Iman Mohammed Jebur et al /J. Pharm. Sci. & Res. Vol. 11(3), 2019, 961-965

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Cloning and expression of l-Asparaginase gene from *Aspergillus terreus* in *E.coli*

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Abstract-
In the present study, L-Asparaginase was isolated from fungal source (Aspergillus terreus) and it is used as the treatment of malignancies. The synthesis of L-asparaginase from two mutant strains, WF and 933 of Serratia marcescens is reported and different media composition alters the levels of enzyme production. The phenotype was conducted by using a light microscope. While the genotype of the strain was also identified. The 18S rDNA sequences of the strain were determined by PCR. The enzyme was purified using ammonium sulphate precipitation, Sephadex G100, CM-cellulose and DEAE Sephadex chromatography.
The molecular weight of the l-Asparaginase from E.coli was determined by SDS-PAGE gel electrophoresis and it was found to be a single protein band with approximately 43 kDa.
Index Terms- cloning, PCR, component, L-Asparaginase.

INTRODUCTION

L-asparaginase is used in the treatment of malignancies of the multiorgans [1]. The enzyme L-asparaginase was found to be responsible for the antitumor activity of guinea pig serum [2, 3]. Further, it was identified that L-asparaginase was also an effective antitumor agent in human clinical trials and now it is regarded as one of the important components of antitumor therapy.
L-asparaginase is the enzyme that hydrolyses asparagine into aspartic acid and ammonia.

STRUCTURE OF L-ASPARAGINASE:

Asparaginase A (ASNase A), the cytoplasmic asparaginase from E.coli forms a tetrameric structure [4]. The crystal structure of Helicobacter pylori, L-asparaginase at resolution 1.4 Å in presence of aspartate can be determined [5].

SOURCES OF L-ASPARAGINASE:

L-asparaginase has been documented to be synthesized from a variety of sources like microorganisms, plants, yeast, fungi and microbial sources from soil and is preferred for therapeutic use.
Bacterial Sources: The synthesis of L-asparaginase from two mutant strains, WF and 933 of Serratia marcescens is reported and different media composition alters the levels of enzyme production.
Yeast and fungi Sources: The production of Asparaginase from different strains of fungi has been reported using a range of media. The production of L-asparaginase by filamentous fungi viz. Aspergillus tamarii and Aspergillus terreus has been reported with the highest L-asparaginase production level in 2% proline medium from A. terreus [6].
Plant Sources: A variety of plant species are described with a significant amount of asparaginase. Green chilies (Capsicum annum L.) and tamarind (Tamarindus indica) contain a certain amount of Lasparaginase and enzyme was purified using ammonium sulphate precipitation, sephadex gel filtration and affinity chromatography [7]. The low temperature inducible cDNA sequence that encodes Lasparaginase was isolated from soybean leaves and expressed in E.coli with almost has 3 times increased activity [8].
L- Asparaginase in soil: The effects of municipal solid waste compost and decomposed cow manure on L-asparaginase activities in submerged rice soil have been studied.

PURIFICATION AND CHARACTERIZATION OF L-ASPARAGINASE

L-asparaginase from Erwinia carotovora was purified using dialysis and DEAE-cellulose anion exchange chromatography. The enzyme activity was found to be 0.31mg/ml/min using L-asparagine as substrate and there was an increase in the affinity of L-asparaginase in presence of dihydropyrimidine derivative [9]. The enzyme was purified from Erwinia carotovora using ammonium sulphate precipitation, sephadex G100, CM-cellulose and DEAE Sephadex chromatography. The enzyme showed maximum activity at pH 7 and temperature 37ºC, inhibited by Ethylenediaminetetraacetic acid (EDTA) and activated by MgCl2. The alkaline pH favours the stability of the enzyme and the apparent weight of the enzyme was found to be 85kDa [10].

MODIFICATION OF THE ENZYME:

The modified form exhibits enhanced resistance to trypsin degradation and has higher thermal stability compared with the wild-type enzyme. The therapeutic properties of L-asparaginase by conjugating the recombinant enzyme from Erwinia carotovora with polyethylene can be improved with modifying reagent Methoxy-p-nitrophenyl carbamate of polyethylene glycol [11].
The enzyme conjugated with oxidized inulin in 2:1 ratio showed decreased antibody (IgG) titer and immunogenicity after repeated injection as compared to the native enzyme.

TREATMENT WITH L-ASPARAGINASE:

Leukemia cells ABT-737 and L-asparaginase together induced greater mitochondrial depolarisation, mitochondrial cytochrome-C release, activation of Bax, Bid and eventually apoptosis than either drug alone [12].
In acute lymphoblastic leukemia, there is the clinical utility of asparaginase antibodies along with some allergic reactions and serum antibodies due to E. coli-asparaginase [13].

Table.1 PCR primer sequence (5'to 3')

Forward primer	5′-GAATTCATGGGTTTCAACATCAAAGCT-3′
Reverse primer	5′-AAGCTTCTAAGCGACAGCCACCTTGGCA-3′

SIDE EFFECTS OF L-ASPARAGINASE

A 7-year-old Thai boy suffering from acute lymphoblastic leukemia developed pancreatic panniculitis when administered with L-asparaginase [14]. Cholesterol level and triglyceride level increases during asparaginase treatment in children with acute lymphoblastic leukemia [15].

Hypersensitivity reactions are caused by some antineoplastic agents caused by L-asparaginase [16].

MATERIALS AND METHODS

Collection of soil sample

The samples were collected in sterilized bottles for isolation of Asparaginase producing *Aspergillus nidulans*.

The composition of media employed

For isolation and the study of fungi, potato dextrose agar medium was used in the present work.

Plate Assay for Evaluation of L-Asparaginase Production

Modified Czapek Dox's medium was supplemented with 0.3 mL of 2.5% phenol red dye prepared in ethanol at pH 6.5 with L-asparagine incorporated in the medium for evaluation of L-asparaginase activity. The media was autoclaved and the plates were inoculated with a 3-d-old culture of *A. nidulans.* The clear zone appeared after 48 h of growth. Uninoculated media served as control.

Morphological identification of fungal culture

Micro slides of fungal culture were prepared in lactophenol-cotton blue, examined under a microscope, observed their morphological characters and identified with the help of the standard keys provided by Rifai, (1969). The measurements of the spore forms and vegetative structures were taken with the help of an ocular micrometer.

The identified fungus was stored on potato dextrose agar slants in the refrigerator at 4^0C prior to use.

Molecular identification of L-asparaginase producing fungus

The morphological analysis was conducted by using a light microscope. The genotype of the strain was also identified. The 18S rDNA sequences of the strain were determined.

DNA extraction

The DNA from one-week old fungal strain was extracted following the Biogene Kit method.

The polymerase chain reaction (PCR) primers ITS-4 and ITS-5, developed by white et al (1990) were used to amplify the internal transcribed spacer regions of ribosomal DNA, which encompass the 18S rDNA gene and both ITS-1 and ITS-2 regions.

PCR amplification products were electrophoretically separated on 1.2% agarose gel prepared in 1×TAE. The gel was run for 45 min at 135 V. Staining was done with ethidium bromide and gels visualized at 300 nm UV and photographed. Gel photographs were scored for the presence and absence of scorable bands with the assumption of positional homology. To establish the genetic relationship of isolate, similarity coefficients were calculated between isolates and dendrogram draw using UPGMA employing the NTSYS-PC, Ver. 2.02 h program (Rohit and NTSYS-PC., 1997; Sneath and Sokal, 1973).

Aspergillus species were designated to the sequenced cultures and analyzed based on similarity with the best-aligned sequence of the BLAST search. The 18S rDNA gene sequence alignments were performed using Clustal × 1.83 software (Thompson et al., 1997).

Amplification of l-Asparaginase gene by PCR:

The primer set used for amplification of l-Asparaginase gene was as mentioned in below Table.1.

Cloning of ASP gene

The amplified PCR products were purified using a QIAquick PCR purification kit (Qiagen, GmbH, Germany) following the manufacturer's instructions.

The purified PCR products were cloned separately into a pGEM-T Vector (Promega, Madison, WI), followed by transformation of the recombinant plasmid into E. coli JM109 Competent Cells. The plasmid DNA was extracted from positive clones using QIAprep Spin Miniprep Kit (Qiagen, GmbH, Germany) and target gene present in the recombinant plasmid was confirmed by PCR. pGEM-T-Easy vector was digested with EcoRI and HindIII restriction enzymes. The cloned insert was purified from the agarose gel after electrophoresis using QIAquick Gel Extraction Kit, subcloned into pET-28a (+) expression vector predigested with EcoRI and HindIII and treated with Shrimp alkaline phosphatase (Promega, USA). The amplified product was subcloned with the plasmid vector pET101. The cloned vector was transformed to competent E. coli DH5α cells. The transformed E. coli cells were grown into an LB agar plate containing 100 µg/ml ampicillin. The plasmid was isolated from E. coli DH5α and was used to transform competent E. coli BL21 cells which were screened with blue/white selection.

Crude enzyme extraction

weighed the quantity of fermented substrate was mixed with phosphate buffer (1:5). The mixture was homogenized and filtered. The filtrate was centrifuged and clear supernatant used for enzyme assay.

Purification of L-Asparaginase

The cells were separated by centrifugation (10000 rpm, 20 minutes), and the supernatant was fractionated by precipitation with ammonium sulfate (80%) and all subsequent steps were carried out at 4°C. The precipitate was collected by centrifuging at 8,000 × *g* for 20 min, and resuspended in phosphate buffer (20 mM, pH 6.5).

Sephadex G-100 Gel Filtration Chromatography

The protein pellet obtained after dialysis was loaded onto a column of Sephadex G-100 (1.5 × 24 cm) (Sigma -Aldrich, St Louis, MO) equilibrated with phosphate buffer (20 mM, pH 6.5). The column was eluted at a flow rate of 60 mL/h

with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. The elution fraction (2.5 mL) were collected and assayed for L-Asparaginase activity and those fractions which shown high activity was collected and used for SDS-PAGE analysis.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In this study, 12% Resolving gel and 4% stacking gel was used to separate the proteins.

RESULT AND DISCUSSION

Screening and Identification of L-Asparaginase Producer

The study with phenol red dye in rapid plate assay method revealed that the presence of dye did not inhibit the growth of *A. terreus* and a clear zone appeared around the colony. A broad zone indicated that the test organism was an efficient producer of L-asparaginase. Gulati et al. *(78)* followed a similar experimental protocol for screening asparaginase producing bacterial and fungal strains.

Morphological characterization

The fungus grows rapidly with immense vegetative mycelium. Colonies are typically plain green in color with dark red-brown cleistothecia developing within and upon the conidial layer (Fig. 1).

Based on morphological characteristics the strain was identified as *Aspergillus terreus* (Rifai, 1969).

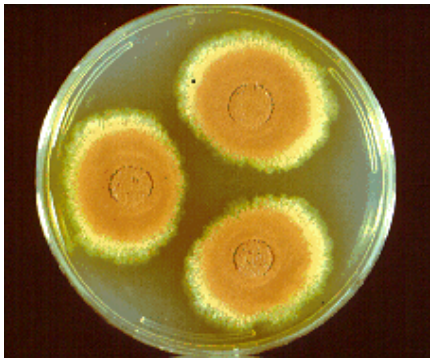

Fig. 1: *Aspergillus terreus* on an agar plate

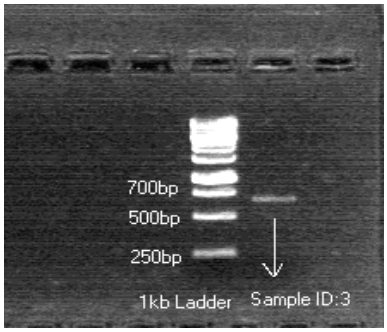

Fig. 2: Agarose gel shows a single band about 700bp.

Molecular Identification

The isolate was characterized as *Aspergillus terreus* based on its morphological and 18S rRNA sequence analysis (Fig. 2). The 18S rRNA (611 bp) sequence of isolate showed high similarity to *Aspergillus terreus* strains. Therefore, it was named *Aspergillus terreus*. The 18S rRNA gene sequence of strain isolate has been deposited in the GenBank database under accession number AB647191.

PCR amplification and cloning of ASP gene:

A PCR product of 1200bp length (fig.3) was amplified using the primer set as mentioned earlier. The results are within the range of ASP gene from Aspergillus and some other fungal species. The results are matching with the findings of earlier studies.

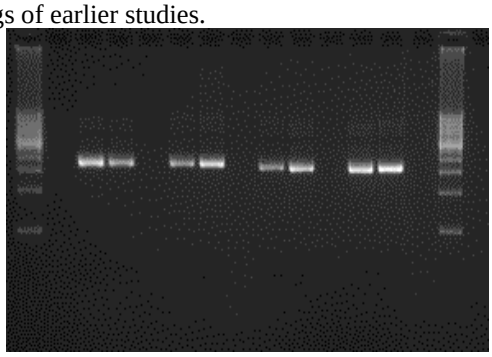

Fig. 3: Agarose gel shows a single band gene about 1200bp.

The ligated PCR product was successfully ligated into the vector and transformed into *E.coli* cells (fig.4). The plasmid was isolated from the E.coli cells to confirm the ASP gene presence by PCR again (Fig. 5 &6).

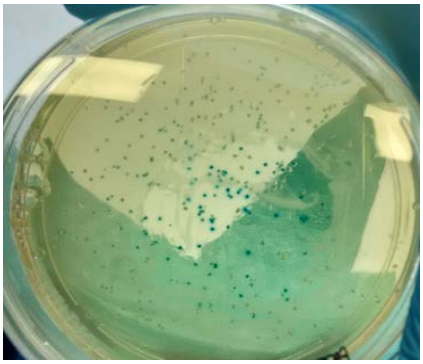

Fig.5 Transformed E.coli cells on LB agar medium.

Purification of l-asparaginase from E.coli:

The L-asparaginase produced by *E.coli* in production media was concentrated by ammonium sulfate (80%) precipitation and purified consecutively dialysis.

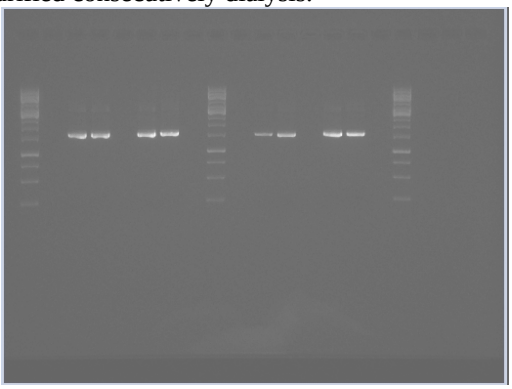

Fig.6: Agarose gel picture of ASP gene ligated pET-28a (+) expression vector (plasmid).

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Table 2. Purification steps of L-asparaginase expressed from *E.coli*

Summary of purification steps was given in Table.2.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	42.35	306.61	7.24	1.0	100
Ammonium sulfate	21.82	288.02	13.2	1.9	51.5
Dialysis	5.8	285.0	49.15	7.3	13.6
Sephadex G-100	2.6	179.6	69.1	16.28	6.13

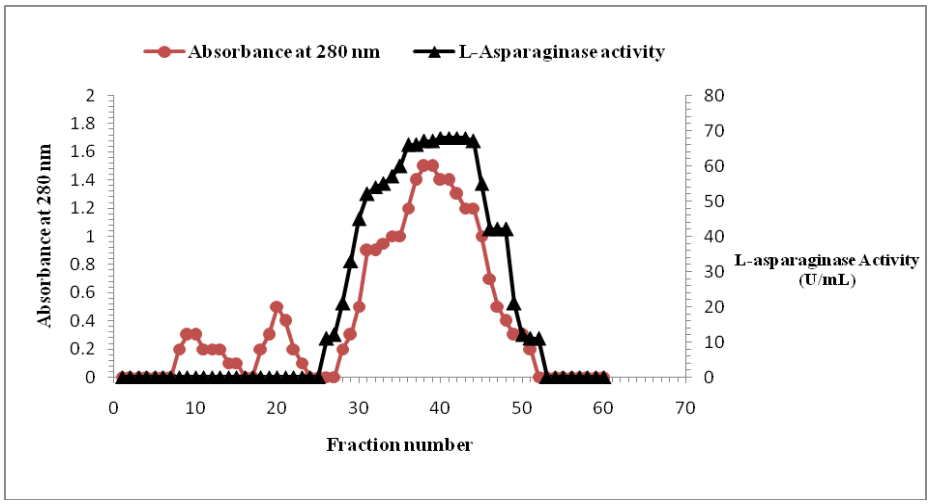

Fig. 3. Chromatogram of the L-asparaginase from *A.terreus* on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with 20 mM phosphate buffer (pH 6.5) at a flow rate of 15 ml/h. Fractions of 3 ml were collected.

The concentrated-active fractions were further purified by a Sephadex G-100 column chromatography. The active fractions with L-asparaginase activity were pooled out and collected separately. The elution profiles of protein and L-asparaginase activity are shown in Fig. 3

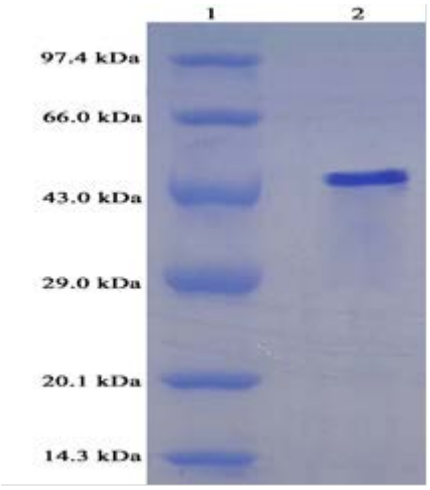

Fig. 4. SDS-PAGE of the purified enzyme; Lane 1: Marker protein, Lane: purified l- Asparaginase

The molecular weight of purified proteins:

The purified L-asparaginase from *E.coli* (lane 2) appeared as a single protein band in SDS-PAGE and with a molecular weight of approximately 43 kDa. (Fig. 4). Similarly, Akilandeswari et al (2012) reported the molecular weight of the L-asparaginase from the *Aspergillus niger* as 43 kDa. Different molecular masses that ranged from 42–46 kDa have been reported for other fungal L-asparaginase as well (Gunaratna and Balasubramanian, 1994; Harighi et al., 2007; De Marco et al., 2004).

CONCLUSION

In the present study, a 1200bp L-asparaginase gene from *Aspergillus terreus* (GenBank accession number: AB647191) was amplified and ligated into expression vector pET-28a. The ligated pET-28a vector was transformed successfully into E.coli cells and the cells were induced to express enzyme L-asparaginase by growing in using L-asparaginase production medium, followed by purification procedure. The enzyme was purified by ammonium sulfate precipitation, dialysis, and Sephadex G-100 gel filtration. The L-asparaginase from *E.coli* was purified

16.12 fold and the apparent molecular weight of the enzyme was found to be 43 kDa by SDS-PAGE. All these data suggest that the L-asparaginase enzyme can be produced at much higher and faster levels using *E.coli* cells with the same activity as that of *Aspergillus terreus* which is a potent producer of the L-asparaginase enzyme.

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