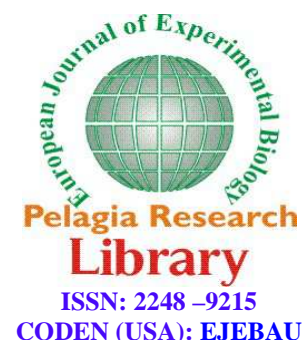


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Optimization of physiological conditions for L-asparaginase production by endophytic fungi (*Fusarium solani*) isolated from *Tinospora cordifolia* (Willd.)
Hook. F & Thomson

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ABSTRACT

Asparaginase converts L-asparagine to L-aspartic acid and has received considerable attention in the recent years for its anti-carcinogenic potential. In the present investigation, the endophytic fungi isolated from *T. cordifolia* were screened for the production of extracellular L-asparaginase during their growth on Modified Czapekdox medium (MCD). The physical and nutritional conditions were optimized for maximum production of L-asparaginase by *F. solani* under submerged fermentation conditions (SmF). The fungi exhibited significant variations in the production of L-asparaginase under the influence of incubation period, temperature, pH, carbon and nitrogen sources. It was observed that maximum enzyme activity was recorded on the 6th day of incubation period at pH 7.0 with an incubation temperature of 30°C at 120 rpm. Sucrose (0.2%) was the best carbon source. Different nitrogen sources at concentrations ranging from 0.1% to 0.5% were assessed. Among them, ammonium nitrate (0.4%) and sodium nitrate (0.4%) showed maximum production of L- asparaginase.

Keywords L-asparaginase, endophytic fungi, *F. solani*, submerged fermentation, optimization parameters.

INTRODUCTION

Endophytes colonize plants without causing any visible signs of disease or morphological changes for at least part or the whole life cycle of the plant [1]. Endophytes are capable of synthesizing bioactive compounds useful for novel drug discovery [2]. L-asparaginase is an extracellular enzyme used as an anti-cancer agent, hydrolyses L-asparagine into L-aspartic acid and ammonia and is a promising candidate for the treatment of acute lymphocytic leukemia. Fungi can be easily cultured and purified for L- asparaginase conveniently [3]. Many fungal strains are known for their L- asparaginase production potential [4, 5]. L-asparaginase is specific in its action and does not pose threat to the survival of normal cells. The production of asparaginase by fungal endophytes has been reported from plant species with anticancer potentials [6]. The medicinal plant *T. cordifolia* (*Menispermaceae*) has antipyretic, anti-asthmatic, hypoglycemic, hypotensive, analgesic, anti-inflammatory and anti-spasmodic properties [7]. Endophytes help the host plant to tolerate biotic and abiotic stress, increase growth rate and extent of reproduction and hence

improve the resistance of host medicinal plants by secretion of bioactive metabolites [8]. The present work focuses on the optimization of cultural conditions for the production of L- asparaginase from *F. solani*.

MATERIALS AND METHODS

2.1. Source of endophytic fungi:

Endophytic fungi were isolated from fresh and healthy wild medicinal plant of *T. cordifolia* (Willd.) Hook. f and Thomson, collected from Western Ghats of Karnataka, India. Herbarium of plant samples were prepared and deposited to National Ayurveda Dietetics Research Institute (Central Council for Research in Ayurveda and Siddha), Department of AYUSH, Ministry of Health and Family Welfare, Govt. of India, (New Delhi) Jayanagar, Bangalore, India. The plant authentication reference number is RRCBI-8976.

2.2. Isolation and identification of endophytic fungi:

The collected *T. cordifolia* (stem, leaf and roots) were washed thoroughly in running tap water followed by double distilled water before processing. The samples were cut into small pieces (5mm x 2mm) using sterile blade and washed with sterile distilled water. The samples were surface sterilized by immersing in 70% ethanol for 1-3 min and then sterilized by dipping into 2% sodium hypochlorite for 2-3 min, rinsed in 70% ethanol for 2 min before a final rinse with sterile water thrice and allowed to surface dry under aseptic conditions. The surface sterilized samples were placed on Potato Dextrose Agar (PDA) plates amended with 50 mg/L tetracycline to suppress the bacterial growth and incubated at $26 \pm 2^\circ\text{C}$ for 2 to 3 days [9]. The hyphal tip of endophytic fungi growing out from the plant tissue was transferred to fresh PDA plates. After incubation at $28 \pm 2^\circ\text{C}$ up to 14 days, the fungal cultures were identified based on the cultural characteristics, morphology of the fruiting bodies and spores, using standard manuals [10].

2.3. Molecular identification of the endophytic fungi:

The fungal isolates were grown in 100ml PDB for 5-6 days at 28°C . The mycelia was harvested and washed with distilled water and ground with liquid nitrogen. The nucleic acid was extracted using the cetyl trimethyl ammonium bromide (cTAB) method. Polymerase chain reaction was carried out using universal primers ITS1 and ITS4. The PCR products were visualized in 0.8% agarose gel using ethidium bromide and UV transilluminator. Prior to sequencing, amplicons were cleaned using Gen Elute™ PCR clean-up kit according to the manufacturer's instructions and sequencing was carried out in an ABI automated DNA sequencer. The sequencing PCR was set up using ABI-Big Dye® Terminator v3.1 Cycle Sequencing Kit [11]. BLAST analysis was carried out in the NCBI database. The fungus was identified and the sequence was submitted to NCBI Gen bank.

3. Determination of L-asparaginase activity from endophytic isolates

3.1. Plate screening of asparaginase producing fungi:

The endophytic fungi isolated from *T. cordifolia* were screened for their ability to produce asparaginase. The mycelial plugs from the isolated endophytic fungi were inoculated on Modified Czapekdox (MCD) agar medium. The incubation was carried at room temperature for 5 to 7 days. The appearance of a pink zone around the fungal colony on a yellow color media is an indication of the L-asparaginase [12].

3.2. L-asparaginase production by submerged fermentation: Submerged fermentation for L-asparaginase production was carried out using MCD medium. The isolates were grown on the MCD plates for 3 days. A 5 mm disc of the inoculum was taken from the culture plate using a cork borer and inoculated into 100 ml of the MCD medium in a 250 ml Erlenmeyer flask. The flasks were incubated at 30°C and maintained up to 8 days. Following incubation, the broth was centrifuged with a cooling centrifuge at 10,000 rpm for 10 mins at 4°C . Uninoculated media served as control. The supernatant was used as the crude enzyme to estimate L-asparaginase activity.

Statistical Analysis: All the experiments were performed in triplicates and the means were analyzed statistically with the SPSS program version 20. The analysis of variance was carried out according to the rules of ANOVA. The significant differences between the means were determined through Duncan's multiple range Test (DMRT).

3. 3. L-asparaginase assay and protein estimation:

3.3.1. L-asparaginase enzyme estimation:

L-asparaginase assay employs the Nesslerization reaction wherein the ammonia liberated from L-asparagine is indicated by the formation of an orange colored solution and L-asparagine is used as the substrate. The reaction

mixture consists of 0.5 ml of 0.5 M phosphate buffer (pH 7), 0.5 ml of 0.04 M L-asparagine, 0.5 ml of distilled water and 0.5 ml of crude enzyme which were mixed together, shaken well and incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.5 ml of 1.5 M trichloroacetic acid (TCA). The ammonia liberated was quantified by calorimetric estimation with the addition of 0.2 ml Nessler's reagent to a mixture of 0.1 ml filtrate and 3.75 ml distilled water. The absorbance was recorded at 450 nm with a UV-spectrophotometer (UV-1800 SHIMADZU, Japan) after a final incubation for 10 min. The blank was prepared by adding the enzyme after the TCA addition. The amount of ammonia liberated by the test sample was calculated using the ammonium sulfate standard curve [13]. One international unit (IU) of L-asparaginase is the amount of enzyme needed to liberate one $\mu\text{mol}/\text{min}$ of ammonia at 27°C. Protein content was estimated by using bovine serum albumin as the standard [14].

$$\text{Units/ml enzyme} = \frac{(\mu\text{mole of NH}_3 \text{ liberated}) (2.5)}{(0.1) (30) (1)}$$

2.5 = Initial volume of enzyme mixture in ml

0.1 = Volume of enzyme mixture used in final reaction in ml

30 = Incubation time in minutes

1 = Volume of enzyme used in ml

International unit: One IU of L-asparaginase activity is defined as that amount of enzyme which catalyses the formation of 1 μmole of NH_3 per minute under the optimal assay conditions

3.4. Optimization of parameters for production of L-asparaginase: The dynamics influencing the L-asparaginase were assessed and the optimizations were carried out by varying one parameter at a time keeping the other parameters unaltered for the maximal production of asparaginase. The optimization of L-asparaginase by *F. solani* depends on several factors such as incubation period, temperature and pH. The effects of different carbon and nitrogen sources on asparaginase production were also investigated.

3.4.1. Effect of incubation period: The L-asparaginase production by *F. solani* was carried out using 100ml of MCD (pH 6) containing 1% asparagine in 250ml Erlenmeyer flasks inoculated with a 5 mm culture disc and were incubated at different growth intervals. The filtrate was assessed at different incubation periods (Day1-8) at 30°C and the enzyme activity was determined.

3.4.2. Effect of pH: To determine the maximum asparaginase production at a particular pH, the medium was adjusted to various pH ranges of 3, 5, 7 and 9 using 1N NaOH and 1N HCl. The endophytic *F. solani* (5mm) was inoculated into the MCD medium and the flasks were incubated for 7 days at 30°C. The clear supernatant was used to determine the enzyme activity.

3.4.3. Effect of temperature: To observe the effect of temperature on the production of L- asparaginase by *F. solani*, the medium was inoculated with a 5mm culture disc and incubated at different temperatures of 28°C, 30°C and 37°C. The flasks were incubated for 7days. The supernatant was used as crude enzyme to calculate the asparaginase activity and protein content.

3.4.4. Effect of carbon source: The influence of different carbon sources on efficient asparaginase production was assessed. Five carbon sources- glucose, sucrose, maltose, lactose and starch (0.2%) were utilized and incubated for 7days at 30°C. The supernatant was utilized for determining the enzyme activity and protein content.

3.4.5. Effect of nitrogen source: A single 5 mm disc of the inoculum from the culture plates of *F. solani* was inoculated into 250ml erlenmeyer flasks consisting of 100 ml of the fermentation medium. The effect of different nitrogen sources (Peptone, Yeast extract, Potassium nitrate, Ammonium nitrate and Sodium nitrate) on asparaginase production was assessed at concentrations ranging from 0.1% to 0.5%, incubated for a period of 7 days at 30°C. The enzyme activity was determined by utilizing the supernatant in order to measure the asparaginase activity.

RESULTS AND DISCUSSION

4.1. Screening for L-asparaginase activity on MCD medium:

A total of 25 endophytic fungi were isolated from *T. cordifolia*, the preliminary screening for asparaginase activity of the endophytic isolates was determined by the plate assay, which revealed the appearance of a pink zone around

the fungal colony indicating the L-asparaginase production (Fig-1). An increase in pH due to ammonia accumulation in the medium results in the change of color from yellow to pink and is suggestive of the fact that the endophytic fungi are able to utilize the asparagine (substrate) by the secretion of the enzyme asparaginase. The plate assay method is a qualitative, simple and rapid screening procedure for L-asparaginase production. The results are depicted in the Table-1.

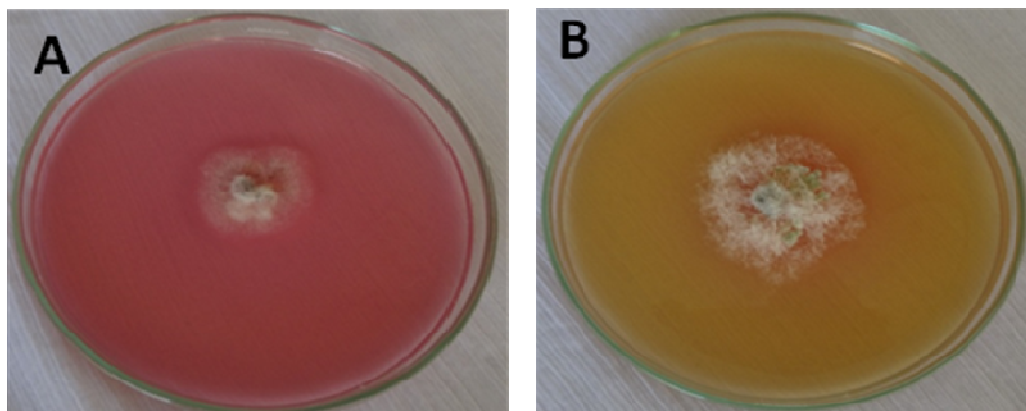


Fig1: Fungal isolate on a Modified Czapekdox plate (MCD)

A) Asparaginase positive plate
B) Asparaginase negative plate

Table-1: Screening of endophytic fungi for L-asparaginase production using plate assay method

Sl. No	Endophytic fungal code	Identification of the isolate	Zone diameter (in mm)
1	ABL	<i>Aspergillus niger</i>	0 ^a
2	ABL1	<i>Fusarium solani</i>	25.00 ^b ±0.59
3	ABL2	<i>Rhizopus sp</i>	0 ^a
4	ABL3	<i>Aspergillus sp.</i>	0 ^a
5	ABLS1	<i>Mucor sp.</i>	0 ^a
6	ABLS2	<i>Mycelia sterilia spp.1</i>	47.66 ^{cd} ±1.30
7	ABLS3	<i>Aspergillus spp.</i>	43.66 ^{cd} ±0.78
8	ABLS4	<i>Mycelia sterilia spp.2</i>	34.66 ^{bc} ±0.29
9	ABPS1	<i>Cladosporium spp.</i>	54.00 ^d ±0.51
10	ABPS2	<i>Penicillium spp.</i>	0 ^a
11	ABPS3	<i>Mycelia sterilia</i>	0 ^a
12	ABS	<i>Penicillium spp.</i>	59.00 ^d ±0.51
13	ABS1	<i>Aspergillus oryzae</i>	0 ^a
14	ABS2	<i>Mycelia sterilia</i>	0 ^a
15	ABS3	<i>Trichoderma asperellum</i>	69.00 ^e ±0.29
16	ABSS1	<i>Cladosporium sp.</i>	0 ^a
17	ABSS2	<i>Aspergillus sp.</i>	23.66 ^{ab} ±0.78
18	ABSS3	<i>Cladosporium sp.</i>	0 ^a
19	ABSS4	<i>Aspergillus sp.</i>	24.00 ^b ±0.78
20	ABSS5	<i>Aspergillus sp.</i>	36.00 ^{bc} ±0.51
21	ABR	<i>Phomopsis sp.</i>	0 ^a
22	ABR1	<i>Mycelia sterilia</i>	0 ^a
23	ABR2	<i>Colletotrichum sp.</i>	0 ^a
24	ABR3	<i>Mucor sp.</i>	0 ^a
25	ABR4	<i>F.solani</i>	76.00^d±0.51

Values followed by the same lower case alphabets in the each column are statistically equivalent ($P < 0.05$) according to the Duncan multiple range test.

Of the 25 endophytic fungal isolates, 11 isolates (44%) were screened positive for the L- asparaginase activity. The isolate ABR4 was selected for the optimization studies based on the initial screening by plate assay method. The isolate was identified as *Fusarium solani* (Fig-2) and the sequence was submitted to Gen bank with the accession no. **KJ729476**.



Fig 2: Pure culture of endophytic fungus *Fusarium solani*

4.2. Optimization of culture parameters for L-asparaginase production:

The various parameters for efficient L- asparagine production were optimized. Different growth conditions have an influence on the production of L-asparaginase, hence necessitating conditions in which the fungi can produce high yield of L- asparaginase without an increase in the cost of production. The physical and the nutritional parameters play a pivotal role in the improvement of the L-asparaginase secretion and hence the incubation period, incubation temperature, pH, carbon and nitrogen sources were optimized.

4.2.1. Effect of incubation period:

The effect of fermentation time was evaluated to note the optimal time period for the best production of L- asparaginase. The time course of the incubation period ranged from Day 1 to Day 8. In the present study, the production of L- asparaginase by *F. solani* was studied when cultivated on MCD medium with 1% L-asparagine as the substrate. The asparaginase activity was determined after each day of incubation up to 8 days in order to determine the optimum incubation period for good production of asparaginase.

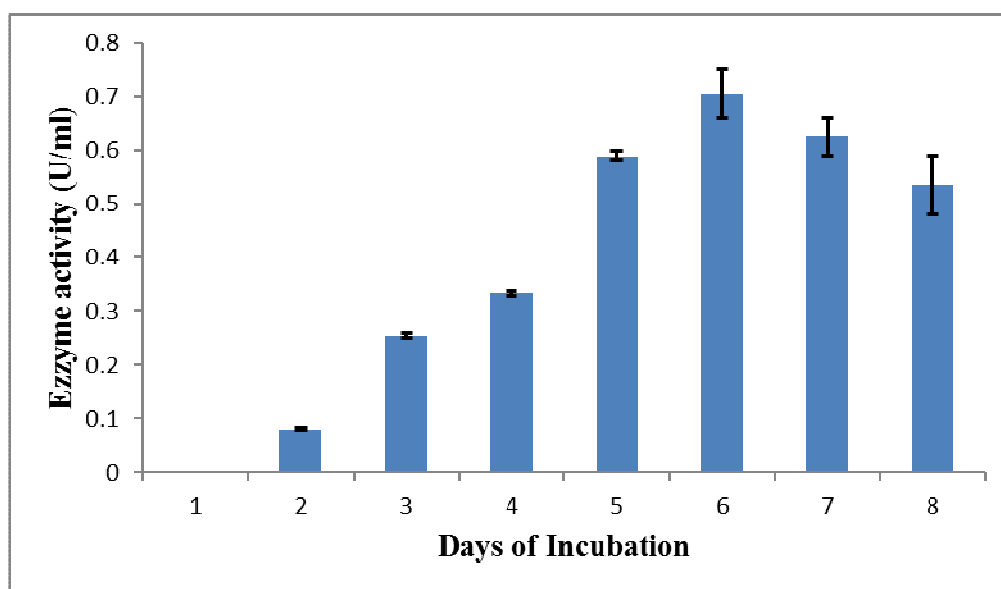


Fig 3: Effect of incubation period on L-asparaginase production by *F. solani*

The maximum amount of L- asparaginase production was noted on the 6th day of incubation period by after which a gradual decline in the enzyme production was noted. Any further increase in the incubation periods resulted in a decreased production of L- asparaginase enzyme. This may be due to the depletion of media nutrients or the

production of metabolites which may result in growth inhibition or a change in pH [15]. Other studies reflect on the shorter incubation period required for L- asparaginase production by *Aspergillus* sp. [16, 17, 18]; *Penicillium* sp. [19]. An incubation period of 120 hrs required for the production of L-asparaginase enzyme by *Fusarium* sp3 [20] whereas efficient asparaginase production by *Aspergillus* sp. ALAA- 2000 was reported on the 6th day of the incubation period by submerged fermentation [21] which is in accordance with our study.

4.2.2. Effect of pH: Microbial enzyme activity is greatly influenced by the surface charges present on the amino acids. Enzyme activity can be either enhanced or inhibited depending on the change in the pH, and hence can influence the growth of microorganisms [6]. The initial pH of the production medium is an important factor affecting enzyme production as it influences the nutrient availability and controls the fungal growth.

The highest enzyme activity was observed at pH 7.0 with an activity of 0.681 U/ml. Similarly, maximum L-asparaginase production at pH 7.0 by *Fusarium equiseti* [15] and *Aspergillus terreus* [16] has been reported. A decline in the enzyme activity noted after the optimum pH may be due to the fact that both acidic and alkaline pH has an inhibitory effect on the growth of *F. solani* and enzyme production. Also, enzyme activity declination may be due to the partial denaturation of the enzyme as a result of dissociation of the ionizable enzyme groups. A change in pH prevents the binding of a substrate to the enzyme owing to change of shape and properties of an enzyme and/or the substrate [22]. A pH of 6.2 as optimum for asparaginase production by *Fusarium* sp. has been reported [20] whereas a pH of 6.0 was the optimal pH for L-asparaginase production in *Penicillium* sp. [23]. Interestingly, a pH of 5.0 was found to be optimal for L-asparaginase production by *F. oxysporum* [24].

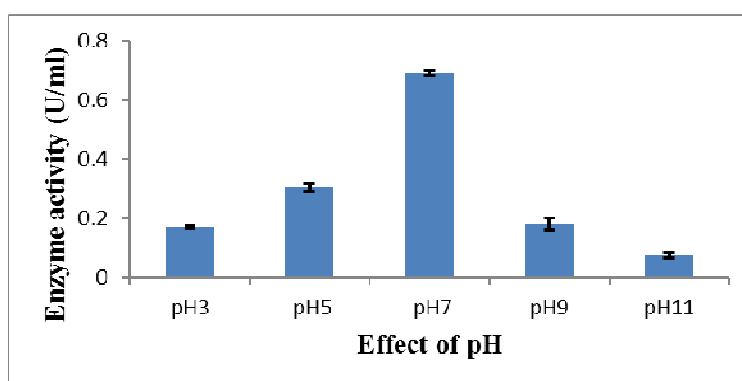


Fig 4: Effect of pH on L-asparaginase production by *F. solani*

4.2.3. Effect of Temperature:

The incubation temperature is a critical environmental factor for L-asparaginase production by microbes as it regulates microbial growth and consequently enzyme secretion. Temperature influences the rate of the chemical reaction thus affecting rate of enzymatic activity. The endophytic fungus *F. solani* was able to grow and secrete the enzyme at all the tested temperature parameters with the maximum production at 30^o C with an activity of 0.416 U/ml. However, the enzyme activity decreased at higher temperature of 35^oC which can be attributed to the partial enzyme denaturation as a result of change in the metabolic activities of the organism. Our results correlate with the reports of earlier workers in *Mucor hiemalis* [25], *Penicillium* sp. [26], *Aspergillus terreus* [27] who have reported maximum activity of L-asparaginase at 30°C. A temperature of 37^oC was found to be optimum for asparaginase production by *A. terreus* [17]. However, highest asparaginase activity has been reported at 45^oC [15].

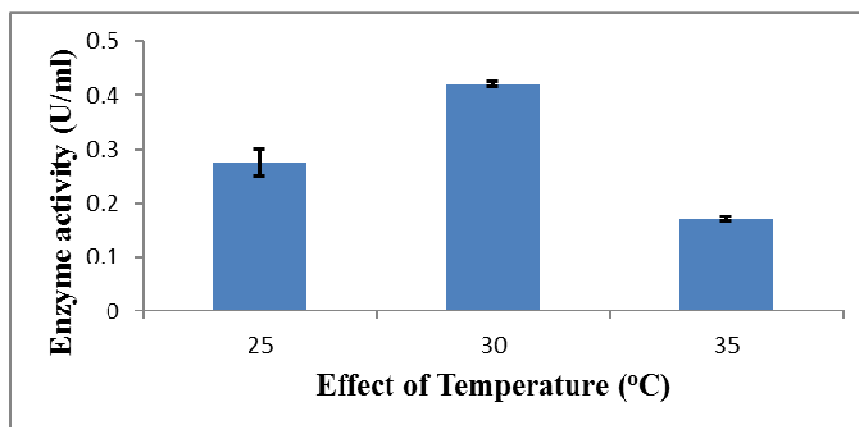


Fig 5: Effect of Temperature on L-asparaginase production by *F. solani*

4.2.4. Effect of Carbon sources: Carbon is the major structural and functional component of microbial cells. Fungi are capable of using wide variety of carbon compounds but most of the fungi prefer simple sugars [24].

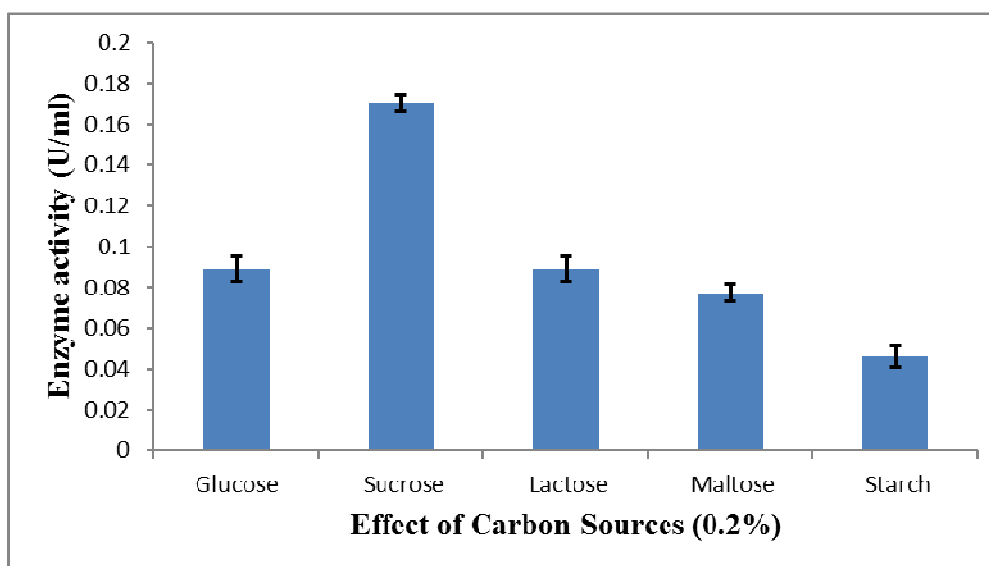


Fig 6: Effect of Carbon sources on L-asparaginase production by *F. solani*

Five different carbon sources were studied for their L- asparaginase production. The present study reveals that *F. solani* could produce maximum asparaginase when sucrose (0.2%) is used as the carbon source (0.166U/ml). Sucrose demonstrates significant production of enzyme due to its inductive effect as an inexhaustible source of carbon as compared to other carbon sources and it also helps in stabilizing the enzyme [28]. Studies have reported sucrose as a good carbon source to induce L- asparaginase production by *F. oxysporum* [24]; whereas highest amount of asparaginase production has been observed in the presence of glucose while sucrose supported only minimum activity by *Fusarium sp.* [29]. A study on L- asparaginase reveals glucose at 0.4% concentration as the best carbon source [25]. Also, earlier reports suggest that glucose served as the best carbon source for the production of L-asparaginase in *F. equiseti* [15] and *A. niger* [30].

4.2.5. Effect of Nitrogen sources: Fungi are reported to exhibit great specificity for nitrogen source present in the medium. The nitrogen source has a profound influence on the metabolism of microorganisms [24]. The influence of different organic nitrogen sources and inorganic sources were studied.

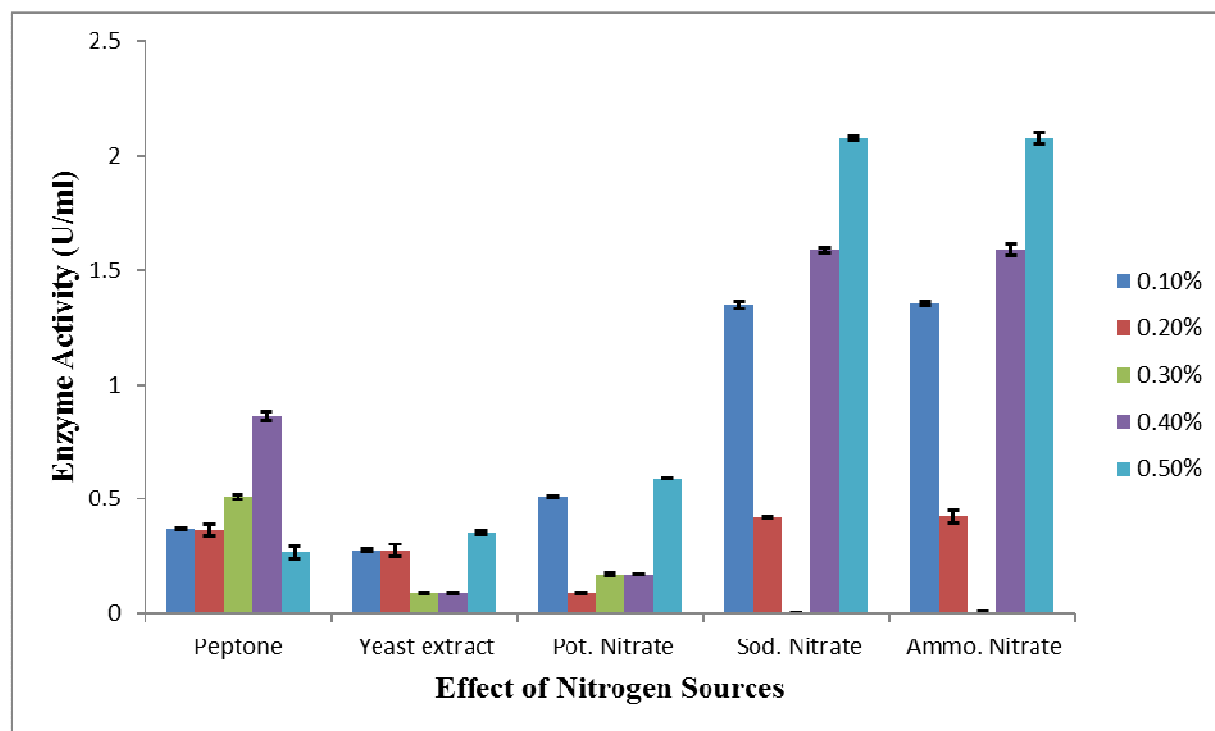


Fig 7: Effect of Nitrogen sources on L-asparaginase production by *F. solani*

Although L-asparagine itself acts as nitrogen source in the growth medium, the influence of supplemented nitrogen source on the activity of L-asparaginase was studied. Each one of these nitrogen sources was added to the MCD medium individually at concentration along with the enzymatic inducer L-asparagine. The different nitrogen sources were supplemented into the medium at concentrations ranging from 0.1% to 0.5% for L- asparaginase production. The graph reveals that sodium nitrate and ammonium nitrate at concentrations of 0.1%, 0.4% and 0.5% demonstrated maximal L-asparaginase production. Peptone (0.4%) revealed significant L-asparagine production as compared to yeast extract and potassium nitrate which produced insignificant amount of L- asparagine (Fig 6). However, yeast extract and potassium nitrate showed lowest production of L-asparaginase by the strain *F. solani*. Incorporation of sodium nitrate in the production medium favored increased L-asparaginase production in *Bacillus* KK2S4 [31]. The substrate L- asparaginase has been found to be the best source for the biosynthesis of L-asparaginase. However, sodium nitrate reported the lowest production of L- asparaginase by *Aspergillus* sp. ALAA-2000 strain [21]. A concentration of 0.25% ammonium nitrate has been found efficient in asparaginase production [15].

CONCLUSION

This report highlights the asparaginase production from endophytic fungi isolated from *T. cordifolia*. The L-asparaginase producing fungus was detected by a plate assay method. Among 25 isolates, ABR4 (*F. solani*) exhibited maximum L-asparaginase production by submerged fermentation. The findings of the study lay emphasis for the further optimization efforts for large scale production and efficient process development for effective enzyme production.

Acknowledgements

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