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Production of anticancer enzyme asparaginase from endophytic *Eurotium* Sp. isolated from rhizomes of *Curcuma longa*

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ABSTRACT

Endophytic fungi generally reside asymptomatically in the tissues of higher plants and generally are source of original amidase group of enzymes. Asparaginase is an important anticancer enzyme, endophytic fungi Eurotium Sp. was isolated from rhizomes of Curcuma longa and evaluated for asparaginase production by qualitative plate assay on modified Czapex dox's agar and quantitative analysis by Nesslerization method. Maximum enzyme activity was recorded at 40^{0}C and pH 8.0. Molecular weight for asparaginase was recorded as 14,300 KD using SDS-PAGE electrophoresis. Enzyme found to be stable at different temperature and pH ranges making it suitable for wide range of industrial applications.

Key words: *Curcuma longa,* endophytic fungi, asparaginase, Nesslerization, *Eurotium* Sp.

INTRODUCTION

The word "endophytes" includes a group of microorganisms that grow intra or intercellular in the tissues of plants without causing any visible symptoms on the host in which they live, [1, 2]. Fungal endophytes are living entity in a host plant for at least a part of life, without causing any apparent disease [3]. Such mutualistic interaction between endophytes and host result in fitness benefit for both partners. The endophyte provide defense and survival environment to their host plant by producing a plethora of substances having potential use in industry, agriculture, and medicine [4]. Approximately 300, 000 plant species growing on the earth are host to one or more endophytes [5], and the presence of different endophyte in huge number plays an important role on bionetworks with greatest biodiversity, for example, the tropical and temperate rainforests [5].

Asparaginase (L-Asparagine amidohydrolase; EC 3.5.1.1) belongs to an amidase group which catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia, and found broadly from microorganisms to mammals. The enzyme is considered to participate in major role in the asparaginase metabolism of the cells. It's a likely therapeutic agent for acute lymphocytic leukemia, acute lymphoblastic leukemia and chronic myelogenous leukemia also reported to be an efficient antilymphoma agent in humans. Asparaginase has been investigated since it was found that the enzyme from definite microorganisms has antitumor activity [6]. Fungi are reported as potential producer of asparaginase in comparison to bacteria [7]. Genera such as *Aspergillus, Penicillium* and *Fusarium* are generally reported in scientific literature for asparaginase production.

The present report will describe the results of screening of endophytic fungi *Eurotium* Sp. for production of extra-cellular asparaginase using a rapid plate assay, quantitative measuring for asparaginase activity under submerged culture and attempts to study the optimization of asparaginase production, its purification and characterization.

MATERIALS AND METHODS

Isolation of endophytic fungi

Mature healthy rhizomes of *Curcuma longa* were collected from garden (Jalgaon, India) and immediately brought to laboratory for use within 24 h. Rhizomes were washed in running tap water for 10 min to remove, soil particles and adhered debris, and finally washed with distilled water. Surface sterilization was done using method described by Petrini [8], with some alteration. Samples were immersed in 70% ethanol for 1-3 min and 4% aqueous solution of sodium hypochlorite 1.5 min, again 1min with 70% ethanol and finally rinsed with sterile distilled water. Disinfected samples were used for aseptic cutting using sterile knife and inner tissues were excised such tissues were inoculated on to potato dextrose agar media, for 4 - 25 days at 27^oC with aim to isolate endophytic fungi. Endophytic fungi were isolated and stock cultures were maintained on potato dextrose agar slant. An isolated fungus with potential asparaginase activity was identified from Department of Advance study in Botany, Banaras Hindu University, Varanasi (India).

Qualitative analysis

Modified Czapex Dox's agar medium pH 6.2 was prepared containing (g/l) glucose, 2.0 ; L-asparagine,10.0; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4.7H_20$, 0.52; $CuNO_3.3H_2O$, 0.001; $ZnSO_4.7H_2O$, 0.001; $FeO_4.7H_2O$, 0.52 ; $CuNO_3.3H_2O$, 0.001 ; agar, 20.0 and 0.009 % phenol red as indicator, control plates were without asparagine. The plates were inoculated with (0.5 x 0.5 cm^2) disk of 3-4 days old mycelium of *Eurotium* Sp. and incubated for 4-5 days at 37^0C. Pink zone radius and colony diameter were measured [9].

Quantitative assay

An 5mm disc of mycelium from positive agar plate was used as inoculum for asparaginase production under liquid state using modified Czapex dox's broth, for 120 h at 37^0C with shaking at 200 rpm. The activity of enzyme was determine in filtrate by means of Nesslerization as described by Imada [7].The substrate was prepared in 0.05M tris (hydroxymethyl) amino methane (tris HCl) (pH 7.2), giving final concentration of 0.04M. The reaction mixture contains 200µl of 0.04 M asparagine in 0.05 M tris HCl buffer, 100µl of 0.05 M tris HCl buffer (P^H 7.2), 100µl distilled water and 100µl of crude enzyme which was obtained from the culture filtrate .The samples were incubated at 37^0C for 60 min and to stop reaction 100µl of 1.5M tichloroacetic acid (TCA) was added.100µl of mixture was than mixed with 750µl distilled water and than nesslers reagent was added and for all incubation was allowed at 20^0C for 20 min. All reactions were measured spectrophotometrically at 450nm.One unit of asparaginase is the amount of enzyme which catalyses the formation of 1µmol of ammonia per min at 37^0C. Asparaginase was partially purified by following steps (A-D)

Step A

Asparaginase activity was found to associate with fraction precipitation at 40-60% saturation. The crude enzyme extract was brought to 45 % saturation with ammonium sulphate (2.6g) at pH of 8.4 and samples were kept overnight in cooled temperature. After equilibration is achieved supernatant was subjected to centrifugation at 5000 rpm for 10 min at 4^0C, after which supernatant was brought to 85% saturation with ammonium sulphate (5.8g) and again centrifuge at 5000 rpm for 10 min. The precipitate was collected separately and store at 4^0C until next step was done.

Step B

The precipitate collected from step A was than dissolved in 1M Tris HCl buffer and dialyzed and these samples were use for further purification steps.

Step C

The samples obtained by dialysis were dissolved in 0.05M Tris HCl (pH 8.4) buffer and loaded on column pre-equilibrated with 0.05M Tris HCl Sephadex G 50. It was eluted with 0.05M Tris HCl (pH 8.4) buffer also containing 0.1M KCl, and fractions were collected at the flow rate of 50ml/25min. Fraction viewing high activity were pooled and asparaginase was assayed by previously described assay method.

Step D

The fraction from Sephadex G 50 gel filtration having highest activity was subjected to SDS-PAGE (Bangalore gene India) to determine molecular weight.

pH and Temperature studies for asparaginase

Asparaginase activity was evaluated at different pH and temperature values. Crude and partially purified enzyme was incubated with 0.04M asparaginase and 0.05M buffers of pH 4-10 under suitable assay condition and amount of

ammonia liberated was find out. The optimum temperature for enzyme activity was studied by incubating the assay mixture at temperature ranging from 10-100^{0}C.

RESULTS AND DISCUSSION

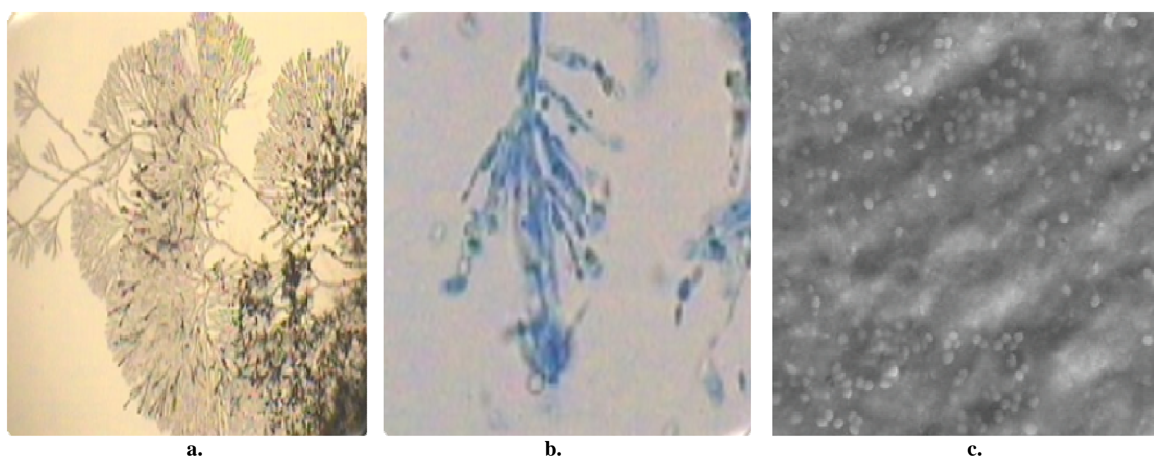

Figure 1. a. Colony morphology of endophytic *Eurotium* Sp in petri plate, b.branche mycelium under 100X of light microscope, c. fungal spore structure under 3000X of electron microscope

Morphological apperance of *Eurotium* Sp. shows ovale shape colourless spores,with branched mycelium, cottony at young stage and brown on maturity along with exudates.

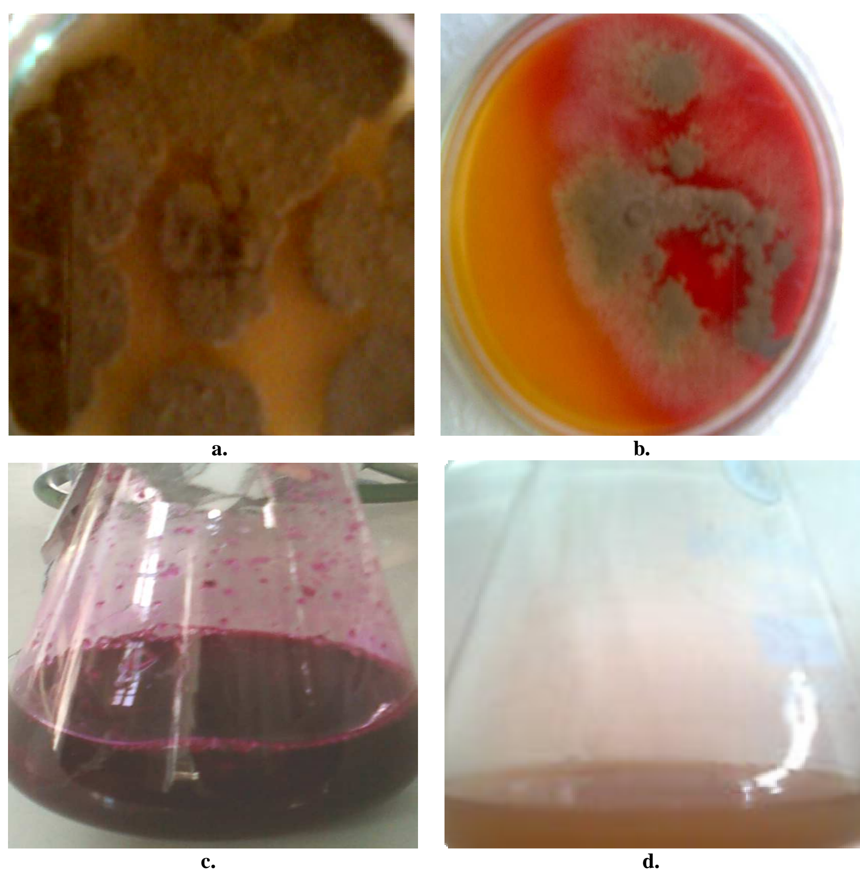

Figure 2.a. Endophytic *Eurotium* Sp., b. Positive asparaginase activity by qualitative plate assay, c.asparaginase production in liquid fermentation medium, d.negative control

Endophytic fungi *Eurotium* Sp. was isolated from rhizomes of *C.longa*. To our knowledge these is first report on isolation of *Eurotium* Sp. as endophytic fungi from rhizomes of *C.longa* and production of asparaginase. Fungi could grow on Czapex dox's agar with phenol red, a dye indicator that changes from yellow (in acidic condition) to

pink (in alkaline condition). The pink zone around fungal colony indicates the pH alteration which originated owing to ammonia accumulation in the test medium and asparaginase production can be examined by plate inspection.

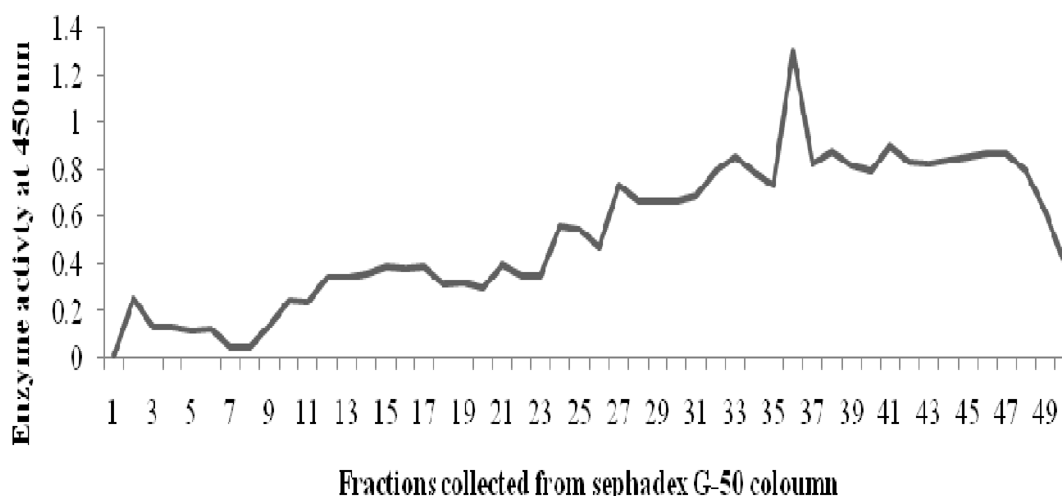

Figure 3.Gel filteration chromatography of asparaginase (Sephadex G-50)

Above figure 3. shows presence of highly active fraction, with enzyme activity of 1.304 units /ml by Nesslerization. One unit of asparaginase is the amount of enzyme which catalyzed the formation of 1 μmol of ammonia per min at 37^0C. The reaction mixture was assayed in triplicate. Sample was then evaluated for production of protein,fraction number 36 showed high content of protein as shown belowe figure 4.

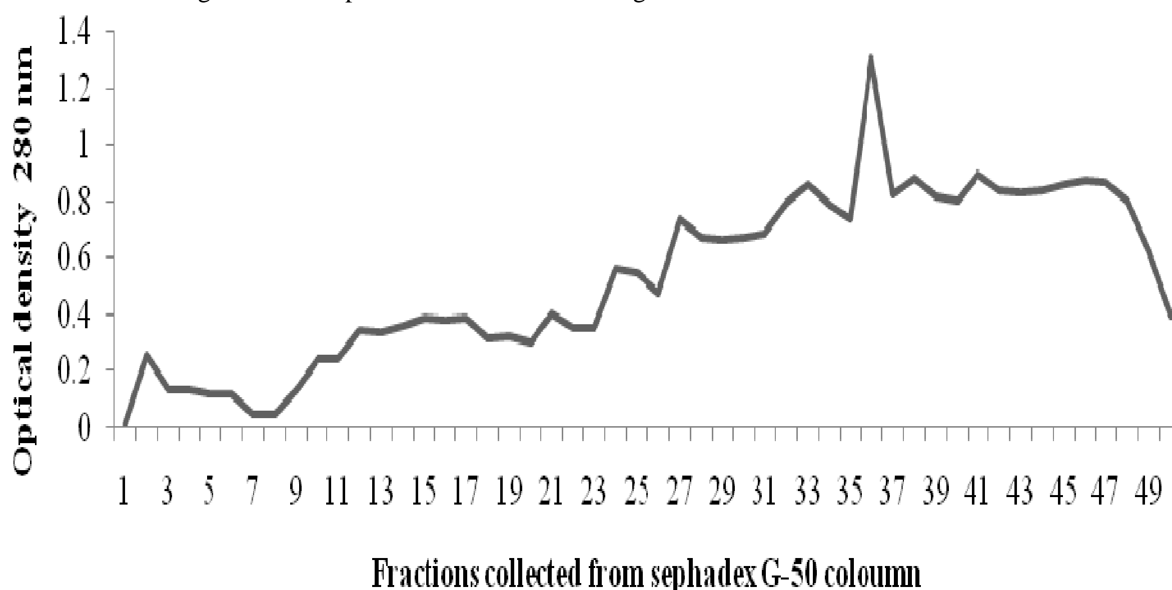

Figure 4. Protein contain at optical density 280nm

Our research showed that enzyme production was more effective in liquid condition, these finding matches with Holker [10], showing that ability of fungi to produce enzyme in solid and liquid state was different. Enzyme activity was found to be 2.3cm and 1.304 units /ml qualitatively and quantitatively respectively.

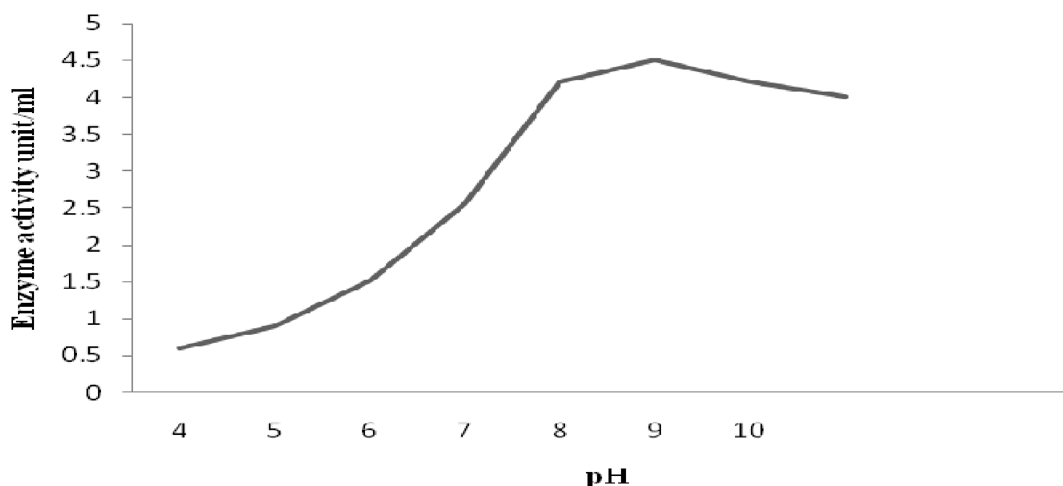

Figure 5.Optimization of pH for asparaginase activity

The activity of asparaginase was evaluated at different pH values under assay conditions and the amount of ammonia liberated was determined calorimetrically. Enzyme activity increased with increase in initial pH and reaches optimum at pH 9 thereafter, it decreased as shown above in figure 5**.**

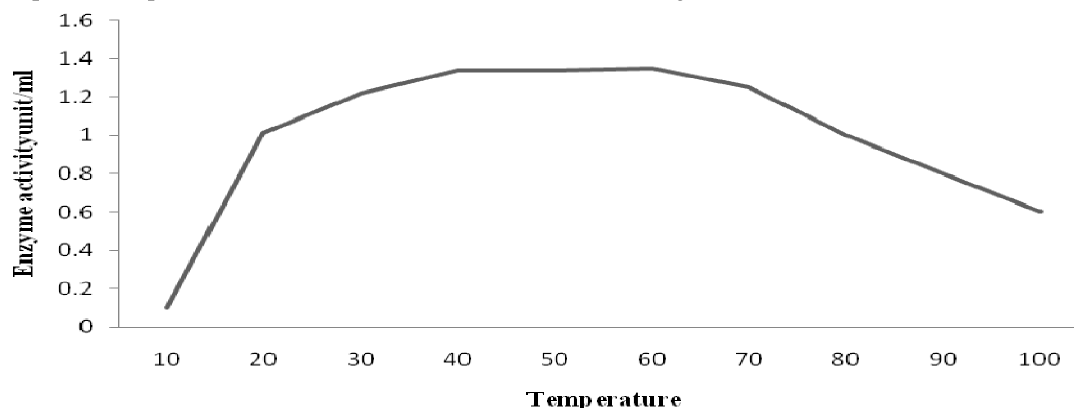

Figure 6.Optimization of temperature for asparaginase activity

Results obtained from above graph (Figure 6**.**) indicate that optimum temperature for asparaginase activity was between 40^{0}C to 60^{0}C concluding that enzyme activity was stable at wide array pH and temperature range.

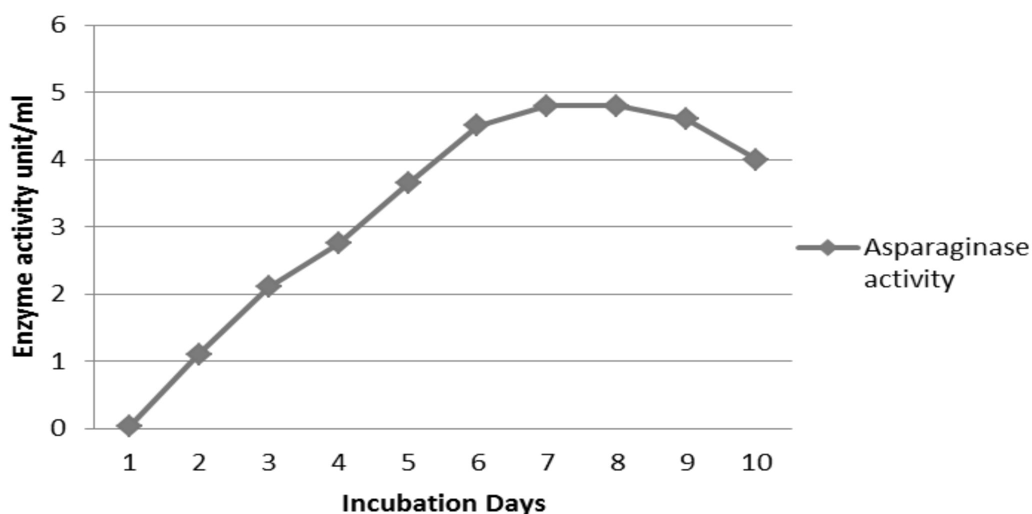

Figure 7.Asparaginase production in liquid media

Production of asparaginase starts after 24 h reaching maximum on 7th day after 144 h of incubation and being stable up to 9th day after which it starts to decline.

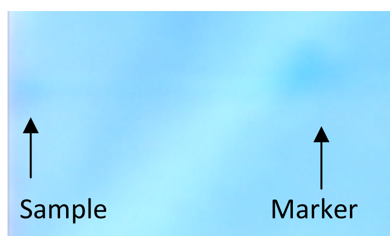

Figure 8. SDS PAGE electrophoresis for enzyme asparaginase

Molecular weight of asparaginas was determined by 7.5% SDS-PAGE. Mobility of band correspond protein marker of molecular weight 14,300 KD approximately (Figure 8.).Hence, can conclude that endophytic fungi *Eurotium* Sp. bears strong potential for production of enzyme asparaginase.Stability of enzyme at different temperature and pH range making it suitable for wide array of pharmaceutical applications.

Anticancer activity on HeLa cell line

Anticancer activity by enzyme asparaginase (H6) was confirm on HeLa cell line using adriamycin as standard as shown in (Figure 9.)

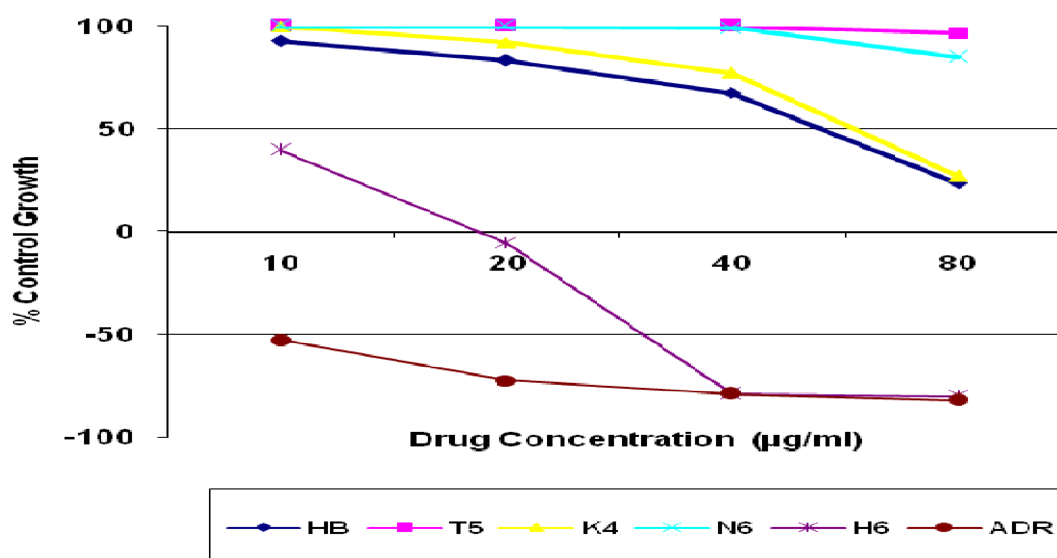

Figure 9.Anticancer activity by asparaginase on HeLa cancer cell line

The activity of asparaginase was evaluated at different values of pH and temperature under assay conditions and the amount of ammonia liberated was determined. Fungal mycelium was separated by filtration crude enzyme was collected from broth and used for further purification process. Asparaginase yield increased with increase in initial pH of the medium and thereafter it decreased and enzyme activity was stable at wide array temperature ranging from 40^0C to 60^0C. It is suppose that changes in optimum pH and temperature of asparaginase may caused by the charge of a water-insoluble carrier, a chemical alteration of the enzyme, or some of the enzymatic reactions.

Purification of asparaginase was carried out by four steps as mentioned in methodology, fractioned collected from Sephadex G50 column were analyzed for asparaginase activity and protein content. Asparaginase Production was studied in some fungi such as *Aspergillus tamarii* and *Aspergillus terreus* these fungi were grown in different medium having different sources of nitrogen and it was found that *A. terreus* showed the highest asparaginase activity (58 U/L) with 2% proline medium [11].

In present study, asparaginase activity found as 1.304 units/ml by *Eurotium* Sp. from rhizomes of *C.longa,* which is less as compared to 3.71 IU/ml by endophytic *Fusarium* sp. KLIVRb9-1 from Thai medicinal plant [12]. This may be due to inappropriate culture conditions, needs or ability of microbe for enzyme production. Some endophytes like

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Fusarium Spp., Xylaria Sp., *Phomopsis* Spp. and some filamentous fungi were reported previously as producer of asparaginase [13]. Isolated *Eurotium* Sp. need more research in concern to asparaginase production.

The marketable accessibility of anticancer enzyme asparaginase has revolutionized the molecular analysis of acute lymphocytic leukemia and melanosacroma. Therefore, the necessities of new purified asparaginase are not only of intellectual significance but also of practical importance [14].There are two types of asparaginase, and asparaginase I and II [15,16,17]. Both of these two enzymes differ in their properties, such as location in the cell, solubility in ammonium sulfate and most significantly, their attraction in favor of substrate L-asparagine.

Molecular sequencing and phylogenetic analysis
CTCCCATCCGTGTCTATCTGTACCCTGTTGCTTCGGCGTGGCCACGGTTCCGCCGAAGACTAACATTTG
AACACTGTCTGAAGTTTGCAGTCTGAGTTTTTAGTTAAACAATAATTAAAACTTTCAACAACGGATCTC
TTGGTTCCGGCATCGATGAAGAACGCAGCGAAACGCGATAATTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTG
CTGCCCTCAAGCACGGCTTGTGTGTTGGGCTTCCGTCCCTGGTAACGGGGACGGGCCCAAAAGGCAGT
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CTAGCCTCGCAACCAATCTTTTTAACCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAA
GCATATCAATA

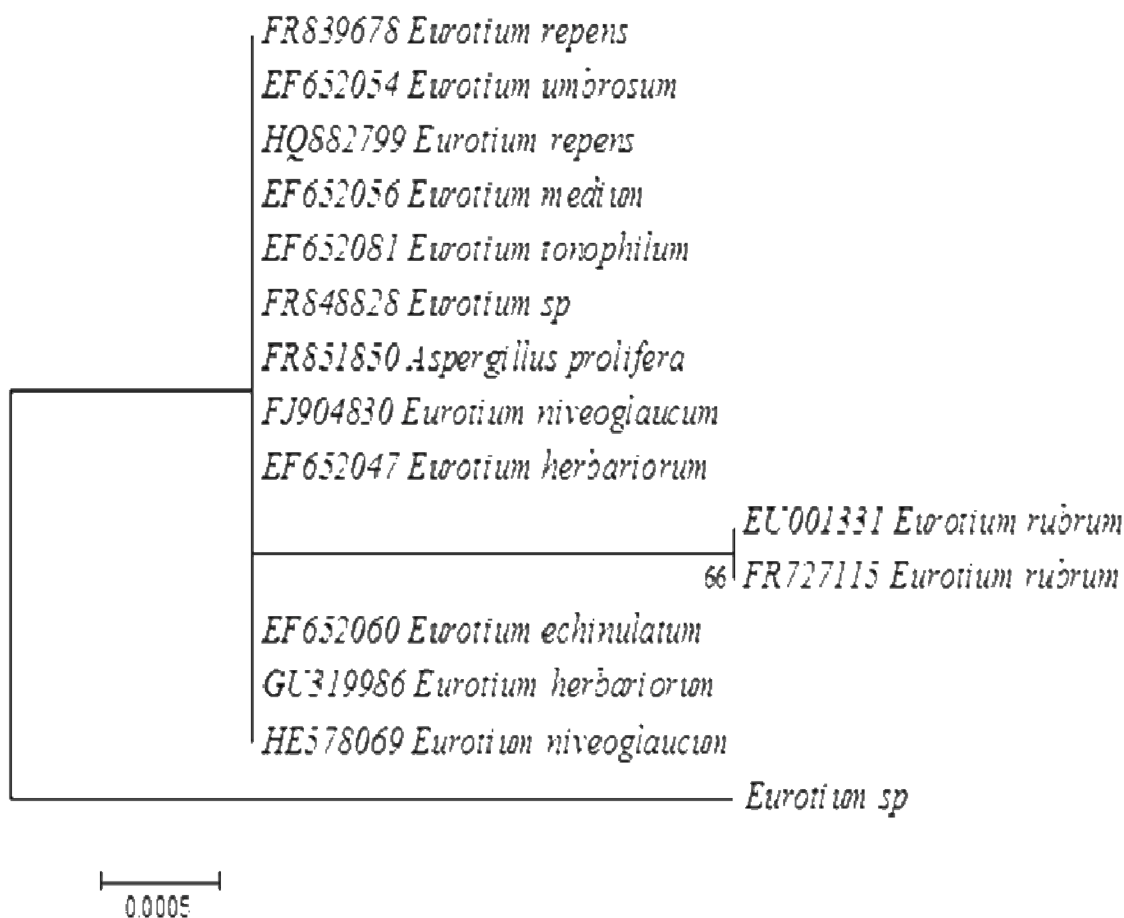

Figure 10 Base pair sequencing and phylogenetic tree of *Eurotium* Sp.

Endophytic fungi *Eurotium* Sp. was sequence on molecular level by 16S RNA using ITS (for internal transcribed spacer) (Figure 10.).With the help of sequence base pairs phylogenetic analysis was done. Isolated fungi forms divergent linkage from cluster of *Eurotium repens, Eurotium rubrum, Eurotium herbariorum, Eurotium niveoglaucum.* Therefore, on the basis of phylogency isolate identified as *Eurotium* Sp. For further characterization up to species level, few more genes have to be sequence.

The present study revealed that, all the selected parameters examined, showed a considerable impact on asparaginase production by endophyte *Eurotium* Sp. its greater catalytic activity at physiological pH and temperature also its stability over a wide range of pH and temperature along with anticancer activity against HeLa cancer cell line, allows it as a favorable enzyme to be exploited anticancer agent.

CONCLUSION

Surface sterilization protocol was suitable for isolation of endophytes, from these present studies, it is clearly indicated that rhizomes of *C.longa* can provide a rich source endophytic fungi producing anticancer enzyme asparaginase.

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