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Screening for the Production and Characterization of L-asparaginase from Endophytic Fungi

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ABSTRACT

L-asparaginase (EC 3.5.11. L-asparagine amidohydrolase) is first enzyme, studied very intensively in human beings with regard to its anti-tumor potential against tumor of lymphoid precursor, acute lymphoblastic leukemia (ALL). The current drugs are suffering from many side effects like immune suppression, infertility, secondary neoplasm. The immunogenic complications associated with its present microbial sources Escherichia coli; Erwinia carotovora limits its medicinal frontier. So there exists a need of switching to novel natural sources to serve as non-immunogenic and better production sources of L-asparaginase. In the present study, four cultures of fungal endophytes viz. TSF-1, TSF-2, TSF-3 and TSF-4 selected on the basis of primary and secondary screening was carried on with L-asparagine as a sole carbon and nitrogen source and phenol red as pH indicator. The maximum protein content was observed to be present in TSF-2 i.e. 2.727 mg /mL and possessed maximum activity of 6.054 Units/ml. Sample was separated by SDS-PAGE, stained by silver staining, showed a single band with molecular weight of approximately ~45kDa.

Keywords- L-asparaginase, fungal endophytes, acute lymphoblastic leukemia.

I. INTRODUCTION

L-asparaginase (IUPAC: L-asparagine aminohydrolase, E.C. 3.5.1.1) belongs to amidase group that hydrolyses the amide bond in L-asparagine to L-aspartic acid and ammonia (Kumar and Verma, 2012). L-asparaginase is an effective antineoplastic agent, used in the Acute Lymphoblastic Leukemia (ALL) chemotherapy (Narta, et al., 2007). The enzyme is widely distributed and is found in animals, plants as well as microorganisms. In recent times L-asparaginase of bacterial origin is used in treatment of ALL. But, in 60% of patients these came up with serious clinical complications such as hypersensitivity reactions, toxicity and instability. Fungal endophytes are group of diverse, polyphyletic microorganisms which are an integral part of the plant micro biome, that internally reside within plants without causing any noticeable infections and live-in mutualistic association with plants for part of their life (Kusari et al., 2012). The rationale behind studying Endophytic fungi is due to the fact that they are potential source of unexplored novel drugs. Fungal endophytes have been recognized as repository of novel secondary metabolites (Strobel et al., 2003). Asparaginase derived from Mucor sp. and Aspergillus terreus, isolated from decomposing vegetable substrate was reported to be non-toxic and possessed myelo-suppressive and immunosuppressive activity (Ali, S.S., 1994). The first and most important benefit is fungal L-asparaginases are non-immunogenic as they are phylogenetically related and post-translational modifications are present in their system, being the eukaryotic microbes and residing inside the plant body so the stability issues are also tackled. The presence of extracellular L- asparaginases in fungi also paved the ways into depth investigations of this enzyme among the various genera of fungi (Nagarethinam et al., 2012). L-asparaginase production throughout the world is carried out mainly by submerged fermentation (SF). This technique however, has many disadvantages. It is cost intensive and has low product concentration. In addition, it generates excess of effluents and consequently needs handling and disposal of large volumes of waste water during downstream processing (EL-Besssoumy et al., 2004). In recent years, the production of enzymes by solid state fermentation (SSF) has emerged. It offers many advantages including high product concentration, less risk of bacterial contamination, ease of product extraction (Lonsane et al., 1995), simple cultivation equipment, low energy requirements and less waste water generation and less environmental concerns regarding the disposal of solid waste (Pandey et al., 2001). This study orange peel used as substrate due to easily availability and has all carbon source for production of enzyme.

II. MATERIALS AND METHODS

Collection of Plant Samples and Surface sterilization

In the present study two plants viz. Azadirachta indica (neem), Ocimum sanctum (Tulsi), recommended for medicine and readily available in field and garden at Bilaspur C.G. and each mature and healthy leaf and stem were used for isolation of endophytic fungi. The samples were brought to the laboratory in sterile bags and processed immediately to reduce the risk of contamination. The healthy plant parts were washed with

distilled water and cut into small pieces. Then tissue pieces were rinsed in 2.5% sodium hypochlorite for 15 min followed by washing in sterile distilled water for 5 min. Surface was sterilized with 75% ethanol for 5 min then rinse in sterile water for three times (Abbas Ahmed et al., 2015).

Isolation of endophytic fungi on Potato Dextrose Agar (PDA) Media

Finally, the pieces were transferred to dishes of isolation media, potato dextrose agar. There are four strains of endophytic fungi were obtained which was screened for L-asparaginase assay.

Screening for L-asparaginase producers

Primary screening of four endophytic fungi was done for L-asparaginase production on Modified Czapek Dox (MCD) agar pH 6.8. The plates were then kept at 28±2°C for 5-7 days. The composition of the MCD agar media is L-asparagine (10g/L), Glucose (2.0g/L), Potassium di- hydrogen phosphate (KH_2PO_4) (1.52g/L), Ferrous sulphate ($FeSO_4.7H_2O$) (0.01g/L), Magnesium sulphate ($MgSO_4.7H_2O$) (0.52g/L), Potassium chloride (KCl) (0.52g/L), Zink Sulphate ($ZnSO_4.7H_2O$) (0.001g/L), Copper Nitrate ($CuNO_3.3H_2O$) (0.001g/L), Agar (20g/L), (Patil et al., 2012). Secondary screening for L-asparaginase producers on 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. L-asparagine acts as sole carbon and nitrogen source. All the positive fungal isolates were grown on L- asparagine agar plates for 7 days at 28°C. The color change from yellow to pink was observed (Gulati et al., 1997).

Enzyme production under Solid state fermentation

The production of L-asparaginase by endophytic fungi under solid state fermentation was performed using orange peel as substrate. Fermentation was conducted in 100 - 250mL conical flasks containing 10 g of solid substrate, separately. Solid substrate was moisture with 10mL distilled water and autoclaved at 121°C, 15 psi for 15 min. Then after cooling 10 6- 108 spores or cells were counted in hemocytometer and 3mL of inoculum was added to each flask under aseptic condition. Then incubate at 30○C for 7 days (Yasser R. et al 2002). Enzymes were extracted by adding 20 mL phosphate buffer in all fermented flask and shaken them at 150 rpm for 2 hours. Then filtered separately with what Mann filter paper, then centrifuged at 12000 rpm for 20 min. After centrifugation supernatant was used as crude enzyme.

L-asparaginase assay

The activity of L-asparaginase was determined by estimating the amount of NH_3 liberated from asparagine during L-asparaginase catalysis using Nessler's reagent. For L-asparaginase determination the reaction mixture composed of 0.5 M phosphate buffer (pH 6.8), 0.4 M L-asparagine and enzyme sample, which incubated for 30 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 1.5 M trichloroacetic acid solution and the liberated ammonia was coupled with Nessler's reagent and quantitatively determined spectrophotometrically at 436 nm (Meghavarnam A.K. and Janakiraman S. 2015).

Purification of L-asparaginase

The purification of L-asparaginase was carried out by salting out method using ammonium sulphate. Solid ammonium sulphate was added to the homogenate to reach a saturation of 70%. The mixture was left overnight at 4°C and then centrifuged at 10,000 rpm for 20 min at 4°C. The precipitate obtained was dialysed and the supernatant was raised to 80% saturation with solid ammoniuam sulphate. The precipitate collected after each of the 70%, and 80% saturation steps were dissolved in minimum volume of 50mM Tris-HCl buffer (pH 8.6) and dialyzed overnight at 4°C against the same buffer (Sindhwad P., and Desai K. 2015). The ammonium sulphate fraction showing the highest enzyme activity was further purified by ion exchange chromatography. Four gram of DEAE cellulose were mixed with 500 mL of 0.02 M Tris HCl an equilibration buffer. The mixture was left for one hour and the buffer was poured off with the small particle in the column. The column was incubated at room temperature overnight to ensure complete swelling (Dura et al., 2002). The dialyzed sample was then loaded on a pre-equilibrated DEAE-cellulose column chromatography (2.6 x 20 cm), and washed with 0.02 M Tris HCl (pH 8.0) buffer. The proteins were eluted with a discontinuous gradient of NaCl (0.1 – 0.25 M) prepared in the same buffer. The flow rate was maintained at 0.5mL/min. with a fraction volume of 5mL. The fraction eluted at each NaCl concentration were separately pooled and tested for protein and L-asparaginase activity determination as mentioned before. The active fractions were used for testing purity by Poly Acrylamide Gel Electrophoresis (PAGE) (Dura et al., 2002).

Detection of proteins by silver staining for SDS-PAGE protein samples

SDS-PAGE is used for characterizing proteins and for molecular weight identification. SDS-PAGE system includes: a tank, lid with power cables, electrode assembly, and cell buffer dam, casting strands, casting frames, combs and glass plates. It is carried under denaturing conditions as SDS is used to give uniform negative charge to the protein (Iwasa et al., 1987).

III. RESULTS AND DISCUSSION

Isolation of Endophytic fungi and their pure culture

Ocimum sanctum (Tulsi) was selected for the isolation of endophytic fungi. Total four fugal endophytes were isolated (figure 1) and maintained on modified Czapek Dox medium. Endophytic fungal were observed under microscope (figure2).

Qualitative screening assay for L-Asparaginase

Four endophytic cultures were subjected for preliminary screening in order to assess their potential to utilize L-asparagine as a nitrogen source. The isolates when grown on modified Czapek Dox agar medium with phenol red as a pH indicator were selected on the basis of their ability to convert yellow plates to pink under alkaline conditions. The changed colour indicated the accumulation of ammonia which resulted as L-asparagine converted to L-aspartic acid in figure 3. All four Endophytic fungi cultures were found to be positive for extracellular L-asparaginase production. Whereas, one isolates of endophytic fungi TSF-2 positive cultures exhibited promising L-asparaginase production and rest were very slow producers being active after 4-7 days. TSF-2 found to exhibit highest activity, as pink color intensity was found to be maximum (Figure 3) followed by TSF-1, TSF-3 and TSF-4 was the slowest L-asparaginase producer among all the selected positive isolates. Table 1 represents the list of fungal endophytes along with their host plant, the part of host plant from where they are collected, place of sampling and their ability as L- asparaginase producers or L- asparaginase non-producers.

Enzyme production

Enzyme production was carried out under Solid state fermentation using orange peel as a substrate. Each flask has 10g of substrate and moisture with 10 mL water and autoclaved. Inoculum was added to each flask and incubated form 6-7days.

After incubation phosphate buffer added to the flask and shaken at 150rpm for 1-2hrs. Crude enzymes were extracted followed by filtration and centrifugation. Crude enzymes were then checked for total protein content and Enzyme activity. The maximum protein content was observed to be present in TSF-2 i.e. 2.727 mg /mL.

The protein estimation of all the isolates was done by Lowry's method as the amount of protein obtained is important for further findings. Amount of protein in test samples was calculated by using the correlation equation obtained by BSA standard curve in graph 1. The total protein content obtained per ml of culture filtrate was shown in table 2.

Quantitative estimation of Asparaginase by Nesslerization

To find the activity of L- asparaginase by quantifying the amount of ammonia released, Standard curve of Ammonia was constructed viz. equation obtained was Y= 0.2426x + 0.0655 with R^2 =0.982, (Graph 2). Then, from this graph the ammonia released in the test sample was calculated using following equation:

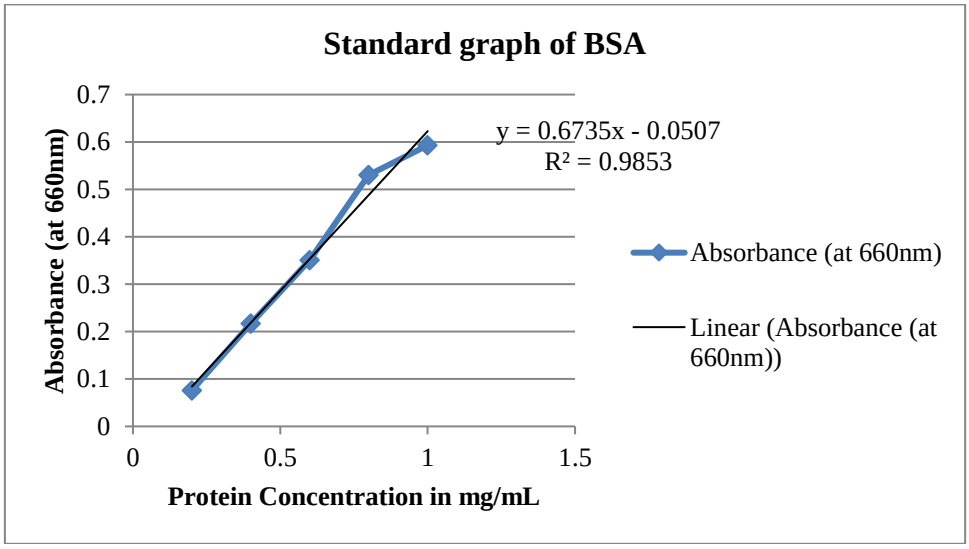
Finally, activity of the L- asparaginase from test fungal isolates was calculated in (Units/ml) using the formula as mentioned previously. In fungal endophyte the crude protein of TSF2 possessed maximum activity of 6.054 Units/ml and the lowest activity was observed in TSF-1 i.e. 5.282 Units/ml (table 3).

Purification of Crude enzyme

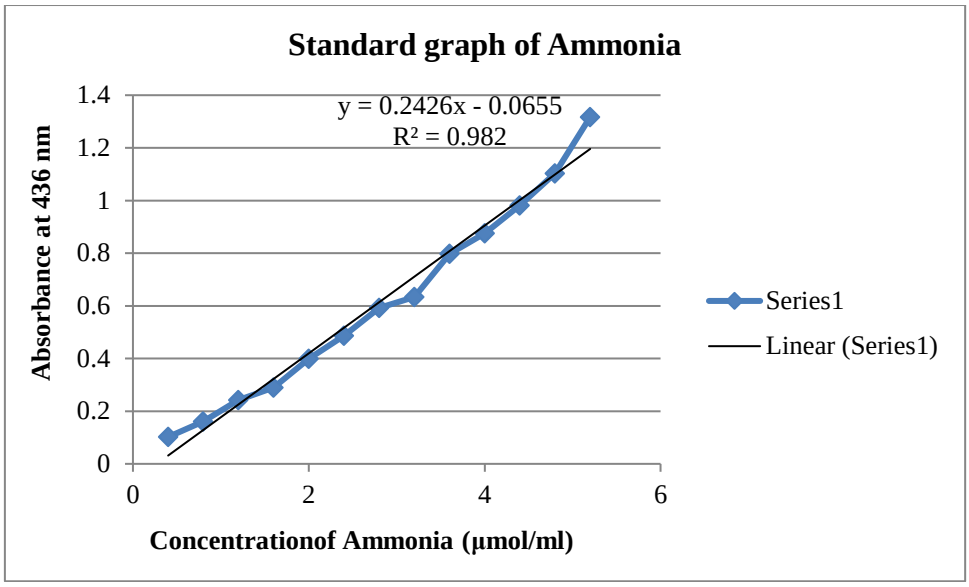
The crude enzyme which shows highest L-asparaginase activity was further purified after large scale production. The purification of L- asparaginase was carried out by salting out method using ammonium sulphate and ion exchange chromatography by using DEAE cellulose column.

Detection of proteins in SDS-PAGE

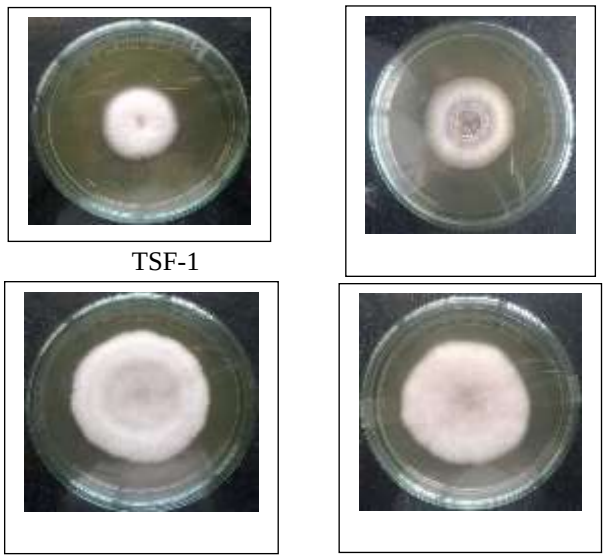
Molecular weight of partially purified enzymes was determined using SDS-PAGE. In lane 1 standard molecular weight markers i.e. Crymotrypsinogen A (24kDa), Ova albumin (45kDa) and BSA (67kDa) and lane 2 protein samples of TSF-2 were loaded**.** SDS-PAGE of protein sample of TSF-2 gives a single band with molecular weight of approximately ~45kDa showing in figure 4.



Graph 1: Standard cure for Bovine serum albumin (BSA)

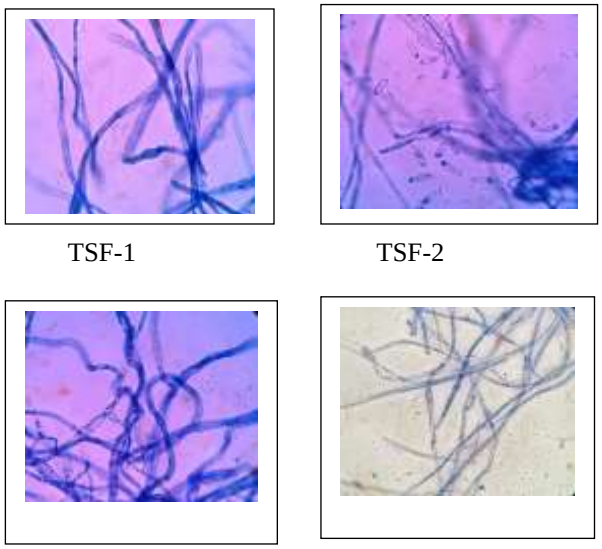


Graph 2: Showing standard curve of Ammonia



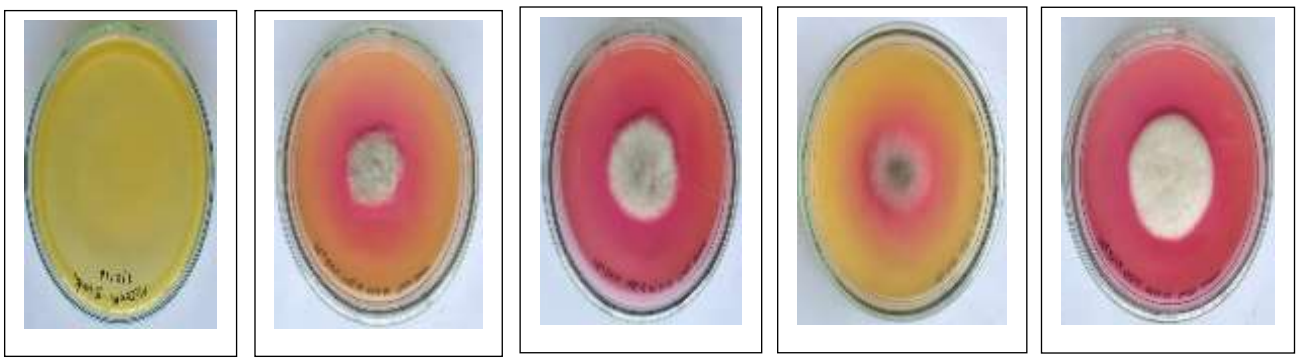
TSF-1

Fig 1: Pure culture of Endophytic fungi



TSF-1 TSF-2

Fig 2: Microscopic view of Endophytic fungi



Control TSF-1 TSF-2 TSF-3 TSF-4

Fig 3: Screening of endophytic fungi on MCD using phenol red indicator.

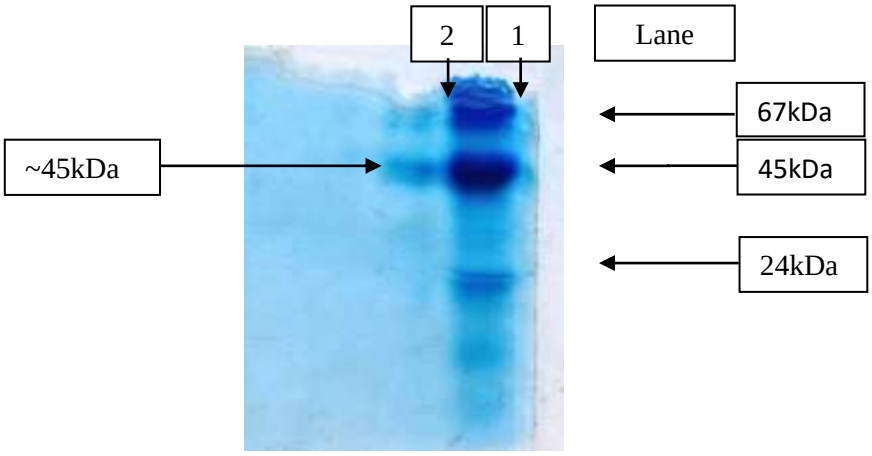


Fig 4: Showing SDS PAGE gel electrophoresis

Table 1: Screening of L-asparaginase producers' endophytic fungi on MCD agar plates

S. No.	Culture Code	Plant part	Host Plant	Place of Sampling	Enzyme activity after 5 Days
1.	TSF-1	Stem	*Ocimum sanctum*	Koni, Bilaspur C.G.	+
2.	TSF-2	Stem	*Ocimum sanctum*	Koni, Bilaspur C.G.	+
3.	TSF-3	Stem	*Ocimum sanctum*	Koni, Bilaspur C.G.	+
4.	TSF-4	Stem	*Ocimum sanctum*	Koni, Bilaspur C.G.	+

Table2: Showing protein content of selected endophytic fungi.

S. No.	Culture Code	O.D. (at 660nm)	Protein Amount (mg/mL)
1.	TSF-1	1.655	2.532
2.	TSF-2	1.786	2.727
3.	TSF-3	1.246	1.925
4.	TSF-4	1.142	1.770

Table 3: Shows the activity of the L- asparaginase of endophytic fungi

S. No.	Culture Code	O.D. (at 436nm)	Enzyme activity U/mL
1.	TSF-1	0.894	5.282
2.	TSF-2	1.034	6.054
3.	TSF-3	0.949	5.586
4.	TSF-4	0.956	5.625

IV. CONCLUSION

The current study describes that beside bacteria, actinomycetes, algae and other known eukaryotic sources; Endophytic fungi are the micro-organisms which can be proved to be better source of L-asparaginase with lower immunogenic response. Out of four endophytic fungi from medical plants of bilaspur, Chhattisgar, India TSF-2 (isolated from *Ocimum sanctum* stem) exhibited maximum potential of L-asparaginase production.

Further profound enzyme kinetics studies, purification and testing of its anti-tumor potential are desired to compare with existing commercial L-Asparaginase producers.

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