

SCREENING AND PRODUCTION OF TUMOUR INHIBITORY L-ASPARAGINASE BY BACTERIA ISOLATED FROM SOIL

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ABSTRACT

In this present study 103 bacterial isolates were obtained from different habitats and screened out for production of L-asparaginase. Among them, forty nine (49) bacterial isolates showed positive L-asparaginase activity on modified M9-medium using phenol red as an indicator dye. However, ten bacterial isolates showed highest enzyme activity exhibiting a pink zone of hydrolysis of around 5.0 cm after 5 days. Our isolate, FS 30 appear to be potent extracellular producer showing 3.369 IU L-asparaginase activity and 877.34 IU/mg of specific activity under submerged fermentation conditions in 3 days.

Keywords: Therapeutic enzyme, L-asparaginase, Acute lymphoblastic leukemia, L-asparagine, Extracellular

INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) was introduced in the therapeutics to treat acute lymphoblastic leukemia¹. However, L-asparaginase is of special significance because of the fact that tumour cells are deficient in L-asparaginase synthetase activity, which restricts their ability to synthesize the normally non-essential amino acid L-asparagine, required for the growth and survival of cancer cells. Therefore, tumour cells are dependent on exogenous supply of L-asparagine from body fluids. Administration of L-asparaginase within the body does not affect the functioning of normal cells because they possess an inherent property to synthesize L-asparagine for their own requirements, but reduces its concentration in the plasma pool. Thus induces a state of fatal starvation in the susceptible tumour cells L-asparaginase is an important therapeutic enzyme and is produced by bacteria including *Erwinia carotovora*², *Pseudomonas aeruginosa* 50071³, *Staphylococcus sp.* 6A⁴, *Pectobacterium carotovorum*⁵; *Bacillus circulans*⁶ and *Bacillus brevis*⁷, actinomycetes^{8,9}, yeast^{10,11} and fungi^{12,13}. The microbial L-asparaginases which are used in clinical practice include ELSPAR, Erwinase and PEG-L-asparaginase¹⁴. However, L-asparaginase used in present therapy is reported to cause problems relating to hypersensitivity in long term usage. Besides its therapeutic value they have also attracted attention of food processing industries for reduction of acrylamide content in fried and baked foods¹⁵.

Therefore, there is a continual need to find out newer microbial sources to obtain high-yielding L-asparaginase producing strains for their therapeutic and industrial applications. Keeping importance of bacteria as a better source of L-asparaginase present investigation was aimed to screen the L-asparaginase activity of novel bacteria isolated from different habitats.

MATERIALS AND METHODS

Test-organisms

A total of 103 bacterial isolates recovered from garden soil (GS), compost (CM) and forest soil (FS) were used as test organisms.

Pre-treatment of Soil Samples

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

Isolation of Bacteria

Isolation of bacteria were performed by the serial dilution technique¹⁶ using nutrient agar medium (Peptone, 5.0 g; Beef-extract, 3.0 g; Sodium chloride, 5.0 g; agar-agar, 20.0 g per litre of distilled water). The sterilization of the media components were carried out in a autoclave at 121°C at 15 lbs pressure for 30 minutes. Approximately 20 ml of media was poured into pre-sterilized

petridishes and allowed to solidify. The sample (1g) was serially diluted upto 10⁻⁶ dilutions. A 0.1 ml of this dilution was aseptically spread over the surface of poured petridishes having nutrient agar medium. The plates were then incubated at 37°C for 48 hours. A colony appeared with characteristics of bacterial morphology was isolated and purified using nutrient agar medium. After purification, all the isolated bacteria were maintained on nutrient agar slants and stored at 4°C for further use.

Screening of L-asparaginase activity on solid medium

All isolates were screened for L-asparaginase activity using modified - M9 medium¹⁷ following tubed agar method with certain modification¹⁸. The medium contained Na₂HPO₄·2H₂O, 6.0 g; KH₂PO₄, 3.0 g; NaCl, 0.5 g; L-asparagine, 10.0 g; 1mol⁻¹ MgSO₄·7H₂O, 2.0 ml; 0.1 M solution of CaCl₂·2H₂O, 1.0 ml; 20% glucose stock, 10.0 ml; agar 20.0 g. per liter of distilled water. The medium was supplemented with 0.005% phenol red dye (prepared in ethanol) and the pH was adjusted to 6.2 using 1N HCl. Nine ml of medium was poured in each tube and were sterilized.

The tubes were inoculated with test organisms by using one loopful culture of bacteria and incubated at 37°C for 5 days. A set of tubes were also run as control without L-asparagine. The modified - M9 medium contains 1% L-asparagine as sole source of nitrogen. L-asparaginases hydrolyses L-asparagine into L-aspartic acid and ammonia. This can be easily detected by the change in the pH of the medium due to production of ammonia. The colour change of the medium (from yellow to pink) indicates positive L-asparaginase production. Whereas, depth of pink zone in tubed agar indicated the extent of L-asparaginase activity. Pink zone developed in tubed agar was measured after 2 days and 5 days of incubation. L-asparaginase activity of each bacteria were recorded by measuring a pink zone in the stabs and depending on the L-asparaginase activity these were categorized as excellent, good, fair, poor and negative producer.

Production of L-asparaginase by Submerged Fermentation

Ten bacterial strains which showed excellent activity during primary screening were further tested for production of L-asparaginase in broth cultures. For this spore suspension of each test bacteria was prepared in modified - M9 broth. The flask having 50 ml broth were inoculated with spore suspension after autoclaving and incubated at 150 rpm for 72 hours at 37°C. Uninoculated medium was served as control. At the end of incubation, culture filtrates were obtained by centrifugation at 8000 rpm for 15 min. The supernatant was then used as crude extract for L-asparaginase activity and stored at 4°C for further use.

Assay of L-asparaginase activity

L-asparaginase activity was determined in culture filtrates by quantifying the ammonia formation using nessler's reagent¹⁹. The enzymatic reaction mixture contains 0.5 ml of 0.04 M L-asparagine

substrate, 0.5 ml of 0.05 M Tris-HCl buffer (pH 8.6) and 0.5 ml of crude enzyme. The enzyme substrate mixtures were incubated at 37°C for 30 min. After the incubation period the enzyme activity was stopped by the addition of 0.5 ml of 1.5 M trichloroacetic acid (TCA). The liberated ammonia was coupled using nessler's reagent incubated at 20°C for 15 min for development of colour. The optical density of the colour produced was measured at 450 nm using a double beam UV-visible spectrophotometer (ECIL model UV- 5704 SS). The liberated ammonia was determined by inference from the standard curve of ammonium sulphate. The L-asparaginase activity was expressed in terms of International unit (IU). One unit (IU) of L-asparaginase is defined as the amount of enzyme which liberates 1µmol of ammonia per ml per minute (µmole/ml/min) at 37°C.

Determination of Protein content in culture filtrate

The protein content was determined by the Lowry method²⁰ using bovine serum albumin (BSA) as a standard.

Study of cultural characteristics of isolated bacteria

Cultural characteristics of 10 selected isolates showing excellent L-asparaginase activity were determined. For this the test strains were sub-cultured on SDA plates by streaking a loopful suspension of bacteria. The plates were then incubated at 37°C for 48 hours and the cultural characteristics of the colonies were noted using standard protocols²¹. The colonies were then studied for their characteristics shape, edge, elevation and colour using a hand lens.

RESULTS AND DISCUSSION

Isolation

A total of 103 bacteria were isolated, these include 56 from garden soil, 18 from forest soil and 29 from compost. The details of number of bacteria found positive for L-asparaginase activity are given in Table 1. Overall 47.57% of bacteria were found positive for L-asparaginase activity in the studied habitat. Maximum numbers of bacterial isolates were isolated from the samples of garden soil, whereas, only 18 test bacteria were recorded from samples of forest soil. Occurrence of more actinomycetes in garden soil samples can be attributed to high nutrient availability in soil.

Table 1: Occurrence of bacteria in different habitats

S. No.	Habitats	No. of bacteria	No. of positive bacteria	Percentage
1.	Garden Soil(GS)	56	25	44.64
2.	Forest Soil(FS)	18	17	94.44
3.	Compost (CM)	29	7	24.13
Total		103	49	47.57

Table 4: Colony characteristics of excellent L-asparaginase positive bacterial strains

S.No	Strain No.	Colour	Shape	Elevation	Margin
1.	GS 24	Cream	Punctiform	Flat	Curled
2.	FS 30	White	Circular	Flat	Filamentous
3.	FS 31	Cream	Circular	Raised	Curled
4.	FS 42	White	Circular	Raised	Curled
5.	CM 43	Cream	Filamentous	Flat	Filamentous
6.	CM 49	White	Irregular	Flat	Curled
7.	GS 56	Cream	Circular	Raised	Filamentous
8.	FS 93	Yellow	Filamentous	Raised	Filamentous
9.	FS 95	Yellow	Round	Convex	Curled
10.	FS 102	Yellow	Filamentous	Flat	Filamentous

Screening of L-asparaginase activity on solid medium

The summarized results of L-asparaginase activity are given in Table 2 and reported that out of 103 test bacteria, forty nine test bacteria were found positive for L-asparaginase activity. Excellent activity of this enzyme was noted in 10 test bacterial isolates, i.e., GS 24, FS 30, FS 31, FS 42, CM 43, CM 49, GS 56, FS 93, FS 95 and FS 102 exhibiting a pink zone of hydrolysis around 5.0 cm after 5 days of incubation. While, 17 test bacteria showed good activity of this enzyme producing a zone of hydrolysis between 3 to 4 cm. Eleven test bacteria showed fair activity of this enzyme. While 54 test bacterial isolates were found to be negative for L-asparaginase activity and 11 test bacteria showed poor L-asparaginase activity.

Table 2: Screening of L-asparaginase activity on Modified -M9 medium.

Category	Pink zone in cm.	No. of bacteria found positive	Percentage
Excellent	4-5	10	9.708
Good	3-4	17	16.504
Fair	2-3	11	10.679
Poor	1-2	11	10.679
Negative	No pink zone	54	52.427
Total		103	

L-asparaginase production by 10 selected strain is given in Table 3. A perusal of data indicates that except test isolate No. CM 43 all showed remarkably good to excellent activity of L-asparaginase even within 2 days of incubation (while) all the other selected bacterial isolates showed excellent production after 5 days of incubation.

Table 3 : Bacterial isolates showing excellent L-asparaginase activity

S.No.	Strain No.	L-asparaginase activity*	
		After 2 Days	After 5 Days
1	GS 24	4.2	5.0
2	FS 30	4.3	5.0
3	FS 31	4.0	5.0
4	FS 42	3.8	5.0
5	CM 43	1.8	5.0
6	CM 49	3.0	5.0
7	GS 56	3.0	5.0
8	FS 93	4.5	5.0
9	FS 95	3.5	5.0
10	FS 102	4.5	5.0

*Depth of pink colour in the tubed agar in centimeter

Identification

The cultural characteristics of the excellent L-asparaginase producer are given in Table 4. The cultural characteristics are valuable in identification because morphological feature depend on the expression of many genes, and are usually stable²².

Table 5: Quantification of L-asparaginase activity of bacterial strains

S.No.	Strain No.	L-asparaginase activity (IU)	Protein Concentration ($\mu\text{g/ml}$)	Specific Activity (IU/mg)
1.	GS 24	0.493	4.220	116.82
2.	FS 30	3.369	3.840	877.34
3.	FS 31	0.832	4.320	192.59
4.	FS 42	0.325	7.540	43.10
5.	CM 43	0.577	3.840	150.26
6.	CM 49	0.352	4.140	85.02
7.	GS 56	0.634	4.480	141.51
8.	FS 93	0.380	4.840	78.51
9.	FS 95	0.422	4.220	100.00
10.	FS 102	1.550	3.640	425.82

Production of L-asparaginase by Submerged Fermentation

Maximum L-asparaginase activity of 3.369 IU was noted in culture filtrate of FS 30 (Table 5). Specific activity of this enzyme was found to be 877.34 IU/mg protein in culture filtrate. Culture filtrate of strain FS 102 showed 1.550 IU activity of this enzyme. There has been report on 3.64 U/ml L-asparaginase activity in *Aspergillus sp.* (VS-26) through submerged fermentation²¹.

From the above results it is concluded that the test bacterial strain i.e. FS 30 could be a potential source for production of L-asparaginase which can be used for the application in food and pharmaceutical industries.

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