified and preserved on master plates and on LB slant for further study.

Isolation of micro organism-

- 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.
- Different dilution from 10^{-2} to 10^{-5} was streaked on LB, XLD and Mac Conkey agar.
- Colonies were isolated on the basis of morphological characters , purified and preserved on LB slant for further study.

Screening of L- Asparaginase enzyme activity: For screening for L -Asparaginase enzyme activity, Modified Czapek Dox's medium (5) 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 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 Agar were added (pH 7 ± 1). A 2.5% 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 were observed and compared to select bacteria with maximum L- Asparaginase activity.

Agar well diffusion technique: 8µl of cell free culture was poured into agar with well diameter of 5mm prepared in plates containing modified Czapek Dox's media.The filterate was allowed to diffuse for 24hr at 37_0C. The color of plates were observed to see L- asparaginase activity (6)

Identification of isolated culture: Identification of isolated culture was done by PCR using Universal primer for 16SrRNA. Genomic DNA was isolated using Insta gene ™ matrix Genomic DNA isolation kit. Using universal primer 27FAGAGTTTGATCMTGGCTCAG and 1492R TACGGYTACCTTGTTACGACTT ,16S rRNA gene fragment was amplified by MJ Research Petlier Thermal cycler. PCR product were purified by using Montage PCR clean up kit (Millipore).PCR product were purified and single pass sequencing was performed using 785F GGATTAGATACCCTGGTA and 907 R CCGTCAATTCMTTTRAGTTT by Yaazh Xenomics. Sequence data was aligned and analyzed for identifying the sample by Yaazh Xenomics, Madurai. The program MUSCLE 3.7 is used for multiple alignments of sequences (3,4). Sequences were submitted to Gen bank and micro organisms were identified.

3. RESULTS

Identification of Micro organisms: 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* **(TableI).**

Primary and secondary Screening for L- Asparaginase enzyme

In the present study 10colonies 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. 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. **(Fig. I).**

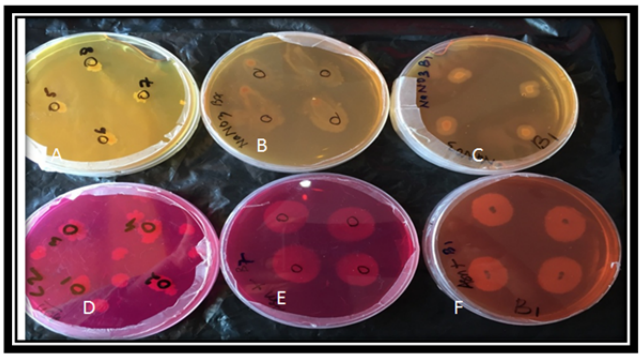

Fig. I Isolated Micro organism showing L- Asparaginase activity. A, B and C, Control with $NaNO_3$ as nitrogen source. D,E and F with L- Asparagine in media . Red zone indicates positive result for L- Asparaginase enzyme presence.

Later on 16srRNA sequencing analysis confirmed five organisms from these eight micro organisms, so data is given for these 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. **(TableII , Fig. II).**

Table I: Identification of microorganism by 16SrRNA gene sequencing

SNo.	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*

Table II: 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 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

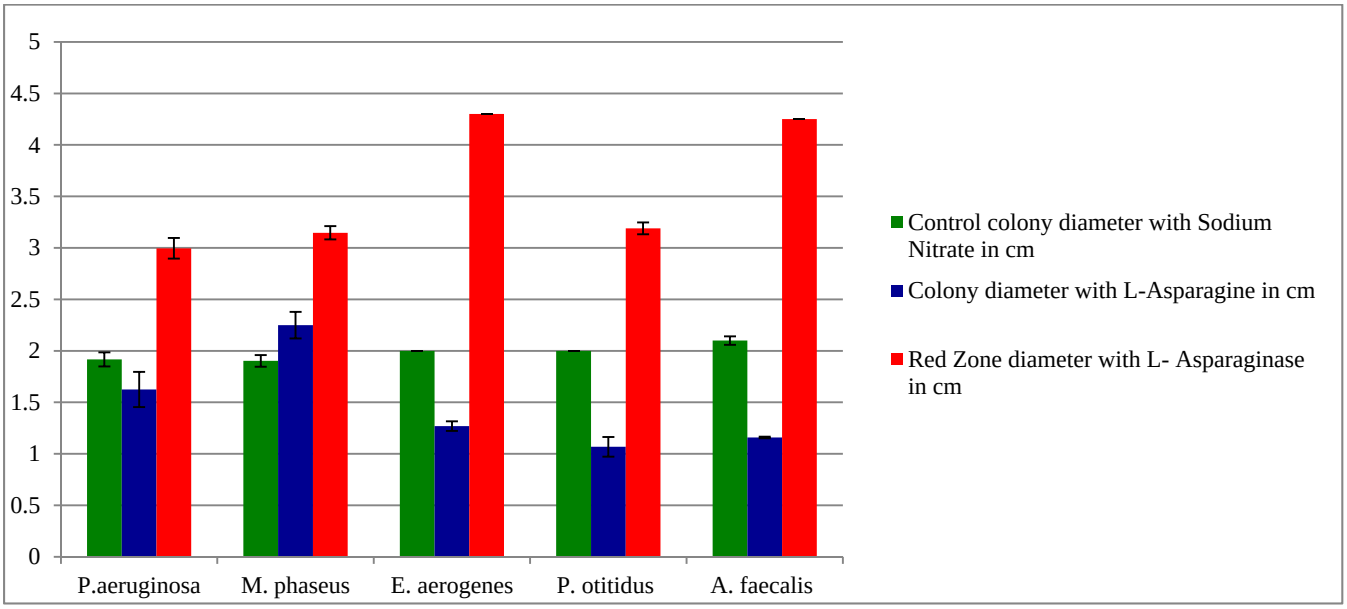

Fig. II: Graph showing screening of micro organism for L Asparaginase enzyme presence (Red zone diameter (cm) and cell growth ring diameter(cm) along with control

4. 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 *Pseudomonas aeruginosa* are reported earlier also for high enzyme activity and showing two type of L- Asparaginase I and II (7,8). 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 are preferable organism for further study.

5. CONCLUSION

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.

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