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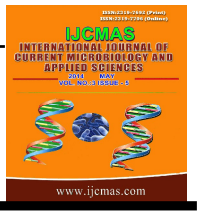

Original Research Article

Exploration of the potential L-asparaginase producing bacteria from the soil of Gwalior (India)

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A B S T R A C T

In recent years, microbial L-asparaginases have drawn particular attention because of their potential antineoplastic properties and significant application in food industries. The present study was undertaken to establish microbial species that produces L-asparaginase, which might have even more efficient antineoplastic activity. Out of 67 isolates 46 were screened for the production of L-asparaginase on a Modified M9 Agar Medium with phenol red as an indicator. Quantitative estimation was done by Nesselerisation. Among all positive asparaginase producing strains, nine were identified as *Bacillus cerus*, seven *Bacillus subtilis*, five *E. coli*, seven other *Bacillus* species, one *Proteus*, four *Pseudomonas* , four *Arthobacter*, two *Lactobacillus* and four *Salmonella species* on the basis of morphological and biochemical characterization. Among them the most significant activity was shown by *Pseudomonas species* (1.6 IU/ml). Further, upon molecular characterization, it was identified as *Pseudomonas proteolytica* strain S13D, with Accession ID KC407594, a new potential strain for L-asparaginase production.

Keywords

Anti-neoplastic activity, Nesseler-isation, L-asparaginase, *Pseudomonas proteolytica*

Introduction

Enzyme L-asparaginase (L-asparagines amidohydrolase EC 3.5.1.1) has the physiological function of hydrolyzing amide group of the side chain in L-asparagine to produce L-aspartate and ammonia. This spectacular property of the enzyme is utilized in chemotherapeutic treatment of certain kinds of lymphoblastic malignancies, mainly in acute lymphoblastic leukaemia (ALL) and lympho-sarcoma for nearly 40 years (Lee *et al.*, 1989; Verma *et al.*, 2007).

In 1963 it was revealed that antineoplastic activity of guinea pig sera was due to the presence of L-Asparaginase (Broome, *et al.*, 1963). Broome's statement was confirmed by partially purifying L-Asparaginase from the sera of guinea pig (Yellin *et al.*, 1966). *L-Asparaginase* activity was also identified from *E.coli* cells as it was demonstrated that *E.coli* steadily produces *L-Asparaginase* under anaerobic conditions (Cedar *et al.*, 1968, Mashburn *et al.*, 1964). Presence of *L-*

Asparaginase was also observed in *Erwinia aroideae* (Peterson *et al*., 1969). Later on, *L-Asparaginase* was produced, purified and crystallized from *Proteus vulgaris* (Tosa *et al*., 1972). A variety of microorganisms has been shown for their *L-Asparaginase* producing potency, viz. *Enterobacter aerogenes* (Mukherjee *et al*., 1999), *Psuedomonas stutzeri* (Manna *et al*.,1995), *Pseudomonas aeruginosa* (El-Bessoumy et al., 2004), *Serratia marcescence* (Boyd *et al*,1971), *Wolinella succinogenes* (Lubkowski *et al*.,1966), and *Staphylococcus* species (Prakasham *et al*.,2007).

Asparaginase is present in many animal tissues, bacteria, plants and in the serum of certain rodents but not in mankind. Although L-Asparaginases are broadly distributed among various living organisms, however, microorganisms are more efficient and inexpensive. The principle source of L-asparaginase for clinical trials is the bacterium *Escherichia coli* (Asselin *et al*., 1993) and *Erwinia* (Aghaiypour *et al*., 2001). Literature indicated that the enzyme's biochemical and kinetic properties vary with the genetic nature of the microbial strain used (Eden *et al*., 1990), suggesting that there is a need to locate new sources of L-asparaginases those are serologically different but have similar therapeutic effects.

This requires screening of samples from various sources for isolation of potential microbes which have the ability to produce the enzyme with novel properties. The present investigation aimed at isolation of bacterial cultures from various soil sources and their qualitative production of L-Asparaginase, which might have even more efficient antineoplastic activity.

Materials and Methods

Sample collection

Soil samples were collected in the sterilized poly bags from the depth of 30cm from different sites viz. Garden, farm and muddy soil of Gwalior (India) as the method described by Prescott (1999).

Isolation of bacteria

Isolation of bacteria was performed by the serial dilution technique, 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). Morphologically different colonies were selected and obtained the pure culture by repetitive streaking method. Pure culture were maintained on nutrient agar slants and stored at 4°C for further use.

Primary screening

The L-asparaginase producing strains were initially screened by rapid plate assay method, based on their capability to form a pink zone around colonies on agar plates of modified M-9 medium (Gulati *et al*., 1997). The medium contained $Na_2HPO_4.2H_2O$, 6.0 g; KH_2PO_4, 3.0 g; NaCl, 0.5 g; L-Asparagine, 10.0 g; 1mol-$MgSO4.7H_2O$, 2.0 ml; 0.1 M solution of $CaCl_2.2H_2O$, 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. Plates were then incubated at 37°C for 24-48 hrs. A set of tubes was also run as a control without L-asparagine. The strains having potential for L-asparaginase production were selected on the basis of pink zone formation and retained for further screening.

Secondary screening

Isolated microorganisms from the previous screening were cultured in liquid media (TGY-Broth with 1% asparagine) at pH 7.0 in 250 ml Erlenmeyer flasks. Un-inoculated medium served as negative control. All experiments for quantitative screening were done in triplicate and data expressed as average values with standard deviation (Calculated from Graph pad Prim 6). After incubation on a rotary shaker (37°C, 120 rpm) for 48 hrs, the culture broth was centrifuged at 10,000 ×g for 10 min, supernatants were collected and enzyme assay done was by Nesselerisation (Imada *et al*., 1973).

Characterization of microorganisms

All 46 selected bacterial isolates showing excellent L-asparaginase activity were characterized on the basis of morphological and biochemical characteristics and the results were interpreted according to Bergey's Manual of Determinative Bacteriology & PIBWIN software version 19.2.(Bryant,2004;Holt, 1994).

Molecular characterization of the potent asparaginase producing strain

Out of all positive isolates, the maximum asparaginase producing isolates were selected and processed for Isolation of genomic DNA, 16S rDNA sequencing and phylogenitic analysis. Amplification of 16S rDNA by PCR was done using Universal bacterial primer 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). Sequencing of 16S rDNA of the isolates was done in Indian Oil Corporation Ltd. Faridabaad.

Phylogenetic analysis

BLAST search was done for partial 16S rDNA of the isolates by submitting queries to NCBI- BLAST (http://www.ncbi. nlm.nih.gov/blast/Blast). Homologous sequences, obtained by standard nucleotide-nucleotide BLAST (blastn) were aligned with the isolate sequence using Clustal program (Higgins *et al*., 1988). The Evolutionary distance matrix was obtained according to Jukes *et al.*, (1969). Evolutionary trees for the data were obtained by neighbour joining method (Saitou *et al.,* 1987) using neighbour joining program (Kumar *et al*., 1993).

Results and Discussion

Based on primary screening, 46 were found to have potential for L- asparaginase production out of 67 isolates. (Fig. 1 & 2). Two control plates were also prepared (a) without inoculum (b) phenol red (PR^{-}) to ascertain L- asparaginase activity.

Secondary screening for enzyme production in Table1, it is clear that bacterial isolate S13D has shown highest production potential (i.e. maximum enzyme activity of 1.6±0.3 U/mg).

Table 2 shows sequential steps of characterization of potent L-asparaginase producing strains. Among all the positive asparaginase producing strains, nine isolates were identified as *Bacillus cereus* (AT, AX, AO, AQ, AH1, AH, AD, AB, AA), seven *Bacillus subtilis* (AC, AI, AZ, AJ, AM, AG, AS), seven other *Bacillus* species (AE, AC1, AC2, AK, AV, AF, AL), five *E.coli* (Z15D, Z15E, Z14A, Z9A, Z5A) one *Proteus*, (T13D) four *Pseudomonas* (S13D, S15H, SI6G, S15D), four *Arthobacter* (B1A, B1B, B1C, B1D), two *Lactobacillus* (AO1, T11C) and four *Salmonella species* (Z15E, Z9D, Z30c, Z14A).

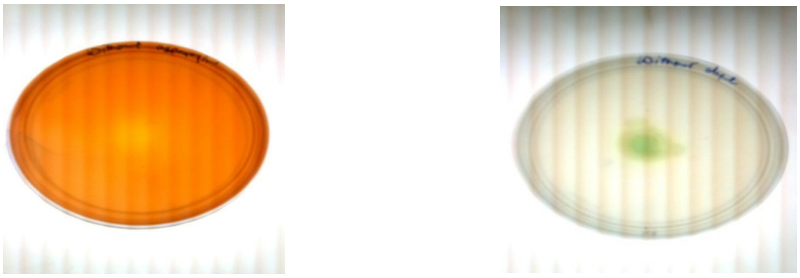


Fig. 1. Plate showing (a) un-inoculated control PR+, LASP+ (b) without phenol red

Fig. 2. Plates showing production of L-asparaginase by microorganisms

TABLE 1- A COMPARETIVE CHART FOR L-ASPARAGINASE ACTIVITY PRODUCTION BY DIFFERENT BACTERIAL ISOLATES

S.D.=STANDERD DEVIATION

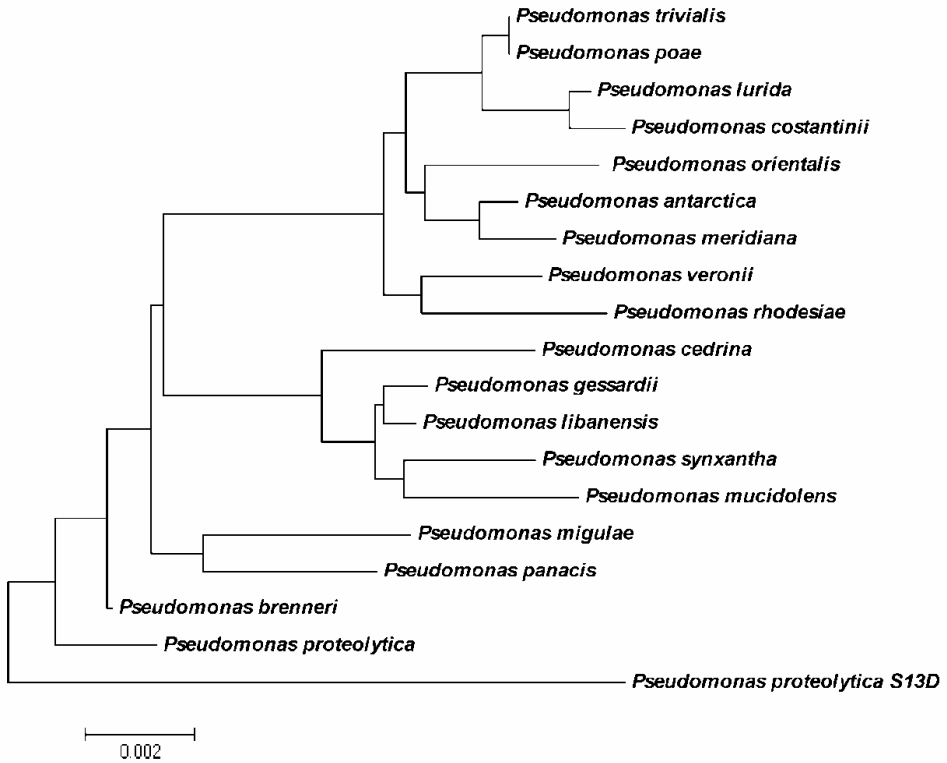
S.No.	CULTURE CODE	ENZYME ACTIVITY IU/ml with S.D. AFTER 24 HOURS	AFTER 48 HOURS	AFTER 72 HOURS	S.No.	CULTURE CODE	AFTER 24 HOURS	AFTER 48 HOURS	AFTER 72 HOURS
1	AA	0.54 ± 0.01	0.59 ± 0.03	0.40 ± 0.02	24	AX	0.59 ±0.06	0.65 ± 0.01	0.70 ± 0.03
2	AB	0.18 ± 0.07	0.33 ± 0.01	0.21 ± 0.001	25	AZ	0.32 ± 0.02	1.27 ± 0.32	0.81 ± 0.007
3	AC	0.66 ±0.02	0.56 ± 0.08	0.49 ± 0.01	26	B1A	0.32 ±0.01	0.39 ± 0.0001	0.17 ± 0.06
4	AC1	0.2 ± 0.01	0.10 ± 0.01	0.13 ± 0.04	27	B1B	0.23 ± 0.04	0.31 ± 0.01	0.29 ± 0.02
5	AC2	0.2 ± 0.03	0.32 ± 0.02	0.22 ± 0.02	28	B1C	0.34 ± .001	0.53 ± 0.005	0.32 ± 0.02
6	AD	0.24 ±0.01	0.30 ± 0.02	0.14 ± 0.02	29	B1D	0.27 ±0.06	0.30 ± 0.02	0.16 ± 0.02
7	AE	0.29 ± 0.03	0.22 ± 0.001	0.27 ± 0.02	30	B1E	0.22 ± 0.01	0.30 ± 0.02	0.29 ± 0.02
8	AF	0.45 ± 0.01	0.87 ± 0.01	0.82 ± 0.06	31	S13D*	0.96 ±0.1	1.67 ± 0.02	0.82 ± 0.06
9	AG	0.26 ± 0.01	0.20 ± 0.005	0.05 ± 0.02	32	S15D	0.63 ± 0.02	0.80 ± 0.02	0.77 ± 0.01
10	AH	0.67 ± 0.03	0.59 ± 0.01	0.42 ± 0.03	33	S15H	0.5 ± 0 .02	0.61 ± 0.07	0.34 ± 0.005
11	AH1	0.32 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	34	S16G	0.58 ± 0.04	0.69 ± 0.01	0.51 ± 0.03
12	AI	0.34 ± 0.01	0.75 ± 0.01	0.50 ± 0.005	35	T11C	0.53 ±0.02	0.69 ± 0.05	0.78 ± 0.02
13	AJ	0.33 ± 0.01	0.40 ± 0.01	0.37 ± 0.005	36	T12C	0.37 ± 0.04	0.50 ± 0.01	0.41 ± 0.03
14	AK	0.19 ± 0.01	0.24 ± 0.04	0.17 ± 0.05	37	T13D	0.76 ± 0.06	0.90 ± 0.04	0.74 ± 0.03
15	AL	0.71 ± 0.03	0.84 ± 0.06	0.57 ± 0.05	38	Z14A	0.2 ± 0.03	0.48 ± 0.05	0.21 ± 0.03
16	AM	0.35 ± 0.03	0.41 ± 0.02	0.21 ± 0.02	39	Z14A	0.21 ± 0 .04	0.51 ± 0.02	0.31 ± 0.02
17	AO	0.61 ± 0.001	0.56 ± 0.1	0.37 ± 0.03	40	Z15D	0.79 ± 0.03	0.79 ± 0.01	0.59 ± 0.02
18	AO1	0.29 ±0.03	0.22 ± 0.08	0.25 ± 0.2	41	Z15E	0.55 ± 0.09	0.68 ± 0.01	0.44 ± 0.01
19	AQ	0.68 ±0.04	0.39 ± 0.06	0.20 ± 0.001	42	Z15E2	0.25 ± 0.04	0.45 ± 0.05	0.20 ± 0.03
20	AS	0.16 ± 0.06	0.34 ± 0.03	0.16 ± 0.03	43	Z30C	0.62 ± 0 .04	0.58 ± 0.01	0.40 ± 0.2
21	AT	0.40 ± 0.02	0.88 ± 0.01	0.72 ± 0.03	44	Z5A	0.55 ± 0.1	0.85 ± 0.01	0.72 ± 0.01
22	AU	0.42 ± 0.01	0.69 ± 0.02	0.63 ± 0.01	45	Z9A	0.71 ± 0 .001	0.49 ± 0.01	0.31 ± 0.01
23	AV	0.18 ± 0.05	0.21 ± 0.05	0.21 ± 0.02	46	Z9D	0.4 ± 0.06	0.52 ± 0.01	0.32 ± 0.001

TABLE -2 – BIOCHEMICAL TEST RESULTS OF BACTERIA SCREENED FOR L-ASPARAGINASE PRODUCTION

ANALYTICAL TEST		RESULTS								
	CULTURE CODE	AT,AX,AO, AQ,AH1,AH ,AD,AB,AA	AC,AI,A Z,AJ,A M,AG,A S,	B1A,B 1B,B1 C,B1D ,	S13D,,S15 H,SI6G,S1 5D	Z15D,Z15E, Z14A,Z9A,Z 5A,	Z15E2,Z9 D,Z30C,Z1 4A	AO1,T11C	T13D,T12C	AE,AC1,AC2,A K,AV,AF,AL
MORPHOLOGY	GRAM STAINING	+	+	+	–	–	–	+	–	+
	SHAPE	RODS	RODS	RODS	RODS	RODS	RODS	RODS	RODS	RODS
	MOTILITY	+	+	–	+	+	+	-	+	+
	ENDOSPORE	+	+	-	-	-	+	-	-	+
B	CATALASE	+	+	–	+	+	-	-	+	+
I	OXIDASE	D	+	+	-	-	-	-	-	-
O	INDOLE PRODUCTION	–	–	ND	+	+	–	-	+	-
C	METHYL RED	-	-	ND	-	+	+	D	+	+
H	VOGES PROSKEUR	+	+	ND	+	–	–	D	-	–
E	CITRATE UTILISATION	+	-	ND	+	–	+	D	+	-
M	CASIEN HYDROLYSIS	+	D	D	D	+	+	-	+	+
I	NITRATE REDUCTION	+	+	–	–	+	-	-	-	+
C	UREASE	-	D	+	-	–	–	-	+	ND
A	MANNITOL SALT AGAR	-	+	D	+	-		-	-	+
L	GLUCOSE FERMENTATIO N	+	+	D	+	+	+	+	+	+
T E S T	SUCROSE FERMENTATIO N	+	+	+	+	–	–	+	-	+
	MALTOSE FERMENTATIO N	+	-	+	-	–	+	+	+	+
	LACTOSE FERMENTATIO N	–	-	+	D	–	+	+	+	+
	FRUSTOSE FERMENTATIO N	–	D	+	-	–	–	-	D	+
		Bacillus cerus	*Bacillus Subtilis*	*Arthro bacter sp.*	*Pseudomo nas sp*	*E.coli*	*Salmonella sp*	*Lactobacill us sp*	*Proteus sp*	*Bacillus sp*

+=Positive Results,- =Negati ve Results, D=differential, ND=Not Determined

Fig-3 Phylogenetic tree of *Pseudomonas proteolytica* strain S13D



L-Asparaginase production from microbial sources through fermentation has been a unique method, owing to its cost-effectiveness and eco-friendly nature. The increasing importance of L-Asparaginase in recent years for its therapeutic applications as well as extensive uses in food industries prompted to utilize newer microbial sources for L-Asparaginase production. In the present study, 67 different strains were isolated from soil and screened for L-Asparaginase production. Out of them 46 were found to be positive. Their ability to metabolize L-Asparagine, present in the culture medium, was selected as the criteria for the secretion of L-Asparaginase enzyme. After qualitative and quantitative estimation, 46 different bacteria were identified as asparaginase producer, which belong to eight different genera (table 2). After generating 16S rDNA sequence from efficient L-Asparaginase producer bacterial isolate, using an automatic sequencer, the sequence obtained (1476 nucleotides) was compared with known sequences from one of the number of databases available on the internet, viz. the Ribosomal Database Project or NCBI (National Center for Biotechnology Information). The query sequence was submitted to NCBI BLAST (Basic Local Alignment Search Tool) for nearest homologue. BLAST search gave sequences with their percentage of match between each member (fig 4). Sequences obtained from Blastn (nucleotide blast) were obtained in FASTA format and relation between each sequence could be known by multiple sequence alignment using a software CLUSTAL algorithm. BLAST search for this isolate result in production of 44 related sequences with the closest match to *Pseudomonas* group. The output file of this alignment was saved in the PHYLIP file format. The phylogenetic affiliation of different sequences recovered could be determined by placing them into a phylogenetic tree using one of a variety of software packages designed for this purpose. The methods used in phylogeny included distance matrix, parsimony and likelihood methods. The phylogenetic programs were controlled through a menu, which allowed us to set different options, and start the computation. TREEVIEW software was used to generate a phylogenetic tree for the L-Asparaginase producing bacterial isolate (fig 5). This resulted in placing the 16S rDNA sequence of this bacterium in a range of *Pseudomonas* group those comprised of *Pseudomonas proteolytica* strain CMS 64, *Pseudomonas gessardii* strain CIP 105469, *Pseudomonas libanensis* strain CIP and some other unidentified *Pseudomonas species*. However, till date there are no reports on *Pseudomonas proteolytica* for its activity of hydrolysing asparaginase. Thus, the *Pseudomonas proteolytica* strain S13D obtained during this study is a novel of its type with unique characteristics of hydrolysing asparaginase. Therefore a new strain name *Pseudomonas proteolytica* strain S13D was assigned to this efficient L-Asparagine hydrolysing bacterial isolate. *Pseudomonas proteolytica* strain S13D to be a good producer of L-asparaginase. A further exploration on the regulation of physico-chemical parameters to increase the L-Asparaginase production potential is underway.

Based on plate assay protocol, 67 bacterial isolates were initially screened for the enzyme production. After quantitative estimation, 46 were found to produce asparaginase, were categorized to eight different genera. Among them maximum activity was shown by Strain S13D, which was identified as *Pseudomonas*

proteolytica on the basis of 16s-rDNA. It is envisaged that further work on purification and characterization of enzyme must be done from the potential isolates. Experiments in cell culture and animal model may also be required to determine their therapeutic efficacy and safety.

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