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PRODUCTION, OPTIMIZATION AND PARTIAL PURIFICATION OF L-ASPARAGINASE FROM ENDOPHYTIC FUNGUS *Aspergillus* sp., ISOLATED FROM *Cassia fistula*

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ABSTRACT

Endophytes are relatively less studied microbes which in future may serve as potential sources of novel secondary metabolites for use in medical, agricultural and pharmaceutical industries. In present study six fungal endophytes were isolated from the leaves, stem and fruit of *Cassia fistula* and screened for asparaginase production on modified Czapek Dox agar supplemented with L-asparagine as a substrate and phenol red as indicator. L-asparaginase production by *Aspergillus* sp. using variable carbon and nitrogen sources, temperature and pH revealed maximum asparaginase production with xylose as carbon source and ammonium nitrate as nitrogen sources at pH 6.0 with incubation temperature of 30°C. The disc size optimization for enzyme revealed maximum activity with 10 mm size disc (0.22 IU mg^{-1} enzyme activity). The endophyte studied showed maximum activity under stationary condition (2.7 IU mg^{-1}) than under shaking condition. Various purification steps included salt precipitation, dialysis, followed by separation on Sephadex G-100 gel filtration. The partially purified enzyme showed the activity of 4.9 IU mg^{-1}. It may be concluded that the isolated *Aspergillus* sp. may serve as a vital source of L-asparaginase for future use.

Keywords: Cancer, L-asparaginase, *Cassia fistula*, leukemia, lymphoma

INTRODUCTION

Cancer is a group of diseases characterized by unregulated growth and spread of abnormal cells, which may result in death if uncontrolled. It is caused mainly by the environmental factors that mutate genes encoding critical cell-regulatory proteins. Therefore, research and development on anticancer agents has become a major focus in both private and public institutions (Itharat and Ooraikul, 2007). John Beard, an English scientist, was the first to use pancreatic enzymes to treat cancer in 1902. He proposed that pancreatic proteolytic enzymes, in addition to their known digestive function, induce body's main defense against cancer. Therapeutic enzymes have a broad spectrum use as oncolytic, thrombolytic and anticoagulant, and as a replacement for metabolic deficiencies. The information on the use of microbial enzymes for therapeutic purposes is scarce and the available reports are largely focused on some anticancer enzymes (Sabu, 2003). Some important therapeutic enzymes are asparaginase, glutaminase, arginase and tyrosinase which are used as antitumor agent. The present

study was focused on the production of L-asparaginase from an endophytic fungus isolated from a medicinal plant, *Cassia fistula.*

L-asparaginase (L-asparagine amino-hydrolase, EC 3.5.1.1) catalyzes the breakdown of asparagine into aspartic acid and ammonia. In recent years, asparaginase has received much attention because of its potential medicinal value and use in food industries. Microbial enzymes are preferred over plant or animal sources due to their economic production, consistency, ease in processing, modification, optimization and purification. They are relatively more stable than the corresponding enzymes derived from plants or animals. A wide range of microorganisms such as filamentous fungi, yeasts and bacteria have proved a good source of this enzyme. Commercially, L-asparaginase is obtained mainly from bacterial sources. However, fungal asparaginase is more promising as it is safe and non-allergic. Conventionally asparaginase producing fungi are isolated from soil. Many medicinal plants serve as a repository of fungal endophytes capable of producing novel metabolites of medicinal and pharmaceutical importance (Goveas *et al*., 2011; Thirunavukkarasu *et al*., 2011; Patil *et al*., 2012; Chow and Ting, 2015). Bacterial L-asparaginase could cause allergic reaction like skin rash, difficulty in breathing, decrease in blood pressure, sweating or loss of consciousness, etc. It may interfere in blood clotting, raise blood sugar levels, raise liver enzyme and cause liver disease in some patients. However, the asparaginase produced by fungi are reportedly non-toxic and have myelosuppressive and immunosuppressive actions (Campbell *et al*., 1976; Baskar and Renganathan, 2011).

Cassia fistula (Amaltas) is a traditional ayurvedic medicinal plant used in the treatment of various diseases. The plant harbours many endophytic fungi, some of which have the ability to produce L-asparaginase enzyme (Pawar *et al.*, 2017). L-asparaginase has attracted the attention because of its anticarcinogenic potential, especially in the treatment of acute lymphoblastic leukemia (mainly in children), Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma treatment, reticulosarcoma and melanosarcoma (Orabi *et al.*, 2019). This significance has led to search alternative cheap sources of asparaginase production. Some eukaryotic microbes like yeast and filamentous fungi such as *Aspergillus, Penicillium, Fusarium* etc. reportedly produce asparaginase with less adverse effects (Sarquis *et al*., 2004; Chow and Ting, 2015). L-asparaginase is biodegradable and non-toxic; and can be administered at local site quite easily. Other agents are quite painful when administered to the patients and cost-effective (Lee *et al.*, 1989). The metabolites from microbial endophytes are likely to be less toxic as they are harboured by eukaryotic hosts. The objective of present study was to screen endophytic fungus *Aspergillus* sp., isolated from *Cassia fistula,* for L-asparaginase enzyme production as well as partially purification and optimization of enzyme production conditions.

MATERIALS AND METHODS

Collection of plant material

The stem, leaf and fruit of *Cassia fistula* plant were obtained from Ambala (India). The samples were brought to the laboratory in sterile bags and processed immediately.

Isolation and identification of endophytic fungi

Endophytic fungi were isolated as per the method described by Hallmann *et al*. (2007). The leaf, stem and fruit samples were washed with running tap water to remove any adhered dust and debris. The samples were surface sterilized with 70% ethyl alcohol for 1 min., immersed in 4% sodium hypochlorite solution for 3 min. and finally rinsed with deionized sterile distilled water and blot-dried on sterile tissue paper. The samples were cut under aseptic conditions using a sterile scalpel and placed in Petri-dishes containing potato dextrose agar (PDA) medium supplemented with 100 µg streptopenicillin mL^{-1} for the growth of endophytic fungi. The Petri-dishes were sealed with parafilm

and incubated at 30°C for 5 days. The fungi growing on PDA medium were isolated and sub-cultured on sterile PDA plates by needle inoculation or disc transfer method. The most promising endophytic fungal isolate was tentatively identified on the basis of colony characteristics (colour, exudates production and growth of colony) and sporulating structures (conidial head, types of conidiogenous cells, arrangement of conidia, sporangial head, type of spores) (Gilman, 1967; Alexopoulos *et al.*, 1996; Kirk *et al.*, 2001; Aneja and Mehrotra, 2011).

Qualitative assay for L-asparaginase

A modified Czapek Dox medium [glucose 2 g, L-asparagine 10 g (Sigma-Aldrich), KH_2PO_4 1.52 g, KCl 0.52 g, $MgSO_4.7H_2O$ 0.52 g, $FeSO_4.7H_2O$ 0.01 g, agar 20 g, distilled water 1000 mL] was used in plate assay. A 2.5% stock solution of phenol red was prepared in ethanol (pH 6.2) and 3 mL of it added to 1000 mL of Czapek Dox medium. A mycelial plug disc (6 mm) cut from the growing margin of colony of an endophyte was placed in a Petri-dish containing 20 mL medium. After 72 h incubation at 30°C, the appearance of a pink zone around the fungal colony in medium indicated L-asparaginase activity.

Protein determination

A series of bovine serum albumin (BSA) solution were set up in 6 test tubes using 0.2 to 1.0 mL stock solution and distilled water was added to make up the volume to 1 mL, alkaline copper solution (5 mL) was added to it and incubated for 10 min at room temperature, followed by addition of 0.5 mL Folin-Ciocalteau reagent and incubation in dark for 30 min. Distilled water was used as blank. Absorbance was taken at 660 nm by using spectrophotometer after setting to zero absorbance with the blank (Singh *et al*., 2011).

Optimization for enzyme activity

Effect of carbon sources: Three carbon compounds (0.2%) *viz*., xylose, sucrose and starch (in place of usual C source) were added to Czapek Dox broth. The endophytic fungus was inoculated on modified Czapex Dox broth at 30˚C for 5 days (Hosamani and Kaliwal, 2011).

Effect of nitrogen sources: Three inorganic nitrogen sources (ammonium chloride, ammonium sulphate, ammonium nitrate) were tested as N source. The endophytic fungus was inoculated on modified Czapek Dox broth at 30˚C for 5 days (Hosamani and Kaliwal, 2011).

Effect of pH: To study the effect of pH on enzyme activity, the endophytic fungus was inoculated in 20 mL modified Czapek Dox medium adjusted to different pH levels (3, 6 and 8) and assayed for enzyme activity. The culture was incubated at 30°C for 5 days in an incubator (Kumar *et al*., 2012).

Effect of temperature: To study the effect of temperature on enzyme activity, the endophytic fungus was inoculated in 20 mL Czapek Dox broth and incubated for 5 days at different temperature (25˚, 30˚ and 35˚C) and assayed for enzyme activity.

Effect of fungal disc size: To assess the effect of disc size on enzyme activity, the endophytic fungus was inoculated in 20 mL Czapek Dox broth of variable disc size (6, 8 and 10 mm) and incubated at 30˚C for 5 days before the enzyme activity assay.

Effect of stationary and shaking conditions: Endophytic fungus was inoculated in 20 mL modified Czapek Dox broth with xylose as carbon source, ammonium nitrate as nitrogen source, optimum temperature 30˚C, pH 6 and disc size 10 mm and keep on shaking and stationary growth conditions for 5 days, before enzyme assay.

Partial purification of L-asparaginase

L-asparaginase enzyme was partially purified by ammonium sulphate precipitation, followed by dialysis and gel filtration (Dhevagi and Poorani, 2005). For ammonium sulphate precipitation, the crude enzyme was brought to 45% saturation with ammonium sulphate at pH 8.4 and kept overnight at 4˚C. After equilibration, the supernatant was centrifuged at 4200 rpm for 10 min at 4˚C and supernatant brought to 85% saturation with ammonium sulphate and again centrifuged at 4200 rpm

at 4˚C for 10 min. The precipitates were collected separately and stored at 4˚C for further purification. For dialysis, the pre-treated dialysis tubes were used for collection of precipitates as in earlier step. The precipitates were dissolved in 1M tris HCl buffer, dialyzed and the samples used for protein and enzyme assays.

Sephadex G-100 gel filtration

Ammonium sulphate precipitated samples were tested for enzyme activity and protein and further subjected to gel filtration-using Sephadex G-100. For preparation of gel-column, a chromatography column made up of glass tubing of 2.2 cm dia. and 60 cm high was used. Tris-HCl buffer (0.05 M, pH 8.5) was used as eluent. The eluent was stored in tightly stoppered brown bottles and brought to same temperature as that of gel bed to prevent bubble formation within gel bed. For preparation of gel slurry, 10 g Sephadex was suspended in 400 mL 0.05 M tris-HCl buffer and kept as such at room temperature for 24 h to swell. The column was packed with Sephadex and stabilized. Ammonium sulphate precipitated samples were poured continuously into the column and fractions collected in 5 mL vials. The collected fractions were randomly assayed for enzyme activity and protein, and the fractions showing better enzyme activity were pooled (Patro and Gupta, 2012).

Statistical analysis

All the assays were conducted in triplicate and the data analyzed as per Gomez and Gomez (1984) were presented as mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

In present study six endophytic fungal strains were isolated from the leaves, stem and fruits of *Cassia fistula* plant. These endophytic fungal isolates were identified as *Aspergillus* sp. on the basis of their morpho-cultural characteristics and with the help of available literature (Gilman, 1967; Alexopoulos *et al*., 1996; Kirk *et al*., 2001; Aneja and Mehrotra, 2011). All strains showed good colour intensity in agar plugs and grew well on modified Czapek Dox (CD) agar medium with phenol red, a dye indicator that changes from yellow (acidic condition) to pink (alkaline condition). Asparaginase causes hydrolysis of L-asparagine into L-aspartic acid and ammonia. The release of ammonia results in pH change. The indicator used in bioassay (phenol red) at acidic pH is pale yellow and when pH changes to alkaline it turns pink, thereby imparting pink colour to agar plugs. The strains exhibiting high asparaginase activity were cultivated in CD broth. Six asparaginase positive isolates were tested for their asparaginase activity in liquid condition using a Nesslerization assay (Doriya and Kumar, 2016). These isolates exhibited asparaginase activity in the range of 0.33 to 0.46 IU mg^{-1} (Table I). The protein content was determined by Lowery method (Lowry *et al*., 1951) and in the isolates it ranged from 0.002 to 0.04 mg mL^{-1}. L-asparaginase activity was detected on the basis of formation of red colour around the colony. All the isolated endophytic fungi showed positive results for L-asparaginase production.

Table 1: L-asparaginase enzyme activity and protein concentration in crude enzyme

Aspergillus isolates	Protein concentration (mg mL^{-1})	Enzyme activity (IU mg^{-1})
Ca. L1	0.04	0.33
Ca. L2	0.02	0.46
Ca. S1	0.01	0.44
Ca. S2	0.005	0.44
Ca. S3	0.002	0.42
Ca. F	0.08	0.43

On the basis of maximum zone of clearance and enzyme activity of six isolates, the most promising isolate Ca. L2 was chosen to optimize various parameters for laboratory-based scale up of asparaginase activity. Of the three carbon sources *viz*., xylose, sucrose and starch studied for L-asparaginase production by *Aspergillus* sp. isolate Ca L2, the highest activity was observed in CD broth containing xylose (0.45 IU mg^{-1}) and

Table 2: Effect of carbon and nitrogen sources on L-asparaginase activity (in IU mg^{-1}) of *Agpergillus* sp. isolate Ca. L2

Carbon source	Enzyme activity (IU mg^{-1})	Nitrogen source	Enzyme activity (IU mg^{-1})
Starch	0.32 ± 0.06	Ammonium chloride	0.70 ± 0.05
Xylose	0.45 ± 0.02	Ammonium nitrate	0.86 ± 0.03
Sucrose	0.40 ± 0.04	Ammonium sulphate	0.40 ± 0.08

least in the medium containing starch (Table 2). The fermentation broth having xylose turned alkaline, while the fermentation broth containing sucrose and starch remained acidic. The acidic nature of fermentation medium appears to inhibit L-asparaginase biosynthesis. Contrarily, Baskar and Renganathan (2011) have reported that the glucose as the best carbon source for L-asparaginase activity. Glucose reportedly inhibits the synthesis of L-asparaginase in *Escherichia coli, Erwinia carotovora, E. aroideae* and *Serratia marscences* due to catabolic repression (Cedar and Schwartz, 1967). In present study, the CD broth containing ammonium nitrate as nitrogen source showed maximum L-asparaginase activity (0.86 IU mg^{-1}) by *Aspergillus* sp. Ca. L2 (Table 2). L-asparaginase is nitrogen regulated and by altering different nitrogen sources, the enzyme production could be enhanced (Sarquis *et al*., 2004). Hosamani and Kaliwal (2011) have reported the maximum L-asparaginase activity of *Fusarium equiseti* in the medium containing ammonium sulphate as inorganic N source.

The asparaginase enzyme activity was assayed at different pH and temperature levels. The optimum pH and temperature for L-asparaginase production was found to be 6 and 30˚C, respectively (Table 3). The acidic nature of fermentation medium could inhibit L-asparaginase biosynthesis. These results are in agreement with Baskar and Renganathan (2011) who found maximum asparaginase activity at pH 6. Being proteinaceous nature, the enzymes are very sensitive to heat. Asparaginase activity of *Aspergillus* sp. was maximum at 30˚C. Optimization of disc size for enzyme activity revealed maximum activity (0.22 IU mg^{-1}) when 10 mm disc size was used (Table 4). The endophytic fungus showed maximum activity under stationary condition (2.7 IU mg^{-1}) than under shaking condition (Table 4).

Table 3: Effect of pH and temperatures on asparaginase activity of *Aspergillus* sp.

pH value	Enzyme activity (IU mg^{-1})	Temperature (°C)	Enzyme activity (IU mg^{-1})
3.0	0.28 ± 0.04	25	0.20 ± 0.06
6.0	0.33 ± 0.02	30	0.45 ± 0.01
8.0	0.30 ± 0.03	35	0.40 ± 0.02

From the above study, xylose was chosen as carbon source, ammonium nitrate as nitrogen source, pH 6, temperature 30˚C and disc size 10 mm for optimal production of enzyme. Amatto and Guimaraes (2019) found the 35ºC as best temperature and pH 5.0 as optimum pH for enzyme activity. L-asparaginase activity produced by *Aspergillus* sp. was increased by 17% while the activity increased by 58.7% after partial purification.

Table 4: Effect of fungal disc size and incubation condition on L-asparaginase activity of *Aspergillus* sp. strain Ca. L2

Fungal disc size (mm)	Enzyme activity (IU mg^{-1})	Incubation conditions	Enzyme activity (IU mg^{-1})
6.0	1.7 ± 0.02	Shaking	2.7 ± 0.05
8.0	2.0 ± 0.03	Stationary	2.5 ± 0.02
10.0	2.2 ± 0.01		

Table 5: Enzyme activity and protein content of L-asparaginase (obtained from *Aspergillus* sp.) in different purification steps

Samples	Enzyme activity (IU mg^{-1})	Protein (mg mL^{-1})
Crude	2.7	0.25
$(NH_4)_2SO_4$ precipitation	3.0	0.20
Dialysis	3.5	0.15
Sephadex gel filtration G-100	0.00	0.21
Fraction No. 1	0.9	0.28
Fraction No. 2	1.2	0.30
Fraction No. 3	3.5	0.25
Fraction No. 4	4.5	0.20
Fraction No. 5	4.9	0.13
Fraction No. 6	4.7	0.12
Fraction No. 7	3.5	0.18
Fraction No. 8	2.0	0.18
Fraction No. 9	1.1	0.15
Fraction No. 10	0.3	0.10
Fraction No. 11	0.04	0.03
Fraction No. 12	0.00	0.01

The partial purification of L-asparaginase from crude extract was affected by ammonium sulphate precipitation (45-85%) which revealed that most of the enzyme activity was preserved in precipitate. The total protein precipitation also revealed that most enzyme activity was preserved in precipitate. The total protein decreased from 0.25 to 0.20 mg mL^{-1} in ammonium sulphate precipitate.

The specific activity increased from 2.7 IU mg^{-1} in crude to 3.5 IU mg^{-1} in dialysis Table 5). However, in dialysis step the protein concentration decreased from 0.25 in crude to 0.15 mg mL^{-1}. The profile of ammonium sulfate fraction and dialysis showed increase in specific activity from 3.0 to 3.5 IU mg^{-1}. The final purification step carried out by Sephadex G-100 showed that specific activities increased from 3.5 to 4.9 IU mg^{-1}. The protein concentration decreased from 0.15 to 0.01 mg mL^{-1}. In present study, partially purified enzyme showed L-asparaginase activity of4.9 IU mg^{-1} while Kushwaha *et al*. (2012) reported asparaginase activity of 0.4 IU mg^{-1}. Further, the enzyme showed 10 times more activity than the activity observed by Kushwaha *et al*. (2012).

Conclusion: *Aspergillus* sp., isolated from *Cassia fistula*, proved a good source for L-asparaginase enzyme production. Optimum enzymes production was achieved when xylose as carbon source, ammonium nitrate as nitrogen sources, 30°C as incubation temperatures, pH of 6.0 and disc size of 10 mm were maintained. Further research on sequencing, antileukemic activity (*in vivo* and *in vitro*), mechanism of action and determination of protein structure are needed.

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