



OPTIMIZATION OF PHYSIOLOGICAL GROWTH CONDITIONS FOR MAXIMAL PRODUCTION OF L-ASPARAGINASE BY *FUSARIUM* SPECIES

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Abstract: L-asparaginase is an enzyme which catalyzes the hydrolysis of amino acid L-asparagine to L-aspartic acid and ammonia. Due to its utility in pharmaceutical as well as food industries, there has been a paramount interest on the production of this enzyme using microbial sources. We have screened three hundred and sixty four soil fungi for L-asparaginase production utilizing a relatively simple and reliable dye based rapid screening method. Our screening yielded two *Fusarium* species which showed appreciable amount of enzyme activity which were identified as *Fusarium culmorum* and *Fusarium brachygibbosum* by 18s rRNA coding nucleotide sequences. The physiological parameters for these two isolates were optimized for maximal production of the enzyme under laboratory culture conditions. The results revealed that *Fusarium culmorum* and *Fusarium brachygibbosum* showed maximum production of enzyme at shaking condition at 120rpm; temperature 30°C and at 1% substrate concentration. *Fusarium culmorum* showed maximal enzyme activity at pH 7.5 whereas, *Fusarium brachygibbosum* showed maximal enzyme activity at pH 6.0. Citric acid, ammonium chloride, CaCl₂ and glutamine enhanced the production of L-asparaginase in *Fusarium culmorum*, whereas, sucrose, FeSO₄ and arginine enhanced the activity of L-asparaginase in *Fusarium brachygibbosum*. However, sorbitol, yeast extract, casein, lysine and glycine repressed the production of L-asparaginase in both species of *Fusarium*.

Key words: L-asparaginase; *Fusarium culmorum*, *Fusarium brachygibbosum*, Carbon sources; Nitrogen source.

INTRODUCTION

Microorganisms are the main source of industrially important enzymes as they are capable of synthesizing and secreting large amounts of extracellular enzymes. L-asparaginase (L-asparagine amido hydrolase E.C.3.5.1.1) is an enzyme belonging to an amidase group that catalyzes the hydrolysis of the amino acid L-asparagine to L-aspartic acid and ammonia. It has been widely used as therapeutic agent in the treatment of leukemia [1]. L-asparaginase also has significant applications in food industry to reduce the formation of carcinogenic acryl amides in deep fried potato [2].

L-asparaginase does not occur naturally in humans, but, however, distributed among plants, animals and microorganisms. Microbes are a better source of L-asparaginase because they can be cultured easily and the extraction and purification from them are convenient, facilitating the large scale production.

L-asparaginase is produced by a large number of microorganisms, both by prokaryotes and eukaryotes. Microbial strains like *Escherichia coli* [3], *Erwinia caratovora* [4], *Pseudomonas aeruginosa* [5], *Bacillus cereus* [6], *Staphylococcus sp* [7], *Streptomyces albidoflavus* [8], *Streptomyces gulbargensis* [9], *Aspergillus nidulans*, *Aspergillus terreus*, *Aspergillus tamari* [10], *Aspergillus niger* [11], *Penicillium sp.* [12], *Emericella nidulans* [13], *Helminthosporium*, *Paecilomyces*, *Alternaria*, *Cladobotrytis* [14] are the main source of L-asparaginase. But, L-asparaginase from bacterial origin causes hypersensitivity in long term administration, leading to allergic reactions like skin rash, difficulty in breathing, nausea, weight loss, sweating, immunosuppression, acute pancreatitis, decreased blood pressure and loss of consciousness [1, 15, 16]. L-asparaginase from fungal source has fewer side effects [10] and therefore used for commercial use.

The main objective of the present work was to identify few potent fungi producing L-asparaginase and to optimize the growth parameters to achieve maximal production of enzyme under submerged fermentation (SMF). Two soil isolates, *Fusarium culmorum* (KP031673) and *Fusarium brachygibbosum* (KP031674) which showed maximum enzyme activity were taken for the present study.

MATERIALS AND METHODS

Chemicals

Media components used in the experiment were obtained from Hi-media (Mumbai, India). All the chemicals are of AR grade. The substrate L-asparagine was procured from Sigma (USA).

Isolation of Fungi from Soil Samples

One hundred and ten (110) soil samples were collected aseptically in polythene bags from different parts of Karnataka and other states of India. One gram of each soil sample was suspended in 10 ml of sterile distilled water, serially diluted (10⁻⁶) and plated on to Potato Dextrose Agar (PDA) [17] and incubated at 28°C for 96h. Individual fungal colonies were further purified and eventually transferred to PDA slants, incubated at 28°C for 4 days, and stored at 4°C, till further use.

Identification of Fungi

The fungal isolates were identified initially by observing their morphological and microscopic characteristics [17] and molecular identification was done by following the method [18]. Phylogenetic tree was constructed by using Clustal X 2.

Culture media and growth conditions

The culture medium used for the study was modified Czapek-dox medium containing g/l of,

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Glucose,2.0; L-asparagine,10.0; KH_2PO_4 ,1.52; KCL ,0.52; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,0.52; $\text{CuNO}_3 \cdot 3\text{H}_2\text{O}$,trace; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$,trace; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$,trace; Agar,18.0, initial pH 5.6 supplemented with either bromo cresol purple or phenol red dyes both at 0.009% [19]. The cultures were incubated at 28°C under shaken condition (120 rpm) for 4 days.

Effect of pH

To determine the effect of pH on production of L-asparaginase, the pH of the growth medium was adjusted over the range 5.0 to 8.5 before autoclaving by using 1N NaOH and 1N HCl with 0.5 pH increments.

Effect of Temperature

To determine the effect of temperature on the production of L-asparaginase, the incubation temperature of the culture was varied ranging from 25°C to 45°C, with increment of 5°C.

Effect of substrate concentration

To determine the effect of substrate concentration on the production of L-asparaginase, the composition of the growth medium was modified by the addition of different concentration of the substrate L-asparagine 0.5%, 0.75%, 1%, 1.25% and 1.5%.

Effect of Carbon Sources

In order to find out the influence of carbon source on production of L-asparaginase, glucose (0.2%) was substituted in the growth medium by different carbon sources such as arabinose, fructose, mannitol, galactose, pyruvic acid, inositol, ribose, xylose, citric acid, sorbitol, lactose, maltose, starch, sucrose, trehalose, carboxy methyl cellulose, raffinose and rhamnose at same concentration.

Effect of Nitrogen Sources

The effect of various nitrogen sources on L-asparaginase production was studied by altering the composition of the growth medium using different organic nitrogen sources like peptone, beef extract, yeast extract, tryptone, casein and inorganic nitrogen sources like ammonium chloride, ammonium nitrate, ammonium sulfate, ammonium dihydrogen ortho phosphate, potassium nitrate, sodium nitrate, sodium chloride were incorporated at a concentration of 0.5% along with L-asparagine 0.5%.

Effect of trace elements and surfactants

To determine the effect of trace elements and surfactants on the production of L-asparaginase, the growth medium was supplemented with CaCl_2 , CuSO_4 , MnSO_4 , HgCl_2 , FeSO_4 , Ethylene diamine tetra acetic acid (EDTA) and sodium dodecyl sulphate (SDS) at 0.002% concentration.

Effect of Amino acids

To determine the influence of other amino acids on the production of L-asparaginase, the growth medium was supplemented with additional amino acids such as arginine, cysteine, glutamic acid, glutamine, glycine, histidine, lysine, ornithine and tyrosine at a concentration of 0.2% along with asparagine 0.5%.

Assay of L-asparaginase

The activity of L-asparaginase in the culture filtrate was determined using the method of [20]. The rate of hydrolysis of L-asparagine was determined by measuring the ammonia released using Nessler's reagent. A mixture of 0.5ml of enzyme extract, 0.5ml of 0.04M L-asparagine, 0.5ml of 0.05M Tris-HCl buffer (pH 7.2) and 0.5ml of distilled water was incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5ml of 1.5M trichloroacetic acid (TCA). The ammonia released in the supernatant was determined colorimetrically by adding 0.2ml of Nessler's reagent into tubes containing 0.1ml of supernatant and 3.7ml of distilled water and incubated at room temperature for 20 min. The absorbance was read at 450 nm. One international unit (IU) of L-asparaginase activity is defined as the amount of enzyme required to produce 1µmol of ammonia per min under the conditions of the assay.

Statistical analysis

All the experiments were done in triplicates and statistical analysis was performed using SPSS software (version 20). The statistical difference between mean values was accessed by one way analysis of variance (ANOVA) through Duncan's Multiple Range Test (DMRT) at significance level of ($p < 0.05$).

RESULTS AND DISCUSSION

Isolation and rapid screening of soil fungi for L-asparaginase production

A total number of three hundred and sixty four (364) fungi were isolated and among these one hundred and thirty five (135) isolates showed positive for L-asparaginase production as indicated by the change in colour from yellow to purple on bromo cresol purple dye amended plates and from yellow to pink on phenol red dye amended plates, between 48-72 hours of incubation at 28°C [21]. Among the one hundred and thirty five (135) isolates, fifty two (52) isolates were selected for further secondary screening to identify the isolates with significantly increased levels of the enzyme based on dye colour change (color zone 1.5cm or above). Among the fifty two isolates short-listed from the secondary screening, two fungal isolates (Fig. 1) which showed highest enzyme activity in the quantitative assay [20] were selected for optimization studies.

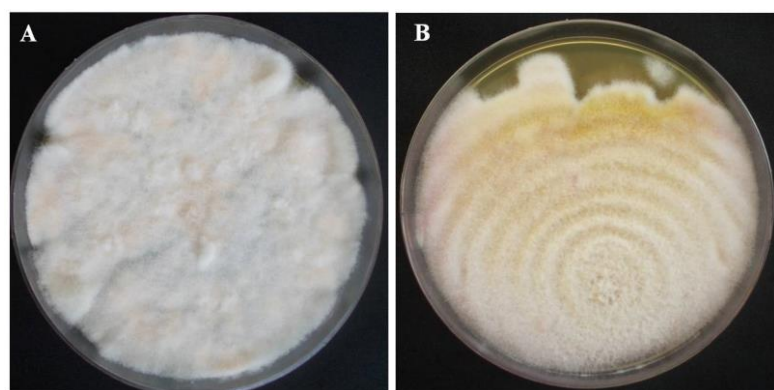


Figure 1: Pure culture of A. *Fusarium culmorum*. B. *Fusarium brachygibbosum*.

Molecular Identification of *F. culmorum*-ASP87 and *F. brachygibbosum*-ASP56

Molecular identification was done by sequencing and characterizing the gene encoding for 18s rRNA (Fig. 2 and Fig. 3). The sequence was queried in nucleotide BLAST search from National Center for Biotechnology Information (NCBI) to find out the homology with the existing species of *Fusarium*. The first sequence showed 100% identity with *F. culmorum* and subsequently coded as ASP-87. The second nucleotide sequence showed 100% identity with *F. brachygibbosum* and subsequently coded as ASP-56. Both nucleotide sequence data were submitted to GenBank and

obtained the accession numbers *F. culmorum* (KP031673) and *F. brachygibbosum* (KP031674).

For optimizing the nutritional and environmental factors for L-asparaginase production in both the *Fusarium* species by submerged fermentation, the control growth condition was maintained at 28°C, pH 5.6 with 0.2% carbon (glucose), and 1% nitrogen concentration (L-asparagine) without addition of any supplements.

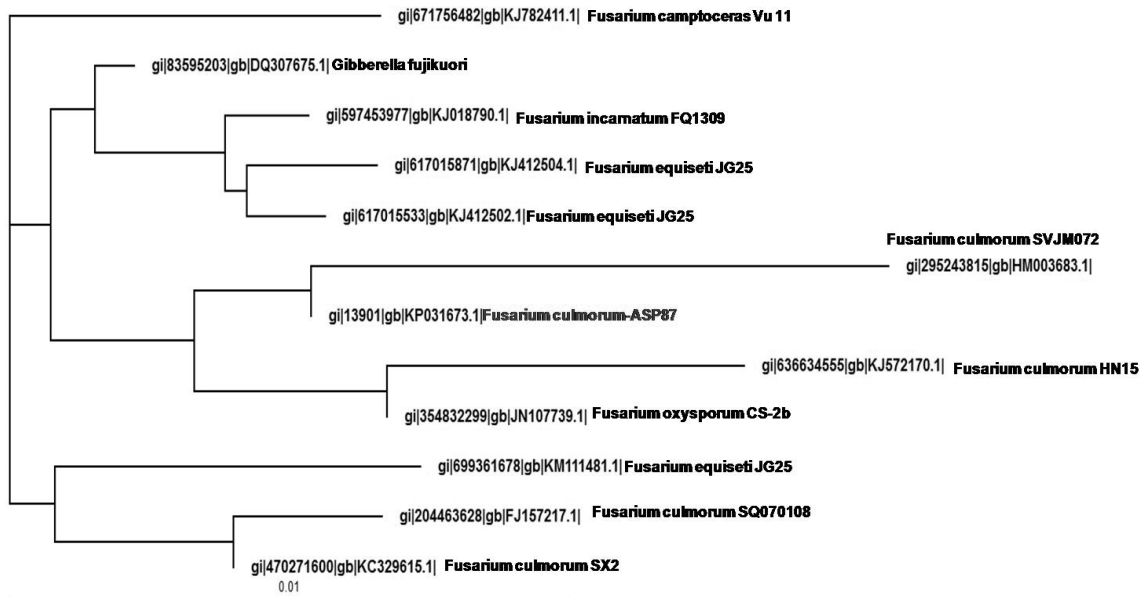


Figure 2: Molecular phylogenetic analysis of *F. culmorum* ASP-87 by Internal Transcribed Spacer (ITS) 18s rRNA sequence analysis.

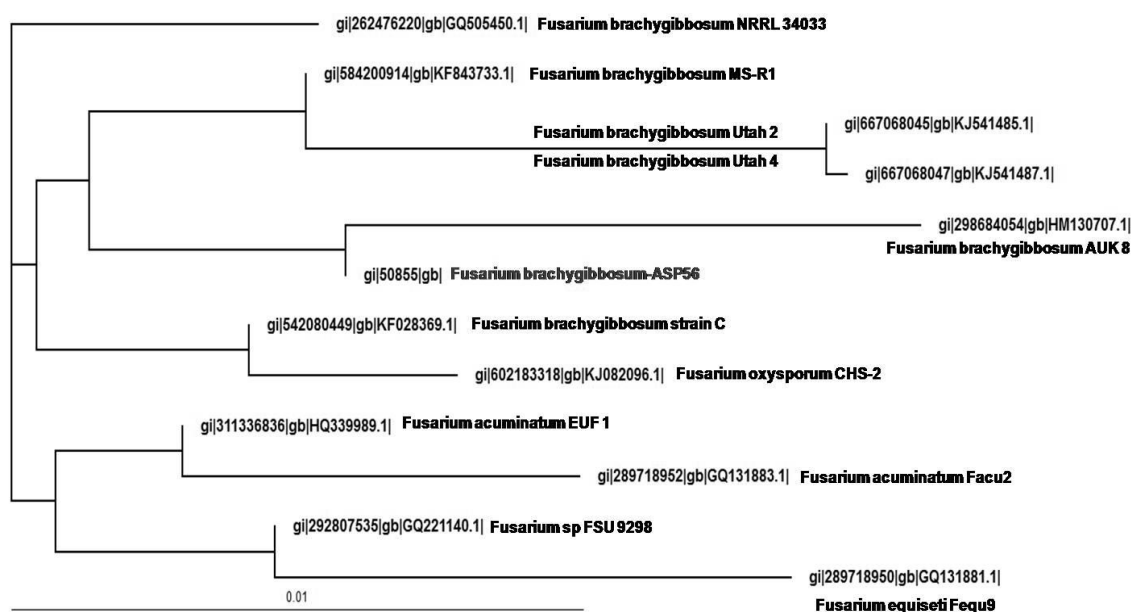


Figure 3: Molecular phylogenetic analysis of *F. brachygibbosum* ASP-56 by Internal Transcribed Spacer (ITS) 18s rRNA sequence analysis.

Effect of pH

The pH of the medium has a profound effect on the production of enzyme. The effect of initial pH on the production of L-asparaginase was investigated from pH 5.0 to 8.0. There were no apparent differences in growth over the range of pH used in the study for the two isolates. However, the pH of initial culture medium pH brought about significant differences on the enzyme production by these two isolates. For example, the maximum activity of L-asparaginase was observed at pH 7.5 in *F. culmorum* (0.40 U/ml) whereas *F. brachyglabosum* showed maximal enzyme activity at pH 6.0 (0.34 U/ml) (Fig. 4). In general, fungal isolates respond differently to change in initial pH of the medium for enzyme production either by providing optimal physiological pH for growth and development or by positively affecting the activity, structural stability in the extracellular matrix environment and solubility of the enzyme. L-asparaginase production in particular, is not an exception and different fungal isolates had different pH optima for L-asparaginase production. For instance, *Aspergillus terreus* produced maximal L-asparaginase activity at pH 7.5 [22], while others reported that pH 6.0 was the optimal pH for L-asparaginase production in *Penicillium* sp. [23] and *Emericella nidulans* [13], similar to our observation with *F. brachyglabosum*. Interestingly, a pH of 5.0 was found to be optimal for L-asparaginase production by *F. oxysporum* [24]. Our results also showed that the activity of L-asparaginase decreased at low (5.0) and high (8.5) pH range for both the species of *Fusarium* (Fig. 4). Presumably, extreme pH cause irreversible denaturation of enzymes by destruction of specific amino acid residues at higher pH and hydrolysis of peptide bonds at lower pH.

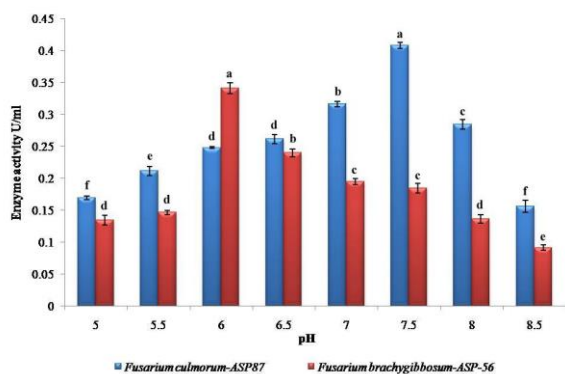


Figure 4: Effect of pH on L-asparaginase production in *F. culmorum* and *F. brachyglabosum*. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to DMRT

Effect of Temperature

The incubation temperature of fermentation process influences growth, metabolic activity and production of enzymes. Both the species of *Fusarium* showed maximum activity of L-asparaginase at 30°C, as reported by earlier workers in *Mucor hiemalis* [25], *Penicillium* sp. [26], *Aspergillus terreus* [27] and *Emericella nidulans* [13]. It is obvious that fungi being mesophilic in nature prefer to grow with high metabolic rate at this temperature. But, on the contrary 35°C was the optimum temperature for the activity of L-asparaginase was reported in *Penicillium* sp. [23]

and *Aspergillus terreus* [28]. Temperature above 45°C drastically reduced the production of L-asparaginase (Fig. 5). This is obviously due to the denaturation of mesophilic enzyme at higher temperature. Furthermore, at low and high temperature the metabolic activities of the microorganisms are expected to slow down and subsequently leading to less production of enzymes [29, 13].

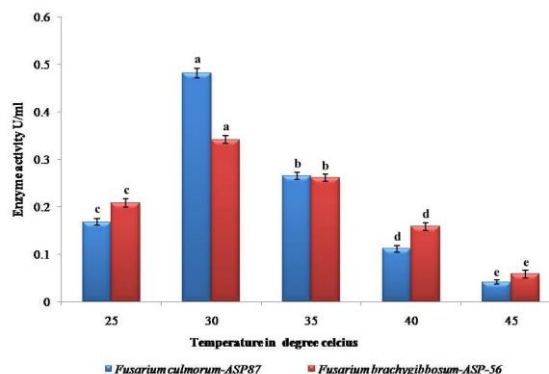


Figure 5: Effect of temperature on L-asparaginase production in *F. culmorum* and *F. brachyglabosum*. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to DMRT

Effect of substrate concentration

The maximum activity of L-asparaginase was found at 1% substrate (L-asparagine) and the activity decreased with increase in substrate concentration (Fig. 6), which could be due to catabolite repression [30]. Similar results were observed in *Bipolaris* sp. [31]. But, however, maximum activity of L-asparaginase was observed at 2% substrate concentration in *Aspergillus terreus* [28].

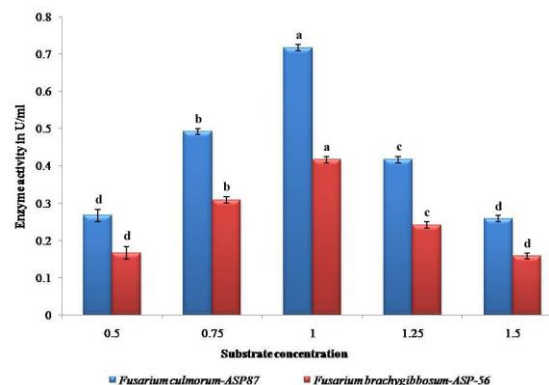


Figure 6: Effect of Substrate concentration on L-asparaginase production in *F. culmorum* and *F. brachyglabosum*. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to DMRT.

Effect of Carbon Sources

Carbon source acts as the major nutrient for the production of enzymes. Our results showed that production of L-asparaginase varied over different carbon sources in both the *Fusarium* sp. Medium incorporated with citric acid as carbon source enhanced the production of L-asparaginase by 3 fold in *F. culmorum* followed by ribose,

starch, mannitol, fructose, xylose, pyruvic acid, rhamnose, galactose, lactose, maltose, inositol, sucrose, arabinose and trehalose (Fig. 7). Whereas, sucrose enhanced the production of L-asparaginase by 3.4 fold in *F. brachyglabosum* followed by xylose, mannitol, trehalose, fructose, citric acid, lactose, rhamnose, pyruvic acid, maltose, CMC, galactose, inositol and starch. Our results were similar to reports of [24] that sucrose as carbon source induced the production of L-asparaginase in *Fusarium oxysporum*. However, sorbitol repressed the production of L-asparaginase in both the species of *Fusarium* (Fig. 7). Similar results were reported in *Fusarium semitectum* [32] and *Aspergillus flavus* [33]. However, earlier reports suggest that glucose served as the best carbon source for the production of L-asparaginase in *Fusarium equiseti* [34], *Fusarium semitectum* [32] and *Aspergillus niger* [11]. This could be due to the fact that fungi prefer to grow and synthesize various metabolites on simple sugars as compared to complex sugars.

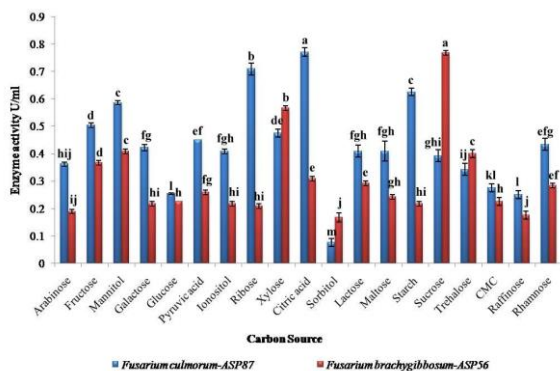


Figure 7: Effect of Carbon source on L-asparaginase production in *F. culmorum* and *F. brachyglabosum*. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to DMRT.

Effect of Nitrogen Sources

The source of nitrogen has a profound influence on the growth and metabolism of microorganisms. Although L-asparagine itself acts as nitrogen source in the growth medium, the influence of additional nitrogen source on the activity of L-asparaginase is studied. Interestingly, we observed that ammonium chloride enhanced the production of L-asparaginase by 12 fold in *F. culmorum* but only 4 fold in *F. brachyglabosum* (Fig. 8). Similar such findings are reported in *Aspergillus terreus* [35]. Whereas, sodium nitrate was the best nitrogen source for the production of L-asparaginase was reported in *Fusarium oxysporum* [24] *Penicillium* sp. [26] and *Fusarium semitectum* [32]. However, earlier reports suggest that yeast extract induced the production of L-asparaginase in *Fusarium equiseti* [34] and *Streptomyces gulbargensis* [9]. But, in our study yeast extract and casein repressed the production of L-asparaginase in both the *Fusarium* sp (Fig. 8). Accordingly we can conclude that nitrogen supplements, especially inorganic nitrogen source in the medium showed significant induction in the activity of L-asparaginase, while organic sources showed no significant increase in the enzyme activity in both the *Fusarium* sp (Fig. 8).

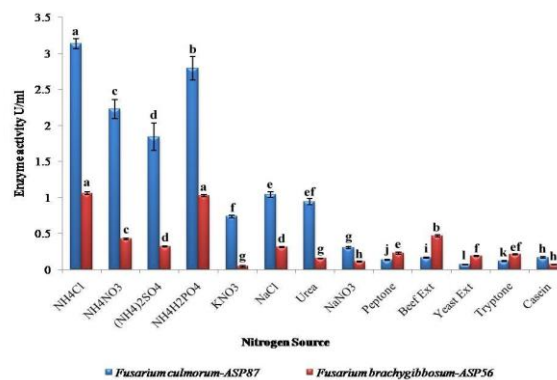


Figure 8: Effect of Nitrogen source on L-asparaginase production in *F. culmorum* and *F. brachyglabosum*. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to DMRT

Effect of trace elements, chelating agents and surfactants

Trace elements play an important role in the biological function of many enzymes. Metal ions take part in the catalytic process of enzymes by serving as either electron donors or acceptors and therefore play an important role in the activity and stabilization of enzyme. Among the various trace elements used $CaCl_2$ (0.43 U/ml) at 0.002% enhanced the production of L-asparaginase by 1.7 fold in *F. culmorum*, whereas, in *F. brachyglabosum* $FeSO_4$ (0.40 U/ml) enhanced the production by 2 fold. Other trace elements such as $MnSO_4$ and $CuSO_4$ did not show any effect on the activity. However, $HgCl_2$, EDTA and SDS repressed the production of L-asparaginase in *F. brachyglabosum* (Fig. 9). However, earlier reports suggest that $ZnCl_2$ and $ZnSO_4$ enhanced the production of L-asparaginase by 1 fold in *Aspergillus terreus* [22] and *Aspergillus niger* [36] respectively.

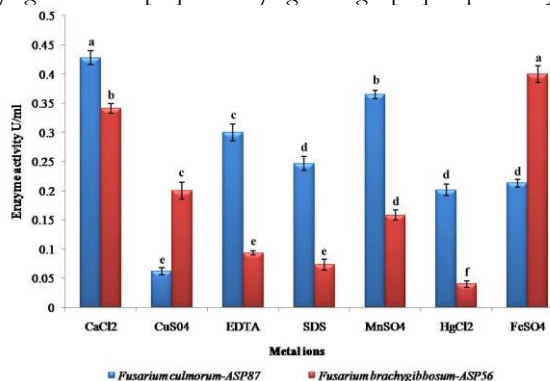


Figure 9: Effect of trace elements, chelating agents and surfactants on L-asparaginase production in *F. culmorum* and *F. brachyglabosum*. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to DMRT

Effect of Amino acids

Amino acids act as the source of nitrogen for the production of L-asparaginase. Addition of amino acids did not influence the production of L-asparaginase. Medium supplemented with glutamine showed very little influence on the production of L-asparaginase i.e. 1.5 fold in *F. culmorum*. Whereas, in *F. brachyglabosum* arginine enhanced

the activity of L-asparaginase by 2 fold (Fig. 10). Other amino acids such as glycine and lysine repressed the activity of enzyme. Although, earlier reports suggest that glutamic acid and proline enhanced the production of L-asparaginase in *Aspergillus flavus* [33] and *Aspergillus niger* [37], our study with *Fusarium* spp. showed any influence on the activity of L-asparaginase.

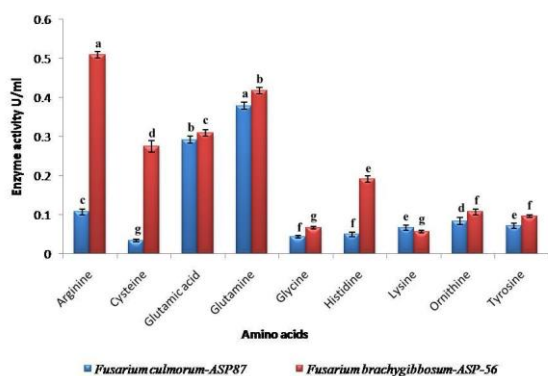


Figure 10: Effect of amino acids on L-asparaginase production in *F. culmorum* and *F. brachygibbosum*. In each series, mean values labeled with the same lower case alphabets are not significantly different (at $p < 0.05$) according to DMRT.

CONCLUSION

Owing to the importance of L-asparaginase in pharmaceutical and food industries, there has been a constant search for microorganisms capable of producing L-asparaginase. In our present investigation, we isolated two *Fusarium* species (*F. culmorum* and *F. brachygibbosum*) showing maximum L-asparaginase activity among the 364 soil fungi screened. Although, both the species of *Fusarium* required identical physiological conditions for temperature (30°C) substrate concentration (1%) and additional nitrogen source (ammonium chloride) for optimum enzyme activity, there were other parameters that were significantly different between the two strains for the induction of L-asparaginase. The enzyme activity in *F. culmorum* was induced by citric acid, calcium chloride and glutamine whereas; sucrose, ferrous sulfate and arginine induced the activity of L-asparaginase in *F. brachygibbosum*. This is the first report demonstrating the production of L-asparaginase from both the species of *Fusarium*. Our preliminary findings presented in this paper represent the optimization efforts in laboratory submerged culture conditions, which in turn, would shed light for future optimization efforts for large scale bioreactors and bioprocess development for therapeutically important L-asparaginase.

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