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CHARACTERIZATION OF L-ASPARAGINASE PRODUCING ENDOPHYTIC FUNGI ISOLATED FROM RIPENED FRUIT OF *CAPSICUM FRUTESCENCE*

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ABSTRACT

The main objective is to evaluate L-asparaginase production of endophytic fungi isolated from ripened fruit of *Capsicum frutescence* var US 341. In the present work endophytic fungal strains were screened for production of extra-cellular L-asparaginase, an anti-neoplastic agent used in the chemotherapy of lymphoblastic leukemia. L-Asparaginase activity of endophytic fungi was detected using modified Czapex dok's medium containing L-asparagine and Phenol red as indicator. In the present study out of 16 fungal isolates, 6 fungal isolates were identified as L-asparaginase positive of which two fungal isolates RFEF-1 and RFEF-2 are identified as *Aspergillus* sp on their morphological characterization of mycelia, conidiospores and conidia under microscopic observation. The effect of growth parameters on biomass and production of L-asparaginase of these isolates have been characterized. Both isolates showed maximum biomass RFEF-1(2.42gms), RFEF-2(3.03gms) and L-asparaginase production RFEF-1(39.7 IU), RFEF-2(37.86 IU) at 35^{o}C and pH -7.0.

Keywords: Anticancer, L-Asparaginase, antineoplastic agent, Endophytic fungi.

INTRODUCTION

Acute lymphoblastic leukemia is cancer of white blood corpuscles (WBC) characterized by the excessive multiplication of malignant and immature WBC (lymphoblast) in bone marrow [1]. Treatment of acute leukemia includes chemotherapy, steroids, radiation therapy and intensive combined treatments including bone marrow or stem cell transplants. The drugs which are employed for chemotherapy are prednisolone, dexamethasone, vincristine, asparaginase, daunorubicin, cyclophosphamide, cytarabine, etoposide, thioguanine, mercaptopurine, hydrocortisone, methotrexate etc [2]. Although varieties of drugs are available today their efficacy in treatment of cancers at third and fourth stage is doubtful [3]. The side effects caused by these chemotherapeutic agents are many such as infertility, secondary neoplasm, nausea and vomiting, immune suppression etc.

Amino acid degrading enzymes are important chemotherapeutic agents for the cure of certain types of cancers [4]. The manufacture or processing of enzymes for use as drug is an important facet of today's pharmaceutical industry [5]. Prior to the discovery of the antileukemic and antitumor effects of the enzyme L-asparaginase, no qualitative difference in nutritional requirement between cancer and normal cells were known and there were no chemotherapeutic agents which were qualitatively specific for any type of cancer cell [6]. A remarkable achievement in the field of medicine was development of L-asparaginase enzyme as an effective antitumor agent [7]. L-asparaginase is broadly distributed among plants, animals and microorganisms.

Recent times, L-asparaginase emerged as potent health care agent for the treatment of acute lymphocytic leukemia [8-10] because tumor cells cannot synthesize L –asparagine and hence take L-asparagine from blood circulation or body fluid [11]. As a chemotherapeutic agent, L-asparaginase degrades L-asparagine present in blood circulation and indirectly starve tumor cells which led to apoptosis [12]. More over L-asparaginase is biodegradable, non-toxic and can be administered at the local site quite easily. However much attention has not been focused on endophytic fungi isolated from medicinally and nutritionally important plants for their anticancer potential. In present investigation endophytic fungi were isolated from ripen fruit of *capsicum frutescence* and evaluated their anti cancer activities.

MATERIALS AND METHODS

Collection of plant material

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Ripened red fruit of *Capsicum frutescence* var US 341 was collected from field at Bapatla, Guntur district, A.P. India and fresh fruits were used for isolation of endophytic fungi.

Isolation of endophytic fungi

Endophytic fungi were isolated as per the procedure described earlier [13] with modification and the whole process of endophytic fungi is carried out aseptic condition [14]. Samples were washed in running tap water to remove dust and debris, dried in air and then cut into 1 cm segments. Surface sterilization was done with 95% ethanol, 10% sodium hypochlorite, 0.1% $HgCl_2$, HCHO, 20% H_2O_2 respectively as follows. Sample segments were soaked in 95% ethanol for 1 min. and washed in sterile distilled water three times and dried in a laminar air flow chamber [15] segments were soaked 10% sodium hypochlorite solution for 3 min, subsequently washed in sterile distilled water three times and dried in a laminar air flow chamber. Segments were soaked in 0.1% mercuric chloride the samples were subsequently washed in sterile distilled water three times and dried in a laminar air flow chamber. Segments were soaked in formalin for one min and samples were subsequently washed in sterile distilled water three times and dried in a laminar air flow chamber. Segments were soaked in 20% hydrogen peroxide for one min and were subsequently washed in sterile distilled water three times and dried in a laminar air flow chamber. After sterilization fruit tissue is homogenized and homogenized sample is diluted according to the standard serial dilution method and cultured on Sabrouds medium by spread plate method.

Screening of L-asparaginase producers

The isolated fungal colonies were screened for L-asparaginase production on modified Czapek dox medium containing L-asparagine and Phenol red as indicator [16].

L-asparaginase producing colonies were selected on the basis of formation of pink zone around the colonies of the medium. Control plates were maintained with modified Czapek-Dox medium without dye and L-Asparagine.

Extraction of L-asparaginase

L-asparaginase enzyme was extracted from endophytic fungal isolates by following the method of Kil JO [17].Endophytic fungal isolates with diameter 6 mm was inoculated into 250 ml Erlenmeyer flask containing 100 ml of Czapek Dox medium and incubated for 7 days. Broth was filtered through whatmann filter paper (NO.1) and centrifuged at 2000-3000 rpm in Kemi hispeed refrigerated centrifuge, model –KHSRC-1, SI.No: 193 for 15 min. The supernatant was use as crude enzyme extract.

Quantitative estimation of L-asparaginase

Quantitative estimation of L-asparaginase was carried as per Imad with slight modifications [18]. 2 ml of reaction mixture containing 0.5 ml of 0.04 M asparagine, 0.5 ml of crude enzyme extract, 0.5 ml of 0.5 M acetate buffer (p^H5.4) and 0.5 ml of distilled water was incubated for 30 min at room temperature and then the reaction was stopped by addition of 0.5 ml of 1.5 M trichloroacetic acid and centrifuged at 10,000 rpm for 10 mins. After centrifugation 0.1ml of supernatant fluid is diluted to 3.7 ml distilled water and treated with 0.2 ml of Nessler's reagent and 1ml of 2N NaoH was added and incubated for 15 to 20 min at room temperature. The O.D was measured at 450nm by using SL 171 Mini Spec Elico Spectrophotometer. Reaction mixture without L-Asparagine was used as blank. The assay procedure was based on direct Nesslerization of ammonia and ammonia liberated is estimated by detecting the optical density at 425 nm using SL 171 Mini Spec Elico.

The amount of enzymes liberated is calculated by the formula given below [19]. One international unit (IU) of L-asparaginase is the amount of enzyme needed to liberate 1 µmol of ammonia in 1 min at 37°C.

Enzymes (Units/ml) = (µ mole of NH_3 liberated) (2.5) / (0.1) (20) (1)

2.5 = Initial volume of enzyme mixture (ml)
0.1 = Volume of enzyme mixture used in final reaction (ml)
30 = Incubation time (minutes)
1 = Volume of enzyme used (ml)

Statistical analysis

All data were statistically analyzed by using one way analysis of variance (ANOVA)

RESULTS

It is the first time report of isolation of endophytic fungi from red fruit of chilli plant for the evaluation of anticancer activities. 16 endophytic fungal isolates were isolated from red fruit of *Capsicum frutescence* var US 341 growing in chilli fields of Guntur district, A.P and cultured on Sabrouds medium.

Screening of anticancer activity

Sixteen Endophytic fungal isolates were examined for L-Asparaginase production on the basis of pink zones around the colony on modified Czapek dox's medium. RFEF-1, RFEF -2 showed positive result by a prominent pink. Based on the result RFEF-1 and RFEF-2 fungal isolates were screened as potential L-asparaginase producers (fig-1).

Identification of Fungal isolates

Morphological nature of mycelium conidiophores and conidial characters were identified under light microscope .Based on colony morphology RFEF-1 and RFEF-2 were identified as two different fungal isolates and based on morphology of mycelium, nature, structure and arrangement of conidia and conidiospores RFEF-1 and RFEF-2 are identified as *Aspergillus* species (Fig.2 and figure-3).

Quantitative estimation of l-asparaginase

L-asparaginase production was estimated quantitatively in IU and the relationship between production of L-asparaginase (IU) and biomass (gms) was analyzed. Results revealed that the production of L-asparaginase was more after 7 days in both RFEF-1 and RFEF-2 isolates but showed variation in their quantity of L-asparaginase (IU) and biomass (gms) as shown in fig 4. In seven days old culture, biomass of RFEF-2 observed high (2.99gm) when compared to RFEF-1 (2.34gms).Whereas L-asparaginase production was high (39.70 IU) in RFEF-1 when compared to RFEF-2 (37.09 IU).

Optimization of growth parameters

The effect of growth parameters temperature and p^H and their influence on biomass and L-Asparaginase production was studied. Both fungal isolates RFEF-1 and RFEF-2 showed maximum biomass at 35^0C. The results revealed 35^0C was optimum temperature for effective growth of endophytic fungal isolates (fig-5a).

Effect of temperature on Biomass and L-asparaginase production

The effects of temperature at a difference of 5^oC ranging from 30^0C to 40^0C on biomass production and L-asparaginase production of endophytic fungi were presented in fig-5a and 5b. Results revealed that biomass production was high at 35^0C in RFEF-1(2.42 gms) and RFEF-1(3.03 g). The growth was low at 30^0Cand inhibited at 40^0C in both the isolates (5a). Yield of L -asparaginase was high at $35\ ^0$C in both isolates and decreased at $40\ ^0$C (fig-5b). Results revealed that RFEF-2 showed maximum L-asparaginase production at 35^0C (39.70 IU) when compared to RFEF-1 (37.86 IU).

Effect of P^H on Biomass and L-asparaginase production

The effect of pH on biomass production was studied at different pH ranging from 5.0-9 and results were presented (fig 5c). Data indicated that the production was increased significantly with increase in biomass from P^H5.0 to P^H7.0 and then decreased at P^H-8.0 and 9.0 in RFEF-1, RFEF-2. Highest biomass was observer at P^H -7.0 in RFEF-1(2.39gms) and RFEF-2(3.34gms).The effects of initial P^H on L-asparaginase production by endophytic fungal isolates (fig 5-d) indicated that the production is increased significantly with increase in p^H from P^H5.0 to P^H7.0 and then decrease at P^H-8.0 and 9.0 . Highest enzyme production was observed in RFEF-2(45.58 IU) at p^H -7.0 when compared to RFEF-1(35.66 IU).

Fig 1. Screening of L-Asparaginase producers from endophytic Fungal isolates

RFEF-1 **RFEF-2**

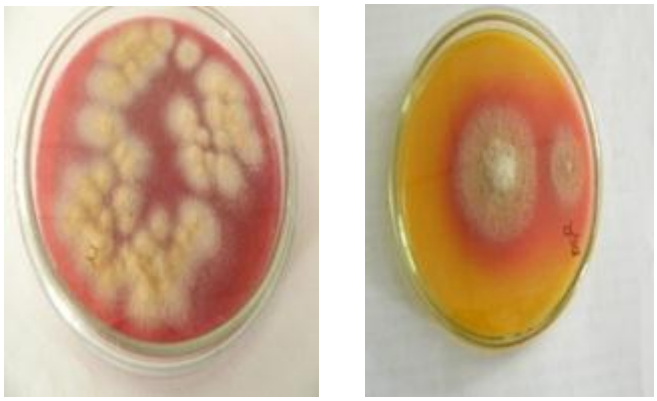
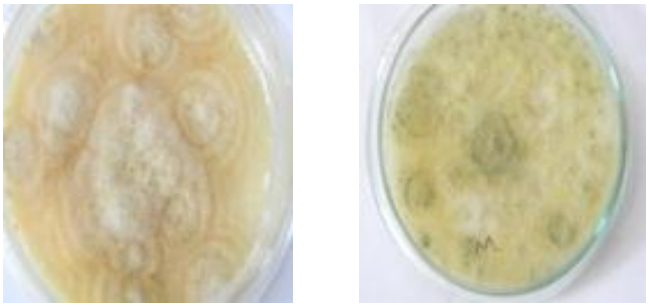


Fig 2. Colony morphology of endophytic fungi isolated from red fruit of *Capsicum frutescence*

RFEF-1 **RFEF-2**



RFEF-1-White Profuse cottony growth with yellow color sporulation

RFEF-2 -White Profuse cottony growth with green color sporulation

Fig 3. Morphological characters of mycelium, conidia and conidiophores of Endophytic fungal isolates observed under Olympus binocular Microscope

Aspergillus RFEF-1 **Aspergillus RFEF-2**

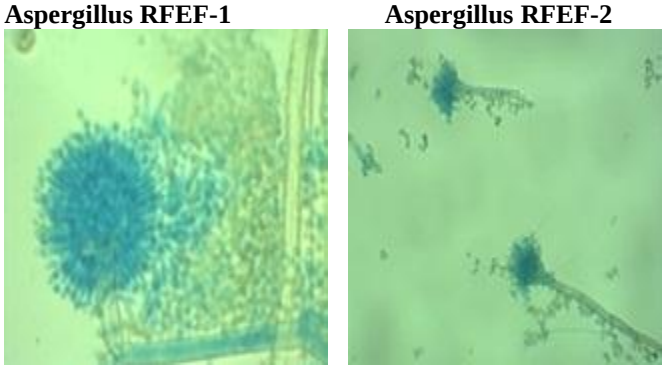


Fig 4. Relationship between L-Asparaginase production and Biomass of Endophytic fungal isolates

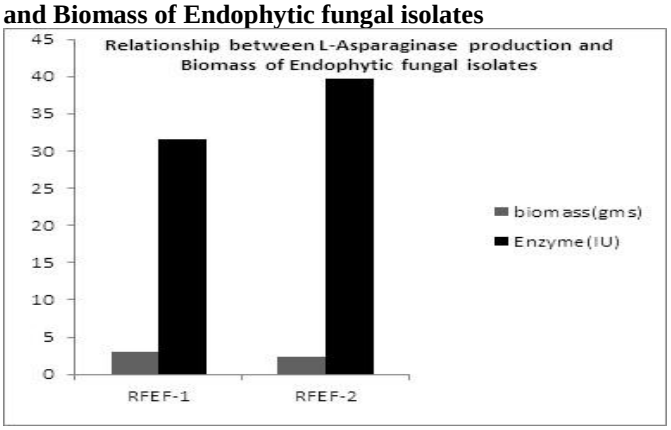


Fig 5. Growth Optimization of endophytic fungal isolates in terms of biomass at varied P^H and Temperature conditions

Figure-5a

Figure-5b

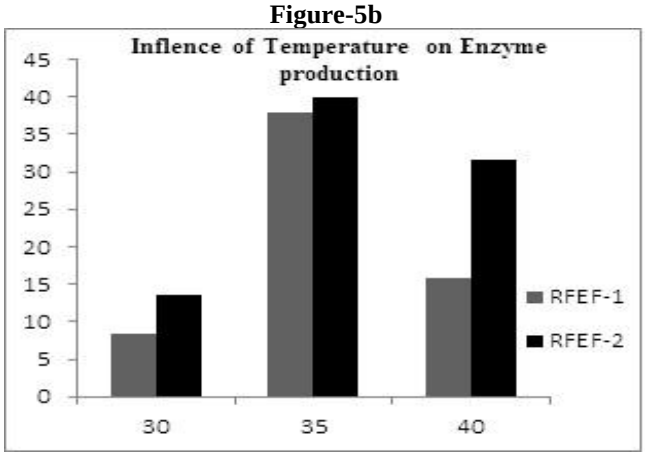


Figure-5c

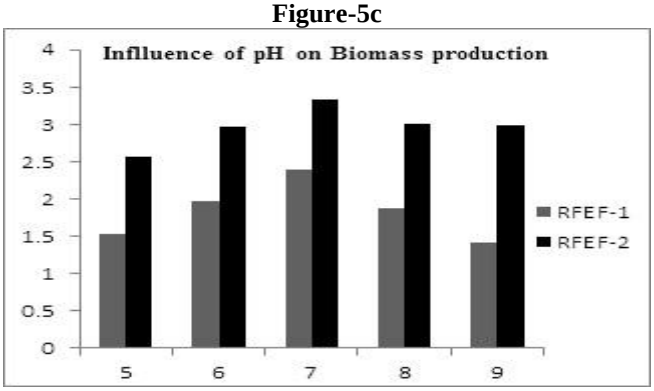
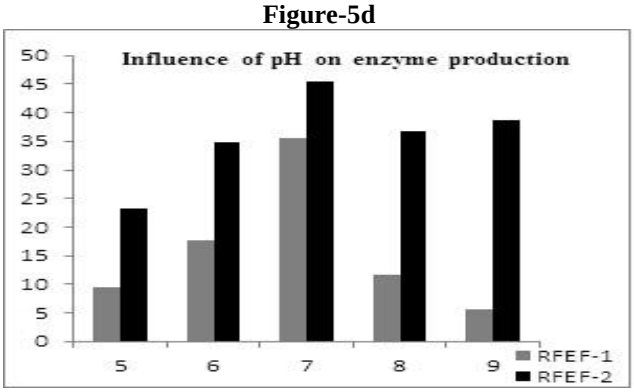


Figure-5d



*Values are mean of 5 replications ± standard deviation

DISCUSSION

Many enzymes have been used as drug likewise L-asparaginase attracted much attention because of its use as effective therapeutic agent against lymphocytic leukemia and other kinds of cancer in man [20].The present study was carried on isolation of endophytic fungi from ripened fruit of Capsicum frutinescence and their potential anticancer and antibacterial activities. 30 % of endophytic fungal isolates were identified as potential L-asparaginase producers. Morphological and microscopic study of mycelia, conidiophores and conidia revealed that the genus Aspergillus was a prominent endophytic fungi in red fruit of chilli. Based on microscopic observation and literature RFEF-2 was identified as *Aspergillus fumigates* [21] and RFEF-1 was unable to identify upto species level. However both isolates were deposited for 18sr RNA Molecular characterization (unpublished).First time an attempt was made to correlate influence of biomass of endophytic fungi over L-asparaginase production and observed that the relationship between biomass and L-asparaginase.

The results revealed that the relation between biomass and L-asparaginase production is highly specific to individual endophytic fungi. In seven days old culture the relationship between biomass production and L-asparaginase production in individual isolate was highly specific. (Fig-3) The relationship of biomass and L-asparaginase production was also collinear.L-aspraginase production of endophytic fungi was not influenced by biomass (fig-3) our results were similar to Siddalingeshwar et al. and Akilandeswari et al. [22,23].

Optimization of growth parameters in terms of temperature and P^H were studied in both species of *Aspergillus* .The effect of temperature on the production of biomass and L-asparaginase are presented in Fig-5a and 5b indicated that 35^0C was suitable for maximum production of biomass and L-asparaginase after seven days of incubation and identified this temperature as optimum range for recovery of maximum enzyme. Temperature beyond optimum temperature (35^0C) was found to have adverse effect on metabolic activity of endophytic fungi. The temperature effect showed variation in L-asparaginase production in individual species. The variation in the yield of L-asparaginase was also observed. Earlier reports also stated that all metabolic activities of endophytic fungi become slow beyond optimum temperature [22,23].

The influence of P^H on the production of biomass and L-asparaginase are presented in Fig-5c and Fig-5d.The variation in optimum P^H was also observed in fungal isolates and may be due to strain specificity. It is clearly indicates that production of L-asparaginase and its optimum p^H mainly depend on the species. Earlier reports revealed that P^H-7.0 was optimum for production of L-asparaginase in *Aspergillus terreus* and P^H 4.5 was found to be suitable *Aspergillus terreus KLSR* strain [24]. Our results also

showed similarity with the earlier reports and also stated RFEF-2 is potential L-asparaginase producer than RFEF-1.

Enzyme supplement are available in the form of pills, capsules and powder. Similarly combination of several different enzymes, John Beard, an English scientist was first to use pancreatic enzyme to treat cancer [25]. Therapeutic enzymes have a broad variety of specific uses as Oncolytic, Thrombolytic and Anticoagulants and as replacement for metabolic deficiencies. Asparaginase is an important enzyme used as an anti-cancer compound for treatment of human cancer and acts as a catalyst in the breakdown of asparagine to aspartic acid and ammonia. Microbial L-asparaginase has been widely used in treatment of cancer mainly acute lymphoblastic leukemia [14], [26]. Normal cells do not require much asparagine to survive when compared to cancer cells because normal cells are able to make required quantity of the asparagine by themselves. Tumor cells, more specifically lymphatic tumor cells, require huge amounts of asparaginase to keep up with their rapid, malignant growth by utilizing asparagine from the diet as well as from its own limited expression .On the other hand tumor cells become depleted rapidly and die if the dietary supply is cut off because they cannot manufacture enough asparagine internally to support their continuing growth L-asparaginase catalyses the conversion of L-asparagine to L-aspartate and ammonia this catalytic reaction is essential irreversible under physiological conditions [23]. Endophytes are the organisms which reside in the internal tissue of the plant without causing any negative effect on plant growth. Adaptations, competition with the host defence mechanism, utilizing the nutrients present in the host internal tissue. However microbes are the best sources of L-asparaginase because they can be cultured easily and the extraction and purification step is also convenient, facilitating the large scale production [27]. Bacterial L-asparaginase could cause an allergic reaction like skin rash, difficulty breathing, decrease blood pressure, sweating or loss of consciousness. The common side effect of this medication is vomiting; in addition it may interfere with blood clotting, raise blood sugar level, raise liver enzyme blood tests and cause liver disease in some patients. L-asparaginase produced by fungal strains is non-toxic and appeared to have myelosuppresive activity [28].

CONCLUSION

Variations in the metabolic activities of various species of endophytic *Aspergillus* isolated from red fruit of chilli helps to evaluate their species specify in the expression of enzyme production in further investigations and also give a scope for scale up the production of anticancer enzyme at optimum growth parameters like p^H and temperature.

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