Indo Global Journal of Pharmaceutical Sciences, 2021; 11(3): 33-38

INDO GLOBAL JOURNAL OF PHARMACEUTICAL SCIENCES
ISSN 2249- 1023

# A Study on Media Optimization, Characterization and Purification of L-Asparaginase Producing *Streptomyces* sp. FSOF Strain Isolated from the Bay of Bengal Coastal Line

Tejaswini Avn, Ramesh Malothu *

*Centre of Microbiology, School of Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological University Kakinada, Andhra Pradesh, India*

**Address for Correspondence:** Ramesh Malothu, ramesh_biotech@jntuk.edu.in

**Received:** 06.06.2020
**Accepted:** 21.08.2020
**Published:** 30.04.2021
**Keywords**
Streptomyces; Asparaginase; Enzyme activity; Phylogeny; Bay of Bengal; Ion exchange chromatography.

**ABSTRACT: Purpose:** The vast marine biodiversity provides abundant opportunities for the isolation and characterization of *Streptomyces* species with therapeutic applications. The present study is focused on isolation, characterization, media optimization of *Streptomyces* sp. FSOF strain for production and purification of L-Asparaginase at an economic level. **Methods:** The isolated strain from the intertidal region of Bay of Bengal coastal region is characterized by physicochemical, genotypic and by phylogenetic methods. The growth medium of *Streptomyces* FSOF strain is optimized for high enzyme activity and further purified by Ion exchange chromatography. **Results and Conclusion:** The FSOF isolate exhibited 4.3 IU/ml enzyme activity in m9 broth. The enzyme activity of the FSOF strain is further analyzed in various growth mediums where 5.9 IU/ml activity is observed in tryptic soy broth. The purified L-asparaginase from FSOF isolate exhibited 23.33 IU/mg specific activity with 3.12 fold purification. The 16s rRNA sequence of FSOF isolate is found to have 99.88% similarity with *Streptomyces albidoflavius* and *Streptomyces odorifer strains.* The sequence is further deposited in GenBank (accession number MN562193.1) for future analysis. © 2020 iGlobal Research and Publishing Foundation. All rights reserved.

**Cite this article as:** Avn, T.; Malothu, R. A study on media optimization, characterization and purification of L-asparaginase producing *Streptomyces* sp. FSOF strain isolated from the Bay of Bengal coastal line. Indo Global J. Pharm. Sci., 2021; 11(3): 33-38. **DOI**: http://doi.org/10.35652/IGJPS.2021.113005 .

## INTRODUCTION

Exploring the immense marine diversity for novel sources and metabolites with major therapeutic properties is a promising approach. Increasing life expectancy and finding a cure for the prevailing conditions challenged by the malignant tumor is of high priority to the researchers. As the available drugs are experiencing resistance buildup, a quest for novel sources of anticancer compounds has arisen. This stands as a significant objective for the present research process. Actinomycetes species are the most research-focused bacteria as they possess habitat, genetic and biosynthetic diversity that endures normal to extreme conditions [1].

The Actinomycetes species are gram-positive, possessing high G+C content, large genome and a vast number of transcription factors that regulate the gene expression [2]. The conversion of L-asparagine by L-asparaginase [3] induces deprival and inability of the tumor cells to synthesize the requisite amino acid eventually triggers apoptosis [4]. The research on the past and present findings on the extraction of L-asparaginase explains that various microbial sources are chosen for its production namely *Bacillus circulans* [5], Lactobacillus sp isolated from Kerala sea coast [6], isolates of Red sea coast [7], *Thermus thermophiles* [8], *Alternaria sp* [9], Erwinia *carotovora* [10], *Salinicoccus sp* [11], *Streptomyces sp* PDK7 [12], *Streptomyces tendae* TK-VL_333 [13], isolate VUK-10 [14], isolates of Koringa mangrove [15], [16]. Attempt to discover new microbial sources for L-asparaginase specifically from marine biodiversity has prime focus due to its therapeutic application precisely in the therapy of acute lymphoblastic leukemia [17].

## MATERIALS AND METHODS

### Isolation

The present study is aimed to isolate the L-asparaginase producing *Streptomyces* species from the intertidal region of the Bay of Bengal coastal line situated along the Kakinada city

(16°57′58″N 82°15′18″E), Andhra Pradesh, India. Foreshore sediment samples are collected at unique areas alongside the coastal line (**Fig. 1**). The unfiltered samples are stored in sterile bottles and transferred to the School of Biotechnology, JNTUK for further analysis.

**Fig. 1: Area of sample collection.**

**Pretreatment of the samples**

The unfiltered samples are transferred to sterile Petri plates to analyze the initial appearance and physical characteristics. The samples are divided into two halves where the first group is dried in an incubator (Remi CIS 24 plus) at 37°C for eight hours (h) while the second cluster of samples are dried in natural sunlight for 8 h. The two different initial incubation phases are chosen to determine the best suitable growth condition. At the end of each incubation period, 1 gram (g) of each untreated sample is transferred to 9 ml of pre-autoclaved water and vortexed for 15 minutes (min) to attain a near homogeneous mixture of the sample and water [18]. The vortexed samples are serially diluted up to $10^{-5}$ dilutions and labeled accordingly [19]. Glucose asparaginase agar (GAA) (Glucose 10 g, L-asparagine 0.5 g, $K_2HPO_4$ 0.5 g, Agar 20 g, Water 1 L, pH 7.4) [20] is used to facilitate the growth of the microorganism. The media apart from glucose is autoclaved at 121°C at 15 lbs pressure for 15 min. The carbon source glucose is filter-sterilized [21] and added aseptically to the above media. 1 ml of each sample is transferred to the culture media under aseptic conditions by spread plate technique [22] and incubated at 30°C for 7 days under continuous observation.

**Primary Screening of the isolates**

Single discrete colonies selected from the seven-day isolates of GAA are inoculated on a modified M9 medium (Agar 20 g, L-asparagine 5 g, $NaNO_3$, 1.25 g, 20% Glucose 10 ml, 1M $MgSO_4$ 2 ml, 0.1M $CaCl_2$ 1 ml, Phenol Red 0.02%, M9 Salts comprising $Na_2HPO_4$ 6 g, $NH_4H_2PO_4$ 3 g, NaCl 0.5 g, Water 1 L) 37°C for 48 h. The isolates are screened by rapid plate assay to identify the L-asparaginase producing strain [23]. The media components consisting of M9 salts, glucose solution, $MgSO_4$, $CaCl_2$ are filtered sterilized and added to the medium under aseptic conditions [24]. The rapid plate technique is a pH, dye-based test used to detect the release of ammonia by the microorganism. The media is enriched with L-asparagine (SRL chemicals) and a few drops of 0.02% phenol (Fisher Scientific) and 100 µg/ml Fluconazole. The presence of phenol enables distinct change in media colour to pink which identifies the enzyme-producing isolate [25],[26].

**Secondary screening and Quantitative estimations of enzyme**

The selected isolate in rapid plate assay is observed for growth in 50 ml of modified m9 broth for a period of 48-120 h. The growth media is maintained at room temperature. The growth media is supplemented with a nitrogen source L-asparagine 5 g and 100 ug/ml fluconazole by filter sterilization to inhibit the growth of fungi. After a duration of 48 h, 1 ml of cell-free supernatant from the fermentation product is then analyzed for enzyme assay by the Nesslerization method [26]. The reaction mixture constitutes 0.5 ml each of 0.05 M Tris-HCl buffer, 0.04 M L-asparagine, crude enzyme. The reaction is brought to an end with 0.5ml of trichloroacetic acid (1.5 M). The enzyme mixture is thus mixed with Nessler's reagent and the observed for 15 min. The amount of ammonia liberated is measured using a UV-Visible spectrophotometer (Biochrom Libra) at 450nm.

**Identification of FSOF isolate**

The isolate found L-asparaginase positive is subjected to morphological analysis and various biochemical characterizations [27]. The isolate colonies are sub-cultured on GAA, Tryptic soy agar (TSA) (Himedia, Pancreatic digest of casein, Papaic digest of soya bean meal, NaCl, Dextrose, $K_2HPO_4$, Agar).

**Genotypic characterization and Phylogenetic analysis**

The genomic DNA isolation is done by adhering to the Biopure™ kits manufacture's guidelines. The FSOF Strain identification is done at Bioaxis DNA Research Centre, Hyderabad, India. Universal primers are used for FSOF 16s rRNA amplification.

- 8F (AGAGTTTGATCCTGGCTCAG)
- 1492R (CGGTTACCTTGTTACGACTT)

The sequential process of PCR is initial denaturation (94 °C-5 min), 35 cycles comprising denaturation (94 °C-30 s), annealing (55°C-45 s), extension (72 °C min) and a final extension (72 °C-7 min). The PCR outcome is analyzed on electrophoresis (1% agarose). The amplicon sequencing is carried on ABI PRISM® 377 Genetic Analyzer. The aligned sequence is searched in the BLAST (Basic Local Alignment Search Tool) algorithm (nr database) [28]. The relationship between the top percentage similarity sequences of the GenBank and the obtained FSOF query is evaluated using MEGA software [29].

**Optimization of FSOF culture conditions**

The ideal growth conditions that enhance the crude enzyme formation are found by altering the pH, temperature, incubation period and substrate as the growth conditions are distinct and vary from one microorganism to the other [30],[31].

**Temperature, pH and incubation period**

The microorganisms are classified to heat stable to temperature liable depending on their growth patterns [32]. The isolate is observed for variation in growth at varied culture conditions thus identifying the optimum conditions that enable high enzyme production. The selected isolate is analyzed for changes in a modified m9 broth. The medium is incubated between 20 °C to 50 °C at constant pH and incubation period. Similarly, the effect of pH and incubation period appropriate for the increased enzyme yield is identified by varying the pH levels from 6 to 9 and 12 h to 72 h respectively.

**Selection of suitable substrate for increased enzyme activity**

The growth, enzyme production and activity are studied by inoculating the FSOF isolate in 50 ml of glucose asparaginase broth (GAB), starch casein broth (SCB) (Starch 10 g, $K_2HPO_4$ 2 g, $FeSO_4.7H_2O$ 0.01 g, $NaNO_3$ 2 g, $CaCO_3$ 0.2 g, Casein 0.3 g, MgSO. $7H_2O$ 0.05 g, NaCl 2 g) [33], LB (Tryptone 5g, NaCl 5g, yeast extract 2.5g, water 500ml), tryptic soy broth (TSB). The media constituents are autoclaved and filter sterilized as per the requirements.

**Purification of the enzyme produced by FSOF strain**

Purification process is performed in a sequential process at 4 °C primarily with ammonium sulphate fractionation. The crude extract of TSB is centrifuged at 10,000 rpm for 10 min. Ammonium sulphate is added to the crude enzyme extracted from FSOF sample to achieve 80% saturation. The precipitate is obtained after centrifuging at 10,000 rpm for 10 min is then subjected to dialysis with 1M Tris-HCL buffer. The active fractions are applied to a DEAE column pretreated with 0.05M TrisHCl (pH 5.6). The active elutions comprising NaCl gradient of 0.1-0.5M and borate buffer is dialyzed accordingly. The presence of enzyme activity[26] along with protein estimation [34] is assayed at every required stage of the purification process. The extracted, active, purified enzyme fraction is analyzed on SDS gel electrophoresis with 10% SDS, 12% resolving gel, 10% APS, and 5% stacking gel.

# RESULTS AND DISCUSSION

**Isolation of the microorganism and screening for enzyme activity**

The samples are named with a three to four-letter code for easy identification. Eleven discrete colonies grown on GAA are transferred to the modified m9 medium. Four isolates from BWS, FWS S1, FWS S2, FWS S3 exhibited high to moderate growth. Isolate from FWS S2 is found to form a larger diameter of the pink zone measuring 2.8 cm. The remaining three isolates remained negative even after 72 h of incubation. The isolate FWS S2 is designated as FSOF for further production and optimization of L-asparaginase.

**Identification of FSOF strain**

The phenotypic characterization of the FSOF strain appeared (front and back view of the isolated growth) to be round with a tough, unequal, elevated creamy-white powdery texture in coincidence with the results by Nagaseshu et al. [35] on SCA medium. The medium is observed free of any pigment production. However, the reverse of the colonies appeared yellow on TSA. The aerial mass color of actinomycetes is found with distinct with different results being reported by other researchers. A study by Shah and Soni [36] stated that the isolates appeared to be yellowish-white, whitish-grey and reddish-purple while Jeffrey et al. [37] have found dark brown, grey, white and yellow colors. The strain FSOF exhibited white aerial mass on the glucose asparaginase agar medium (Fig. 2). The FSOF isolate is found positive towards Gram's staining, starch and casein hydrolysis, catalase, urease, methyl red, whereas negative results are obtained for indole and Voges Proskauer test. The results of phenotypic and biochemical characterization helped in identifying the strain to be a member of *Streptomyces* species. The colonies appeared to be bigger in TSA rather than on GAA (**Fig. 2**).

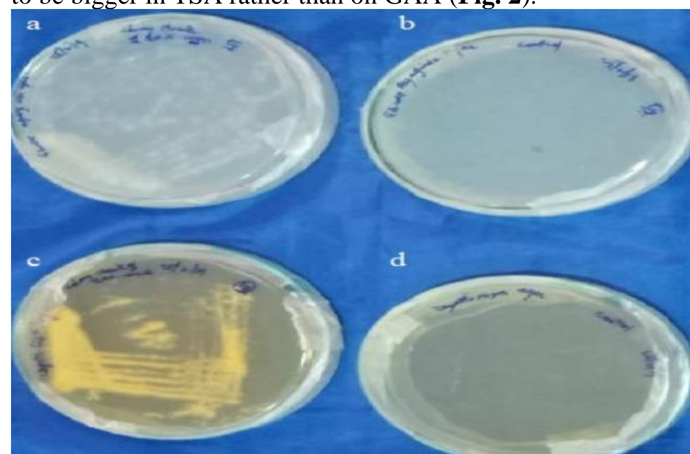


**Fig. 2: a-b: Isolate FSOF growth on GAA; c-d: Isolate FSOF growth on TSA. b and d are control for each medium GAA and TSA respectively.**

**Secondary Screening and Assay of L-asparaginase from FSOF strain**

The crude enzyme extract produced obtained after fermentation is examined for enzyme activity by the Nesslerization method. FSOF has exhibited 4.3 IU/ml enzyme activity in the unfiltered, unpurified extract.

**Molecular Identification of FSOF strain**

The molecular weight of the amplicon is found to be 1.5 kb. The aligned sequence (838 bp) is searched in BLAST algorithm (nr database) [28] showed a high percentage identity between the FSOF query and other generated hits. The *Streptomyces* sp. DNA_24 (KT954947), *Streptomyces globisporus* subsp. *caucasicus* strain NBRC 100770 (NR_112593), *Streptomyces albidoflavus* strain NBRC 13010 (NR_041095), *Streptomyces odorifer* strain DSM 40347 (NR_026535), *Streptomyces griseoviridis* strain K61 (CP029078) are the top five hits with 99.88% similarity with the query sequence. The phylogenetic relationship of *Streptomyces* species and FSOF strain is evaluated using the Neighbor-joining method [38],[39] in MEGA software [29] (**Fig. 3**). The FSOF strain is identified to be a species

belonging to the *Streptomyces* family. The *Streptomyces* sp. FSOF strain deposited in GenBank is assigned with an accession number MN562193.1.

**Fig. 3: Representation of phylogenetic relationship between 16s rRNA sequence of FSOF isolate with highly percentage similarity sequences of Streptomyces species present in GenBank.**

**Optimization of culture conditions**

The pH range between 6 to 9 apart from 7.5 expressed a low-to-moderate level of enzyme activity. It is evident from the results that the enzyme activity of *Streptomyces sp.* FSOF strain is high at pH 7.5 with 4.7 IU/ml (Fig. 4) in similarity with *Streptomyces albidoflavus* [40]. A pH of 7 is ideal for *Streptomyces acrimycini* NGP [41] and *Corynebacterium glutamicum* [42], pH between 8 and 8.5 for *Streptomyces sp.* PDK7 [12], pH of 9 for *Streptomyces gulbargensis* [43]. The enzyme activity is observed to decrease to 4.3 IU/ml, 4.4 IU/ml at 37°C and 45°C respectively representing low and high temperatures as moderately favourable. The temperature ranging from 40 °C to 42 °C (considering minor temperature changes) is found to be highly favourable with 4.7 IU/ml (Fig. 4). The temperature of 40°C is also found promising for *Bacillus subtilis* B11-06 [44] and *Streptomyces griseoluteus* sp. WS3/1 [45]. The study on the incubation period showed an increase in enzyme activity from 12 h to 48 h. The results depict that 4.9 IU/ml is observed at 72 h which is an optimum incubation period (Fig. 4) while the enzymatic activity decreased beyond 72 h. Similarity with the optimum incubation period is expressed by *Streptomyces* strain A2 [46]. Although there is a decline in enzyme activity of *Streptomyces sp.* FSOF strain at 96 h, it is still found favourable next to 72 hr as there is an increase from the original enzyme activity of 4.3 to 4.6 IU/ml.

**Selection of high enzyme yielding medium**

The enzyme activity of *Streptomyces sp.* FSOF strain varied in each culture mediums comprising GAB, SCB, LB, TSB (Fig. 4). The involvement of different mediums (pH 7.5) for the study at 40 °C for 72 h also has an added advantage of finding the ideal requirements of the FSOF isolate. TSB is observed to produce 5.9 IU/ml enzyme activity followed by SCB, LB and GAB. TSB is also utilized for high antifungal metabolite and L-asparaginase production from *Streptomyces albidoflavus* C247 [47], *Pseudomonas aeruginosa* 2488 [48] respectively. SCB is also considered as an enrichment broth [49],[50], for enzyme activity of seawater isolate [51], *Streptomyces griseoplanus* strain [52]. GAB is also preferred for antibiotic production of *Streptomyces hygroscopicus* [53] and *Streptomyces aburaviensis* IDA-28 [54].

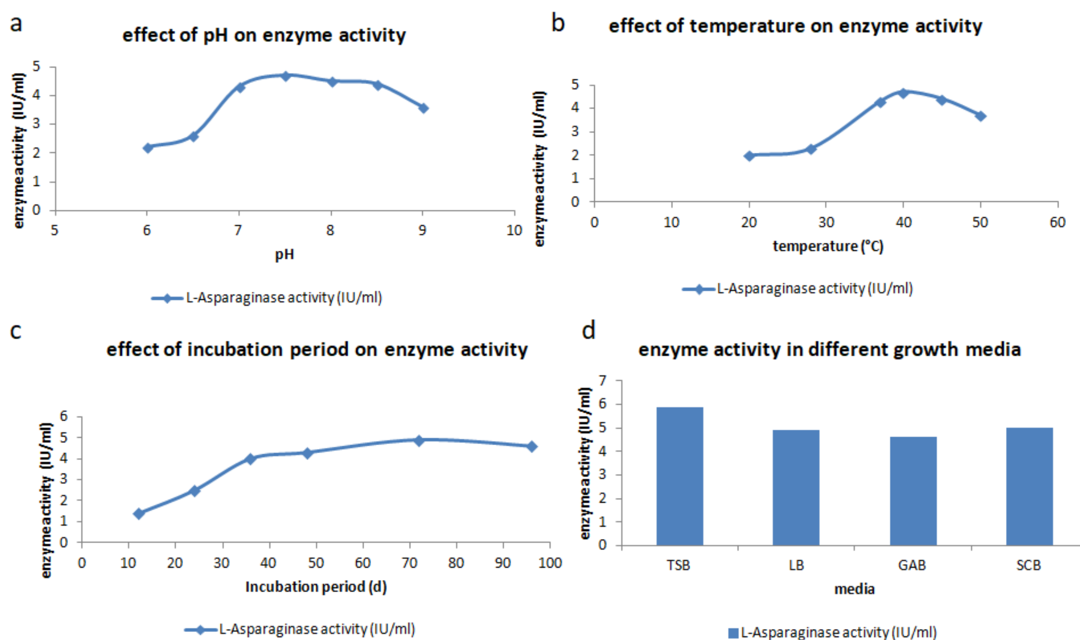


**Fig. 4: a,b,c- Effect of pH, temperature, incubation period on FSOF strain growth and enzyme activity; d- Enzyme activity on different growth mediums.**

**Purification of enzyme produced by FSOF isolate**

The crude enzyme L-asparaginase extracted from FSOF isolate is purified and specific activity is found at every step of the purification process. The crude enzyme extract of 0.92 mg/ml exhibited 6.41 IU/mg activity. Ammonium sulphate fractionation and dialysis resulted in 15.41 IU/mg specific activity, 2.40 purification fold from the crude extract and 17.69 IU/mg with 2.75 purification fold respectively. The application of DEAE column chromatography showed 23.33 IU/mg specific activity with 3.12 purification fold. The specific activity is observed to increase with the progressing purification process from 6.41 to 23.33 IU/ml. Considering the yield of the crude enzyme to be maximum, 62.7% yield is obtained during the $(NH_4)_2SO_4$ fractionation and 23.7 % during DEAE column with a 3.12 increase in purity of enzyme.

# CONCLUSION

The study on the foreshore sediment isolates collected from coastal region progressed towards successful isolation of a strain belonging to *Streptomyces* species. The isolate FSOF is found to be an innate producer of an enzyme L-Asparaginase on controlled conditions. The strain FSOF grown in tryptic soya broth exhibited high enzyme activity is thus considered for purification and further studies. Based upon the results of morphological, biochemical tests, genotypic and phylogenetic analysis the strain is identified to be a member of Streptomyces sp, deposited under MN562193.1 accession number in GenBank. The present study intends to further analyze the protein structure along with enhanced approaches for increased metabolite production.

# ACKNOWLEDGMENT

The authors extend their sincere gratitude to the School of Biotechnology, JNTUK for assistance in the present research work by providing various instrumental facilities.

# CONFLICT OF INTEREST

None

# FUNDING SOURCE

No external funding source has been disclosed.

# DATA AVAILABILITY

Not declared.

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*Indo Global Journal of Pharmaceutical Sciences( ISSN 2249 1023; CODEN- IGJPAI; NLM ID: 101610675) indexed and abstracted in CrossRef (DOI Enabling), CNKI, EMBASE (Elsevier), National Library of Medicine (NLM) Catalog (NCBI), ResearchGate, Publons (Clarivate Analytics), CAS (ACS), Index Copernicus, Google Scholar and many more. For further details, visit http://iglobaljournal.com*