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Molecular Characterization and Cytotoxic Activity on HCT-15 and HepG2 of L-asparaginase Producing Bacteria from *Mugil cephalus*

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Abstract: ***Over the recent years the active role of enzymes in every field of work has tremendously increased. Medicinal field is one such area which harbours the enzyme for the treatment of many diseases. L-asparaginase is a therapeutic enzyme which has the ability to treat acute lymphoblastic leukaemia and lymphosarcoma. It converts the free asparagine to aspartic acid and ammonia. Present paper showed the biochemical analysis and the molecular characterization of maximum enzyme producing bacterial strain LAS-8. 16S rRNA of the strain was sequenced and identified as Enterobacter cloacae. The precipitated enzyme sample was further analysed for its cytotoxic activities.***

Keywords: L-asparaginase, acute lymphoblastic leukaemia, *Enterobacter cloacae*, HCT 15 cell line, HepG2 cell line

1. Introduction

L-asparaginase which was isolated from the microbial origin is of greater use in the clinical purpose [1]. L-asparaginase which has got the chemotherapeutic activity will convert L-asparagine to L-aspartic acid and ammonia [2]. Apart from medicinal purpose, food industry also exploits the usage of this enzyme which prevents the formation of acrylamide when food processing at high temperatures takes place [3]. The reason behind using this enzyme is it is non-toxic, biodegradable and administration is convenient at the local site [4]. Natural products from marine source play a key role in drug development in the recent years [5]. The L-asparaginase from the microbial origin which is in clinical practice includes ELSPAR, Erwinase and PEG-L-asparaginse [6]. Molecular techniques play vital part in the analysis and characterization of microorganisms from various sources. Thus the molecular methods provide the detailed information about the organism which is easy to use and interpret [7].

2. Methodology

2.1 Sample Collection

The fish sample (*Mughil cephalus*) was freshly collected from the Velrampet lake, Puducherryand transferred to a sterile polythene bag. The external surface of the sample was sterilized with 70% ethanol before opening the ventral surface with sterile scissors.

2.2 Gut isolation and their homogenization

The fish sample was dissected and the gut was isolated. One gram of fish intestine was taken, homogenised and suspended in 9ml of sterile water. It is then serially diluted and allowed to grow in modified M9 medium.

2.3 Isolation of marine bacteria

Standard microbiological methods were employed and dissected fish gut was grinded with sterile pestle and mortar, serial dilution was done and the samples were plated on Zobell marine agar which was supplemented with 3% NaCl and it was incubated at 30°C for 24h [8]. Based on the colony morphology and the potential to produce maximum enzyme, these bacterial isolates were chosen for further studies.

2.4 Screening of isolated bacteria for L-asparaginase production

Semi-quantitative rapid plate assay was employed to screen the bacterial isolates for the production of L-asparaginase [9]. On the modified M9 medium the cultures were streaked which is supplemented with L-asparagine (2% W/V) and phenol red (0.09% W/V) as pH indicator. Phenol red appears yellow in colour at acidic pH and turns pink in alkaline pH on incubation. Positive strains showing pink zones were screened after 48 hrs of incubation which promises the production of L-asparaginase [10].

2.5 Biochemical Studies

Morphological characters such as colour and shape of the colonies were examined. Motility and Grams staining were also performed. L-asparaginase producing potential strain was biochemically analysed for the activities of ONPG (O-Nitrophenyl-β-D-galactopyranoside), Lysine utilization, ornithine utilization, Urease, Phenylalanine deamination, nitrate reduction, H_2S production, Citrate utilization, VogesProskauer's, Methyl red, Indole, Esculin hydrolyses, Oxidase test and sugar fermentation tests such as Arabinose, Xylose, Adonitol, Rhamnose, Cellobiose, Melibiose, Saccharose, Raffinose, Trehalose, Glucose, Lactose by standard microbiological methodsand results were compared with Bergey's manual of Systematic Bacteriology.

2.6 Molecular characterization of potent asparaginase producing strain LAS-8

2.7 Genomic DNA isolation

Centrifugation was carried out for 2 min at the rpm of 8000. After removing the supernatant, the cells were washed with 400µl of STE buffer twice. The cells were centrifuged for 2 min at 8000 rpm at 10°C. Re-suspension of the pellets was done in 200 µl of TE buffer followed by 100 µl of Tris – saturated phenol and vortexed for 90sec for bacteria cells to lyse. The samples were again centrifuged at 13000 rpm at 4°C for 5 min to separate the aqueous and the organic phase. To a clean 1.5 ml vial 160 µl of aqueous phase was transferred. To this vial TE buffer (40µl) and chloroform (100 µl) was added and centrifuged at 13000 rpm for 5 min at 4°C. Purification with chloroform extraction was repeated till a white interface was no longer appearing. To the 150µl of upper aqueous phase 1ml of absolute ethanol was added and centrifuged at 13000 rpm for 5 min at 4°C. The pellet thus obtained was dissolved in 40 µl of TE buffer/nuclease free water [11].

2.8 PCR Ampliflication

The genomic DNA of *Enterobacter* strain was isolated [12]. PCR was performed with the Universal primers forward (5′ AGAGTTTGATCCTGGCTCAG3′) and reverse (5′ GGTTACCTTGTTACGACTT3′) and 40 ngof template DNA. PCR was done as follows: denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, temperature for primer-specific annealing is at 53.8°C for 45 sec and extension at 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were electrophoresed in a 1% agarose gel in 1 × TAE buffer which was pre-stained with 1 µg/ml ethidium bromide.

Primer Details

Primer Name	Sequence Details	Number of Base
8F	5'AGAGTTTGATCCTGGCTCAG 3'	20
1942R	5'GGTTACCTTGTTACGACTT 3'	19

2.9 DNA Sequencing and construction of phylogenetic tree

With the same forward and reverse primer, sequencing reactions were performed in both directions. Bio-Edit (Version 7.0.9.0) was used for reassembling the sequences. The sequence homology of the potential isolate in identifying the species was made using the BLASTn program (NCBI). Based on the comparison of 16S rRNA sequences the phylogenetic tree was prepared for *Enterobacter*strains with the other strains of *Enterobacter* that were acquired from the Gen Bank database (http://www.ncbi.nlm.nih.gov). With Clustal Omega all sequences were aligned and phylogenetic tree was constructed using the Neighbourhood - Joining method, the Bootstrap value being 1000 in MEGA5.

2.9.1 Cytotoxic activity

Briefly, 96 well plates were seeded with 200µl of Human Colon cancer cell line **(HCT15)**and **(HepG2)**Humanliver cancer cell lineat a concentration of $1x10^4$cells per well. DMEM media with 1X antibiotics and 10% FBS was used to maintain the cell lines. The cells were incubated with 5% CO_2 at 37˚C in CO_2 incubator. Different concentrations of 20, 40, 80, 200, 400(µl/ml) and 200, 400, 600, 800, 1000 (µl/ml) of L-asparaginase were treated on the cell lines HCT-15 and HepG2 respectively and inoculated for 24 hours to study the cytotoxicity of L-asparaginase. After 24 hrs incubation the cells were washed with sterile 1X PBS to remove unattached cells. Appropriate controls were maintained. To study the percentage cytotoxicity MTT at a concentration of 0.5mg/ml in PBS was prepared and 10µl of MTT solution was added to each well. The cells were incubated in 5% CO_2at CO_2 incubator for 4hrs. On completion of incubation cells were washed with sterile 1X PBS.

The crystal formed was dissolved using 100µl of DMSO and mixed thoroughly. The intensity of formazan formation was calculated by measuring the absorbance at 570nm using a plate reader. The percentage cytotoxicity was calculated using the formula

$$\text{Percentage cytotoxicity} = \frac{A_1-A_0}{A_0} \times 100$$

A_1- drug treatment
A_0- control

3. Results and Discussions

3.1 Screening and isolation of potent bacteria

Isolation was initiated with 153 bacterial strains that were isolated from *Mugil cephalus* gut collected from Velrampet lake Puducherry. 8 microbial isolates showed positive result for L-asparaginase by producing pink coloured zones on Modified M9 medium where phenol red acts as indicator as depicted in Figure 1. The optimum pH (6.5-7) and temperature (30°C) was maintained for obtaining the maximum yield of 69.88IU/mg from the bacterial strain LAS-8 [12]. Hence this strain was selected for further study of biochemical and molecular characterization.

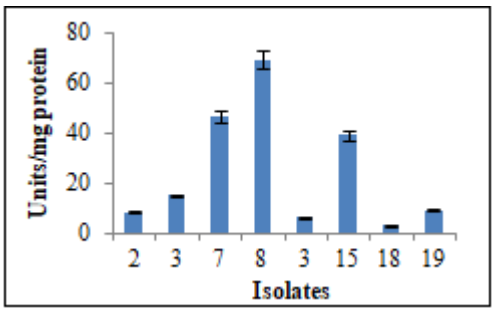

Figure 1: Quantitative Screening of L-asparaginase producing bacterial isolate

3.2 Biochemicalstudies

Morphological and biochemical characteristics of the strain LAS-8 was performed and the results were shown in Table 1. Morphological studies showed the microorganism to be

gram negative, rod and motile. Different biochemical characters have been studied among them ONPG, Ornithine, Nitrate, Citrate, VogesProskauer's, Malonate, EsculIn hydrolysis showed positive results and other tests such as Lysine utilization, H_2S production, Phenylalanine deamination, Urease, Methyl red, Indole, Oxidase were negative. LAS-8 was found to utilize maximum selected sugars such as Saccharose, Xylose, Cellobiose, Raffinose, Arabinose, Trehalose,Rhamnose and Glucose except Adonitol, Melibiose and Lactose. The morphological and biochemical characters confirmed the strain LAS-8 to be *Enterobacter sp*. as shown in table 1[13].

Table 1: Morphological and Biochemical characteristics of LAS-8 isolate

Morphological characters			
Gram staining	Gram negative		
Morphology	Rods		
Motility	Motile		
Biochemical characters			
ONPG	+	Sugar Utilization	
Nitrate reduction	+	Arabinose	+
Ornithine utilization	+	Xylose	+
Urease	-	Adonitol	-
Lysine utilization	-		
Phenylalanine deamination	-	Rhamnose	+
Citrate Utilization	+	Cellobiose	+
H_2S production	-	Melibiose	-
Malonate Utilization	+	Saccharose	+
Oxidase	-	Raffinose	+
Methyl red	-	Trehalose	+
VogesProskauer's	+	Glucose	+
Indole	-	Lactose	-
Esculin hydrolysis	+		

3.3 Molecular characterization

The Genomic DNA was extracted using standard procedure. The gene 16S rRNAwas amplified using the primers 8F (5′ AGAGTTTGATCCTGGCTCAG3′) and 1942R (5′ GGTTACCTTGTTACGACTT3′) and the gel was documented as in figure 2. Thus the bacterium producing maximum enzyme was sequenced and submitted to NCBI Genbank with accession number (MH36811) and phylogenetic analysis shared 100% similarity with *Enterobacter cloacae*.

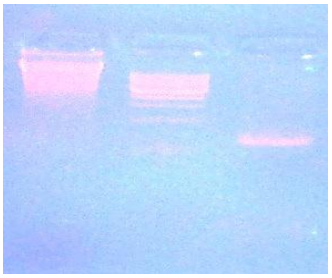

Figure 2: Genomic DNA extracted from the bacterial isolates & PCR amplification of 16SrRNA gene

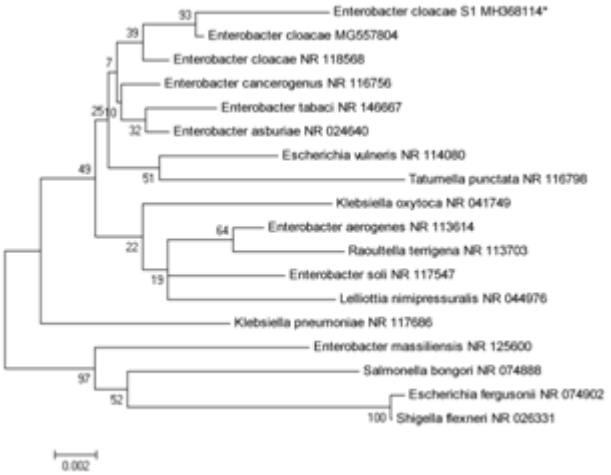

Figure 3: Evolutionary relationships of taxa (Neighbour-Joining method)

By Neighbour-Joining method the evolutionary history of the bacterial strain was inferred [14]. The evolutionary history of the taxa was analysed by the bootstrap consensus tree inferred from 1000 replicates. The percentage of replicate trees of the related taxa were clustered together in the bootstrap test (1000 replicates) is shown next to the branches [15]. Using the Maximum Composite Likelihood method the evolutionary distances were computed that are in the units of the number of base substitutions per site [16]. 18 nucleotide sequences were involved in the analysis. The final dataset contained 1326 positions and the Evolutionary analyses were conducted in MEGA5 [17].

3.4 Cytotoxic activity

The lyophilized L-asparaginase enzyme which was precipitated by ammonium sulphate and partially purified by dialysis was subjected to anti-inflammatory activity *in vitro* on the cell lines of HCT 15 (Human Colon carcinoma) and HepG2 (Human Lung Cancer). MTT was converted to purple coloured formazan byviable cells into a product with an absorbance near 570 nm. When cells die, the ability of viable cells to convert MTT into formazan is lost.

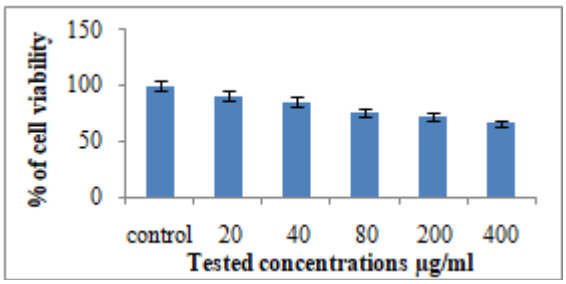

Figure 4: Cytotoxic activity of the enzyme on HCT-15

The cytotoxic activity has shown no significant effect on the Human colon cell line at various concentrations. This is in contrast to the result that L-asparaginase produced from chicken liver has inhibited the growth of HCT-116 [18].

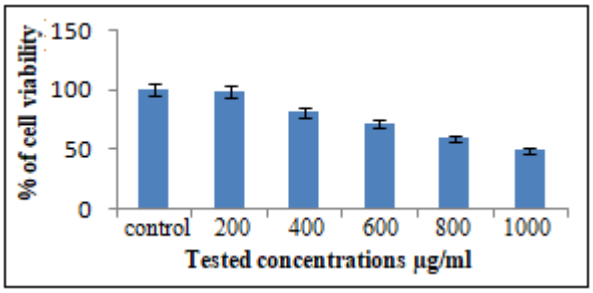

Figure 5: Cytotoxic activity of the enzyme on HepG2

Significant anti-leukemic activity of the enzyme was observed at higher concentrations in the Human Lung Cancer cell lines.

4. Conclusion

The members of *Enterobacteriaceae* family are proved to be the best producers of L-asparaginase [19]. The present study discovered the presence of endo-symbiontic bacteria which is involved in the production of potent anti-leukemic L-asparaginase from the gut of *Mugil cephalus*. The enzyme was subjected to morphological, biochemical and molecular analysis which concluded that the organism was *Enterobacter cloacae* (MH368114-GenBank Accession Number). Studies have demonstrated that the human colon cancer cell line HCT 15was resistant and there is no inhibitory effect seen by the enzyme L-asparaginase whereas there is notable anti-leukemic activity observed in the Lung Cancer Cell Line of human.

L-asparaginase has proved to a potent anti-proliferative enzyme in Acute Lymphoblastic Leukaemia (ALL). Hence the present study focused on producing L-asparaginase from the marine product which is naturally and commonly available. More researches are to be focussed in bringing this enzyme to a greater reach in the pharmaceutical field in controlling the tumorigenic cells.

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