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L-Asparaginase – A prospective anticancer enzyme on MCF-7 cell lines extracted from soil Microflora

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ABSTRACT

This study aims at investigating the soil bacteria for L asparaginase enzyme production and its anti-cancer effect on MCF 7 cell line. In this study, 30 soil samples were screened for bacterial producing L asparaginase enzyme. The soil samples were collected from various places in Ethiraj College, Chennai. Isolation was done using spread plate technique, biochemical and microscopic characterization for the potential isolates revealed the bacterial genera as *Bacillus, Escherichia* and *Pseudomonas* species. Of the thirty samples collected, six isolates produced pronounced L asparaginase enzyme. These samples were further subjected to crude enzyme extraction and estimation. The isolates S2 and Ni5 showed maximum production of enzyme with 1090 units/ml of activity. Dialysis was carried out to concentrate the enzyme and molecular characterization was performed using SDS PAGE to separate the proteins according to the molecular weight which was found to be approximately 100 to 116 kDa. Anti-cancer activity was evaluated on MCF 7 tumour cell line. The cell viability was dose dependent and IC_{50} of S2 was 62.5 µg and Ni5 31.2 µg respectively.

Key words: *MCF 7 cell line, Anti-tumour effect, SDS PAGE, Crude enzyme extraction, M9 medium, Pseudomonas* spp.

Introduction

L -asparaginase, an enzyme plays a predominant role in treating certain types of tumours like lymphocytic leukaemia, Hodgkin lymphoma etc (Egler *et al.*, 2016 and Zheng *et al.*, 2018). L-asparaginase from *E. coli* and *Erwinia chrysanthemi* are approved as a drug for clinical use in the treatment of leukaemia (Salzer *et al.,* 2014 and Thandeeswaran *et al.,* 2016). L Asparaginase enzyme works by breaking down asparagine which is essential for cancer cell growth. In cancer cells, asparaginase is lacking and treating the patient with asparaginase helps in hydrolysing serum asparagines (Cachumba *et al.,* 2016). This enzyme can be produced from different microbial sources such as bacteria, fungi, yeast and actinomycetes (Prasad *et al.,* 2014). It can also be isolated from soil microflora. *Pseudomonas aeruginosa* is one such organism from which L Asparaginase can be isolated (Subhashini and Vani, 2020). Long term usage of this enzyme results in side effects particularly in remission induction phase like hypersensitivity, low blood pressure, stomach cramps, diabetes, drug induced liver disease, pancreatitis etc (Wang *et al.,* 2009). This study process involves screening of L asparaginase enzyme from soil bacterial isolates and to study its antitumor effect on MCF-7 breast cancer cell lines. This paves the way for finding out a new microbial source for enzyme production with lesser side effects.

Materials and Methods

Collection of Soil Samples

Soil samples were collected in rhizosphere region at different locations of Ethiraj College campus and

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named as S1-S10, M1-M10 and Ni1-Ni10 respectively. The samples were collected in a sterile container and stored for further use.

Isolation of soil bacteria

The samples collected were processed by spread plate technique. 1g of soil was taken and diluted in 9ml of sterile saline and serial dilution was made up to the dilution factor of 10^8. 0.1 ml of soil sample from tube 7 and 8 was taken aseptically and transferred onto a sterile M9 minimal medium with asparaginase and spread evenly with the help of sterile L rod. The plates were incubated at room temperature for 48 to 72 hours. The colonies were sub cultured on Nutrient Agar plate and colony morphology of the organism was observed. Preliminary tests and biochemical characterization were done to reveal the bacterial genera.

Enzyme extraction and estimation

The isolates were inoculated aseptically onto sterile M9 Broth and incubated at 37 ºC for 24 hours. 0.5 ml of 24 hour culture was added to 5 ml of M9 medium and kept on shaker bath for 48 hours at 30 ºC as shown in the Figure 1. Then it was centrifuged at 2000 rpm for 20 minutes using cold centrifuge. Cell free supernatant was collected and used further.

Fig. 1. Quantitative Determination of Asparaginase-Enzyme Produced in M9 Medium.

Standard Graph for Ammonia Estimation

132 mg of Ammonium Sulphate was added onto 100 ml of distilled water. 5 ml of stock solution was made up to 50 ml using distilled water (1:10 dilution). Two ml of Tris buffer from the respective dilutions were taken and added to another tube and 1ml of diluted samples were added to it. Then 2.5 ml of Trichloroacetic acid was added to all the tubes. 1ml of NaOH and 0.2 ml of EDTA was added, incubated for 2 minutes and colour development was observed after adding 0.5 ml of Nessler's reagent. Optical density was recorded at 450 nm.

0.5 ml of cell suspension was taken and 1ml of 0.01 ml of tris buffer, 0.5 ml of 0.04 ml asparaginase was added onto it and incubated at 37 ºC. The reaction was terminated by addition of 0.5 ml of 0.1N TAA. Later, it was centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and 1 ml of 1N NaOH, 0.2 ml of 0.1M EDTA was added and kept for 2 minutes. 0.5 ml of Nessler's reagent was added and the colour change was recorded as optical density at 450 nm (Imada *et al.*, 1973).

Calculation

$$\%\text{Units/ml} = \frac{\text{Micromole ammonia released}}{\text{10 minutes / ml enzyme in reaction}}$$

Dialysis

Semi permeable membrane preparation

The membrane was kept in 1L of 2% sodium bicarbonate/1 mm EDTA in 2 L glass beaker. It was allowed to boil for 10 minutes and thoroughly rinsed with double distilled water. In a test tube, 1 ml of sample and 1ml of saturated ammonia sulphate solution was taken and centrifuged at 1500 rpm for 5 minutes. Supernatant was removed and 0.4 ml of 40% ammonia sulphate was added, centrifuged again at 1500 rpm for 5 minutes.

The supernatant was discarded and the white precipitate was collected in eppendorf tube. One end of the semi permeable membrane was tied tightly and the precipitate was loaded onto it and then the other end was also tied tightly.

This set up was tied onto a glass rod, introduced in a beaker containing Phosphate buffered saline and magnetic stirrer. The setup was kept undisturbed until precipitate becomes clear.

SDS–Page

The processed samples were further subjected to SDS-PAGE to study the molecular weight of the enzyme. It was carried out in a vertical slab get Electrophoretic unit. Separating gel, stacking gel and the samples were prepared and electrophoresed. After electrophoresis, gel was stained for 1-2 hours, then it was treated with destaining solution and the bands were observed.

MTT Assay

Antitumor activity was carried out for the sample Ni5 and S2 by colorimetric method (Mosman, 1983).

Cells were introduced in 24 well plates and kept for incubation at 37 ºC in 5% CO_2 atmosphere. Once the cells reach the confluence, different concentrations of the sample were added from 1000 µg/ml to 7.8 µg/ml and incubated for 24 hours. After incubation, samples were discarded by gentle aspiration. 100 µl (5 mg/ml) of MTT was dispensed into each well, including the control well and incubated for 4 hours. Then, 1000 µl of DMSO was added to all the wells for dissolving the formazan crystals. Colour intensity of the crystal is proportional to the cell viability. The absorbance was recorded at 570 nm with reference range at 650 nm using UV Spectrophotometer. 50% inhibition (IC50) was determined graphically. Percentage of cell viability was calculated based on the following formula.

$$\% \text{ Cell viability} = \frac{\text{A570 of treated cells}}{\text{A570 of control cells}} \times 100$$

Results and Discussion

L-Asparaginase Production

Of thirty samples inoculated, pink colouration around the colonies were observed in six samples on M9 minimal medium with 1% asparaginase (Figure 2). Hence 20% of the isolates were positive for L asparaginase production.

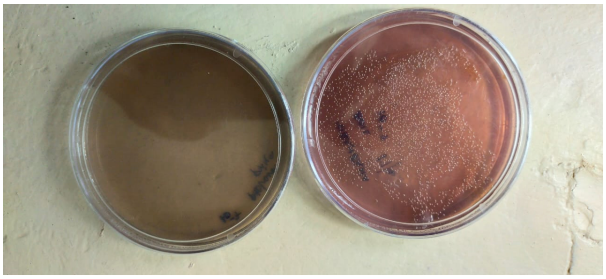


Fig. 2. Plate showing Asparaginase production - pink coloration

Biochemical Characterization

Preliminary identification revealed that the bacterial isolates were from the following genera *Bacillus*, *Pseudomonas* and *Escherichia* (Table 1 and Figure 3).

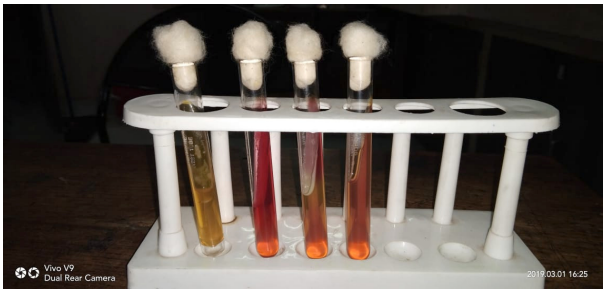


Fig. 3.1. TSI- tube showing A/A gas + (1st tube), K/K (3 tubes)

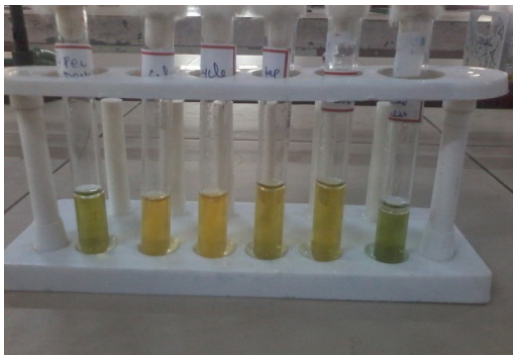


Fig. 3.2. O-F test showing fermentative (yellow) and oxidative results (green)

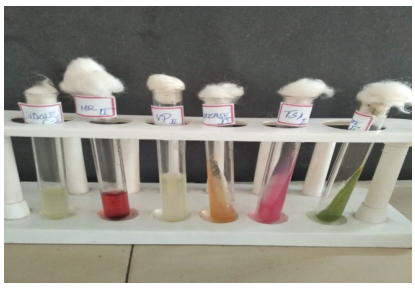


Fig. 3.3. IMViC Test and Urease test

Table 1. Isolates showing prompt asparaginase production qualitatively

Isolate No	Gram Stain	Catalase	Oxidase	TSI	OF	Presumptive identification
S1	Positive	+	-	A/A	Fermentative	Bacillus
S2	Negative	+	+	K/K	Oxidative	Pseudomonas
S3	Negative	+	-	A/A	Fermentative	Escherichia
M1	Negative	+	+	K/K	Oxidative	Pseudomonas
Ni5	Negative	+	+	K/K	Oxidative	Pseudomonas
Ni7	Negative	+	+	K/K	Oxidative	Pseudomonas

Extraction and Estimation of Enzyme activity

Standard graph was calculated according to the concentration of ammonia released and OD values were recorded as depicted in the Table 2 and in Chart 1.

Table 2. Standard Graph – Estimation of Ammonia Released

S. No.	Concentration of Ammonium Sulphate (mM)	OD value at 450nm
1	30	3.5
2	20	2.95
3	10	2.205
4	5.0	1.85
5	2.5	1.134
6	1.0	0.321
7	0.75	0.1
8	0.5	0.05
9	0.25	0.025

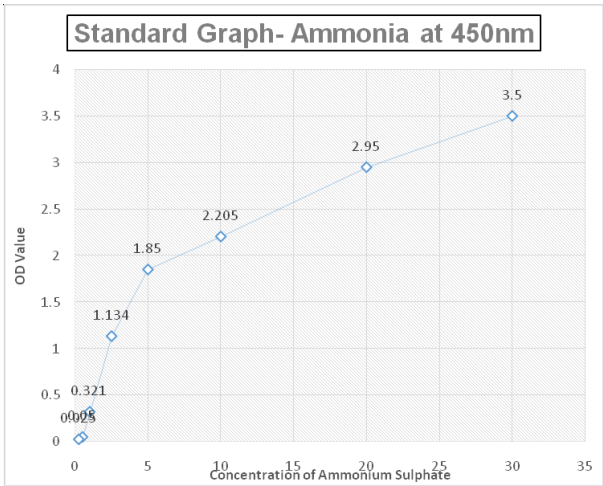


Chart 1. Standard Graph of Ammonia released from Ammonium sulphate

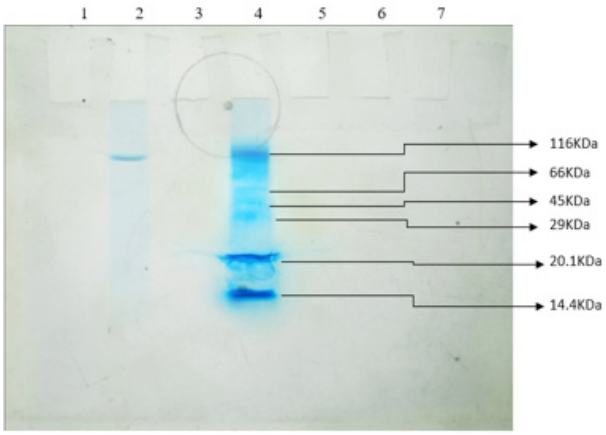
Enzyme from the soil isolates were quantified and it lies in the range of 270 to 1090 units/ml as shown in Table 3 and 4. The soil isolates S2 and Ni5 exhibited the maximum production of enzyme with 1090 units/ml of activity.

Table 4. Enzyme Quantification of the Isolates

Isolate No	Micromoles of Ammonia released (µmoles)	Asparaginase produced in units/ml
S1	5400	270
S2	21800	1090
S3	13800	690
M1	13800	690
Ni5	21800	1090
Ni7	13800	690

Molecular characterization by SDS PAGE

These two isolates were further processed for molecular characterization and the molecular weight of the proteins were in the range of 100-11 6 kDa (Fig. 4).



Lane 2- Sample

Lane 4- Marker

Fig. 4. Molecular Weight determination of Asparaginase by SDS PAGE

Table 3. OD reading of isolates showing higher asparaginase production (crude enzyme)

Isolate No	OD value at 450nm	Concentration (Extrapolated)	Micromoles of Ammonia released (µmoles)
S1	1.702	5.4	5400
S2	3	21.8	21800
S3	2.5	13.8	13800
M1	2.5	13.8	13800
Ni5	3	21.8	21800
Ni7	2.5	13.8	13800

Anti-tumour Activity on MCF 7 cell line

Antitumor activity of L- asparaginase enzyme on MCF 7 cell line showed that the cell viability was dose dependent and IC50 of S2 was 62.5 µg and that of Ni5 was found to be 31.2µg as represented in the Table 5 and 6, Chart 2 and 3 and in Figure 5 and 6.

Table 5. Anticancer effect of Sample Ni5 on MCF 7 cell line

S. No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.120	16.87
2	500	1:1	0.168	23.62
3	250	1:2	0.217	30.52
4	125	1:4	0.266	37.41
5	62.5	1:8	0.315	44.30
6	31.2	1:16	0.364	51.19
7	15.6	1:32	0.412	57.94
8	7.8	1:64	0.462	64.97
9	Cell control	-	0.711	100

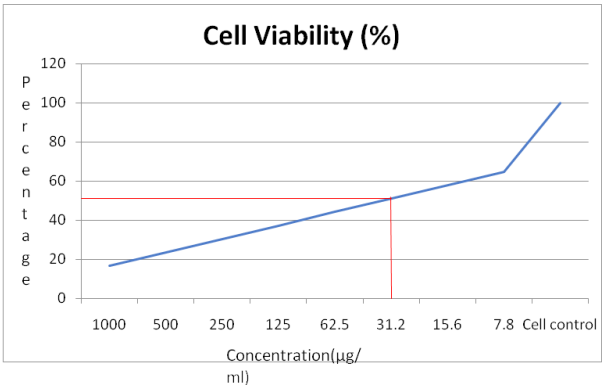


Chart 2. Anticancer effect of Sample Ni5 on MCF 7 cell line

Table 6. Anticancer effect of Sample S2 on MCF 7 cell line

S. No	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.162	22.78
2	500	1:1	0.207	29.11
3	250	1:2	0.253	35.58
4	125	1:4	0.298	41.91
5	62.5	1:8	0.343	48.24
6	31.2	1:16	0.387	54.43
7	15.6	1:32	0.431	60.61
8	7.8	1:64	0.476	66.94
9	Cell control	-	0.711	100

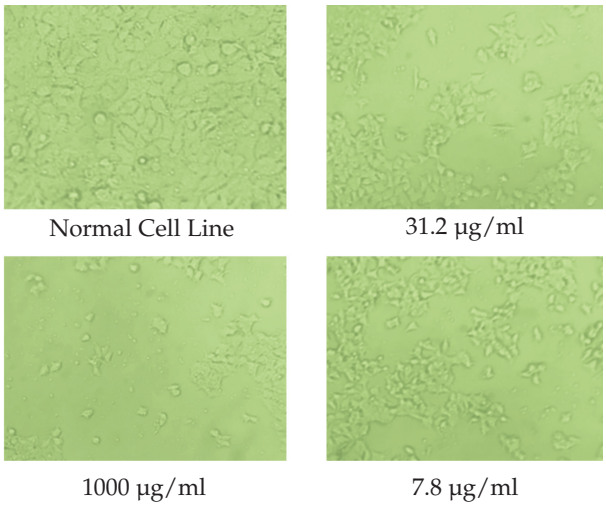


Fig. 5. Anticancer effect of Sample Ni5 on MCF 7 cell line

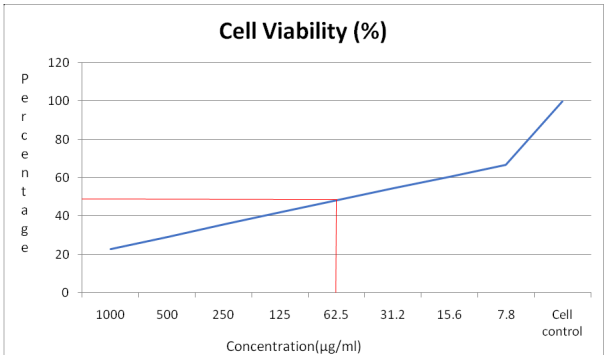


Chart 3. Anticancer effect of Sample S2 on MCF 7 cell line

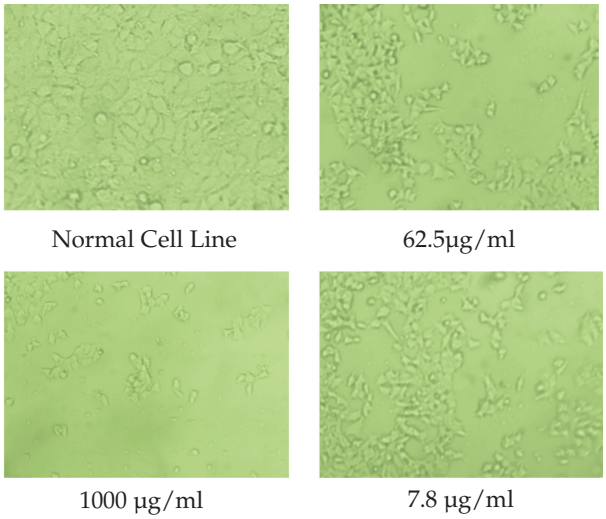


Fig. 6. Anticancer effect of Sample S2 on MCF 7 cell line

Discussion

Asparaginase enzyme has a prime role in treating certain types of tumours. The unique mode of action of asparaginase in acting against cancer cells makes it as one of the important constituents in multi-drug chemotherapy in children and adults (Piatkowska-Jakubas *et al.,* 2008).

Though it is effective, the toxicity of asparaginase leads to hypersensitivity, half short life etc (Rathod *et al.,* 2019 and Fonseca *et al.,* 2021). In addition, asparaginase with glutaminase coactivity results in many complications like leukopenia, hyperglycaemia, pancreatitis etc (Durden *et al.,* 1983). To overcome this lacuna, this study focuses on search of an alternative source of enzyme from soil microflora and studied its efficacy on MCF-7 cell lines.

Soil is rich in microflora and suitable medium for isolation of L-asparaginase enzyme producing bacteria (Fatima *et al.,* 2019 and Mohamed Abdel Raouf *et al.,* 2020). M9 medium with 1% asparaginase used for isolation of enzyme producing bacteria (Devi *et al.,* 2016). Preliminary investigation revealed different bacterial genera and of all the strains *Pseudomonas* spp were dominant and also exhibited maximum enzyme production (Badoei-Dalfard, 2016). Molecular characterization was determined by SDS PAGE to analyse the molecular weight of proteins and were around 110kDa (El-Naggar *et al.,* 2016 and Jimat *et al.,* 2017). Antitumour activity was performed for both S2 and NI5 samples on MCF 7 Cancer cell line and were proved to be effective (Alrumman *et al.,* 2019).

Conclusion

The study revealed that the bacterial isolates from the soil can act as a potent asparaginase producer and has potential anticancer activity on MCF 7 cell lines. The organism belonging to the *Pseudomonas* were predominant in the tested samples and moreover the highest cancer activity was shown by *Pseudomonas* species. This would be a promising agent for treating certain type of cancers in future.

Acknowledgements

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Conflict of Interest

Authors declare no conflict of Interest.

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