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Production of L-Asparaginase By *Fusarium Oxysporum* Using Submerged Fermentation

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ABSTRACT: *An attempt is made in the present study to optimize the cultural conditions for the production of L-asparaginase by Fusarium oxysporum using submerged fermentation. Different fungal species were isolated and identified using standard manuals, screened initially for the production of extracellular L-asparaginase during their growth on Czapek's Dox medium containing L- asparagine as sole source of carbon. Fusarium oxysporum was selected for further studies based on the initial screening by plate assay method. F. oxysporum in the present investigation exhibited significant variations in the production of L-asparaginase under the influence of incubation period, temperature, pH, carbon source and nitrogen sources. The enzyme activity recorded was maximum on 5th day of incubation period with an activity of 182.5 U/ml. At pH 5 L-asparaginase production was high (105.0 U/ml). Optimum enzyme activity was recorded at 25°C (123.2 U/ml). The highest amount of enzyme production by F. oxysporum was observed when sucrose was used as the carbon source (170.0 U/ml). Sodium nitrate as nitrogen source was responsible for maximum production of asparaginase with an activity of 185.0 U/ml. With few prospects, a positive association could be observed between growth and L-asparaginase production and the nutrient source employed.*

KEY WORDS: *L-asparaginase, Fusarium oxysporum, Czapek's Dox medium, submerged fermentation*

I. INTRODUCTION

L-asparaginase (L-asparagine amido hydrolase, E.C. 3.5.1.1) belongs to an amidase group that catalyses the conversion of L-asparagine to L-aspartic acid and ammonia [1]. Asparaginase enzyme is broadly distributed among the plants, animals and microorganisms. The chemical name for L-asparaginase enzyme is mono methoxy polyethylene glycol succinimidyl L-asparaginase. L-asparaginase is modified by covalently conjugating unit of mono methoxy polyethylene glycol (PEG), forming the active ingredient PEG-L-asparaginase (derived from *Escherichia coli*).

$HOOCCHNH_2CH_2CONH_2+H_2O \rightarrow HOOCCHNH_2CH_2COOH+NH_3$

L -Asparagine is an amino acid required both by normal cells as well as cancer cells for the production of protein. Normal cells synthesize this amino acid by the catalytic activity of asparagine synthetase from aspartic acid and glutamine. However, neoplastic cells cannot produce L-asparagine due to the low levels of L-asparagine synthetase [2] and they depend on cellular pools of L-asparagine for their growth. Tumor cells, more specifically, lymphatic tumor cells require huge amounts of asparagines for their rapid and malignant growth. This enzyme has been isolated, purified and experimentally studied in detail as an anti - leukemia agent in human patients [3, 4]. The importance of microorganisms as L-asparaginase sources has been focused since the time it was first discovered from *Escherichia coli* and its anti-neoplastic activity demonstrated in guinea pig serum [5]. Since then several research groups have extensively involved in isolation of microbial strains such as *Pseudomonas fluorescens* [6], *Serratia marcescens* [7], *Erwinia carotovora* [8], *Proteus vulgaris* [9], *Saccharomyces cerevisiae, Streptomyces karnatakensis, S. venezuelae*. Eukaryotic microorganisms like yeast and filamentous fungi – *Aspergillus, Penicillium* and *Fusarium* have a potential for the production of L-asparaginase [10] without side effect. Comparative evaluation of L-asparaginase for its potential activity from different microbial sources revealed that biochemical and therapeutic properties differ with source of strain in addition to enzyme production properties. L-asparaginase is useful for cancer treatment as it interferes with growth of cancer cells, by reducing their growth. It also interferes with the protein synthesis and also with DNA and RNA synthesis specifically in G1 phase of cell division [11]. L-asparaginase causes death of tumor cells which are asparagine dependent induces apoptosis [12]. Therefore, a search for new L-asparaginase which is different from that existing has been greatly desired and to attain this it is essential to optimize the production conditions.

In the present study optimization of L- asparaginase production by *F. oxysporum* using submerged fermentation was carried out using various parameters like incubation period, temperature, pH, carbon source and nitrogen source.

II. MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade and media components of high purity grade. The microbiological media used were dehydrated media (Hi-media Mumbai). Production studies were carried out as batch cultures in 100 ml Erlenmeyer flasks, containing 25 ml of culture media. All the experiments were carried out independently in duplicates.

Isolation

The fungal species were isolated by employing dilution plate technique [13]. Soil samples were collected randomly from the college campus and brought to the laboratory in separate polythene bags to avoid contamination. They were serially diluted as desired (10^0 to 10^{-10}), 0.5 ml of these dilutions were inoculated into sterilized petri-dishes. The sterilized and cooled Czapek's Dox medium was poured gently, by making rotational shakings to ensure uniform distribution of inoculum. Petri dishes thus prepared were incubated for 5-7 days at 28 $\pm$ 2ºC. The colonies of each species appearing in a petridish were identified on the basis of morphological, cultural and characteristic reproductive structures by using standard reference manuals [14, 15].

Substrate

L-asparagine purchased from sigma chemicals co.USA was used as substrate for L-asparaginase production.

Screening or Plate assay for L-asparaginase on Czapek's Dox media

All fungal isolates were screened by plate assay method for their abilities to produce asparaginase during their growth on modified Czapek's Dox media containing L- asparagine as the sole source of carbon. A stock solution of 2.5% of phenol red was prepared in ethanol (pH 6.2) and 3 ml of this was added to 1000 ml of Czapek's Dox medium. A mycelial plug of 5 mm is taken from the growing margin of the colony of fungal species was placed in a petridish containing Czapek's Dox media and the inoculated plates were incubated for 3 to 5 days at 28 ± 2ºC. Positive isolates were detected based on the appearance of a pink zone around the fungal colony in yellow colour medium indicating L-asparaginase activity due to the change in colour of phenol red indicator as NH_3 is liberated. The amount of asparaginase produced was quantified under submerged fermentation condition.

Assay of L - asparaginase enzyme

The amount of asparaginase produced was measured by using 1% L-asparagine as substrate. Soluble protein content of the culture supernatant was determined by the method as described by Lowry *et al*. [16] using bovine serum albumin as standard. L-asparaginase activity was measured by the modified method of [17]. The fungus was grown for 5 days at 28 ± 2°C in modified Czapek's Dox medium. A reaction mixture containing, 0.5 ml of sodium borate buffer (pH 8.5), 1 ml of L-asparagine (0.04M) [200mg/100 ml], 1.0 ml of suitably diluted enzyme source (culture filtrate) and 0.4 ml of distilled water (total volume of 2.0 ml) was incubated at 37°C for 30 min. The reaction was terminated by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA). Blank tubes were prepared by adding the enzyme source after the addition of TCA. After termination of the reaction, 3.7 ml volume of distilled water and 0.2 ml of Nessler's reagent were added to 0.1 ml of the above reaction mixture and incubated for 20 min. The amount of ammonia released during the reaction was determined by measuring the absorbance at 500 nm. The amount of asparagine was calculated from a standard graph using Ammonium sulphate. One international unit (IU) of L-asparaginase is the amount of enzyme needed to liberate 1 µmol of ammonia in 1 min at 27ºC [17].

$$\text{Units/ml enzyme} = \frac{(\mu\text{mole of } NH_3 \text{ liberated})\ (2.5)}{(0.1)\ (30)\ (1)}$$

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

Asparaginase production under submerged fermentation

For asparaginase production under submerged condition, Czapek's Dox broth supplemented with 1% L-asparagine was used. For inoculation of 25 ml of culture of broth, 5 discs each of 5mm in diameter were obtained by using sterile cork borer from Czapek's Dox culture plate containing fungal lawn. Inoculated flasks were incubated at 28 ± 2ºC for 7 days. The culture medium was filtered using Whatman no .5 filter paper, the filtrate was centrifuged at 3000 rpm for 10 min. The clear supernatant was used as the crude extra-cellular enzyme source.

Mycelial dry weight

Mycelial biomass was collected on pre-weighed Whatman filter paper no 5, dried to a constant weight at 60ºC and the difference in weight denoted the mycelial growth of fungus.

Organism

Fusarium oxysporum was used for L-asparginase production based on the screening results. It is a potent phytopathogenic fungi. Czapek's Dox broth supplemented with 1% of L-asparagine was employed for production studies. The pH of the medium was adjusted to 6 with the help of 0.1 N HCl before sterilizing the media. The media was autoclaved at 121ºC for 20 min and incubated at 28 ± 2^{o}C after inoculation.

Inoculum preparation

The fungus (*F. oxysporum*) was routinely grown and maintained on Czapek's Dox agar slants. They were subcultured from old culture on to fresh agar slants. Five days old culture of the organism served as inoculum. The culture medium was filtered using Whatman no.5 filter paper, centrifuged at 3000 rpm for 10 min. The clear supernatant was used as the crude extracellular enzyme source.

Optimization of process parameters

The optimization of medium and cultural conditions was carried out based on step wise modification of the governing parameters for asparaginase production. Cultivation of the organism was carried at different incubation periods, temperature and pH. Effect of different carbon and nitrogen sources on asparaginase production was also investigated.

Effect of incubation period

In this experiment the production of asparaginase by *F. oxysporum* was carried along 7 days of incubation. Three sets of Czapek's Dox broth flasks with 1% asparagine were prepared. One flask was harvested after 3rd, 5th and 7th day of the incubation periods and the enzyme activity was determined.

Effect of temperature

Effect of temperature on asparaginase production by *F. oxysporum* was examined at 10ºC, 25ºC and 37ºC. The flasks were incubated for 3, 5 and 7 days and the supernatant was used as crude enzyme to calculate the asparaginase activity and protein content.

Effect of pH

To determine the maximum enzyme production at a particular pH, the medium was adjusted to different ranging from pH (3-7) prior to autoclaving and the organism was inoculated. The flasks were incubated for 3, 5 and 7 days and supernatant was used for determining the asparaginase activity.

Effect of carbon source

To check the effect of different carbon source on asparaginase production-glucose, sucrose, fructose, lactose and citric acid were employed. Different carbon sources were added at a concentration of 1% to the medium and incubated for 3, 5 and 7 days and supernatant was used for determining the enzyme activity and protein content.

Effect of nitrogen source

To know the effect of nitrogen source on asparaginase production, the fermentation broth was supplemented with both organic and inorganic nitrogen sources. The nitrogen sources used are - proline, potassium nitrate, ammonium nitrate, ammonium sulphate and ammonium chloride. The nitrogen source were used at 1% level by replacing the prescribed nitrogen source of the fermentation medium and was incubated for 3, 5 and 7 days and supernatant was used for determining the asparaginase activity and protein content.

III. RESULTS AND DISCUSSION

The present study mainly focused on the production of L-asparaginase enzyme by *Fusarium oxysporum* isolated from soil sample. Increasing reports mentioning the isolation of newer microbial species for asparaginase production reveal an ever increasing interest by the scientific community. In order to deal with industrial wastes, research and development department have worked towards the establishments of strategies that are totally free from the use of hazardous chemicals and provide the same results as are achieved through conventional methods. A treatment with asparaginase does not create any environmental threat and therefore provides a glimpse of hope to environment. Growth conditions regarding the incubation time, temperature, pH of the medium, effect of media, carbon source and nitrogen source were optimized for the maximum enzyme production. The results for the above parameters are discussed. The fungal strains were stained by using lactophenol wet mount stain. They were identified on the basis of morphological, cultural and characteristic reproductive structures by using standard reference manuals [14, 15]. Five fungal species were identified – *Penicillium citrinum, Aspergillus ochraceus, F. oxysporum, Alternaria alternata* and *Rhizopus sps.*

Screening for L-asparaginase activity on Czapek's Dox media medium

The primary screening for the asparaginase activity of all the fungal isolates was determined by taking the inoculum, which was seeded onto the Czapek's Dox medium. After 5 days of incubation, the plates were observed for the growth of the organism as well as the appearance of a pink zone around the fungal colony. The results are depicted in the Table 1**.** *F.oxysporum* showed the maximum growth (3.5 cm) with zone diameter (pink) of 2.4 cm followed by *A. alternaria* (3.3 cm) with zone of 1.8 cm was observed. *Rhizopus sps* could grow on the media with colony diameter of 3.6 cm but it did not show the pink zone around the colony (Fig 1). Quantitative asparaginase was also done. Overall, maximum asparaginase production was recorded by *F.oxysporum* (165 U/ml) followed by *A. ochraceus* with an enzyme activity of 107 U/ml. On the other hand minimum activity was recorded in *Penicillium* and *Rhizopus* with enzyme activities of 90 U/ml and 92.0 U/ml respectively. Enzyme production in microbes is associated with the growth phase [18]. So to confirm this mycelial dry weight of all the isolates was determined. In the present study the results depicted that the mycelial dry weight for all the fungal isolates contribute much for enzyme production.

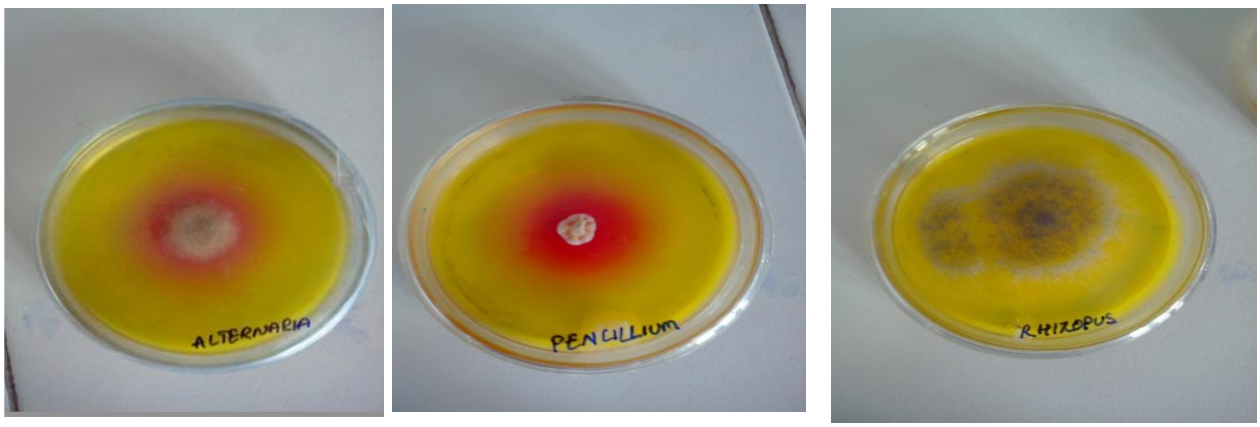
Table 1: Screening /Plate assay method

Name of the Organism	Colony diameter (Cm)	Zone diameter (Cm)
Aspergillus ochraceus	1.4	1.3
Penicillium citrinum	0.7	2.1
Fusarium oxysporum	3.5	2.4
Alternaria alternata	3.3	1.8
Rhizopus sps	3.6	-

--No zone

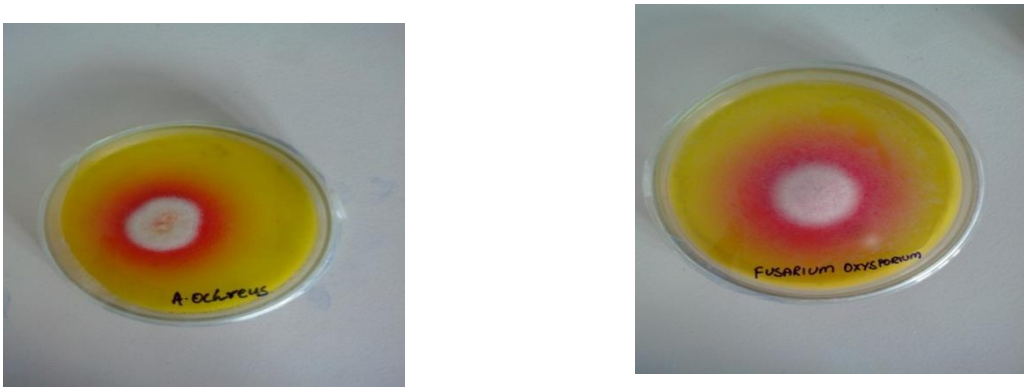
Fig 1: L- asparaginase activity detected by plate assay method [(A). *Alternaria alternata;* (B).*Fusarium oxysporum;* (C). *Rhizopus*; (D). *Penicillium citrinum;* (E). *A.ochraceus*

(A) (B) (C)



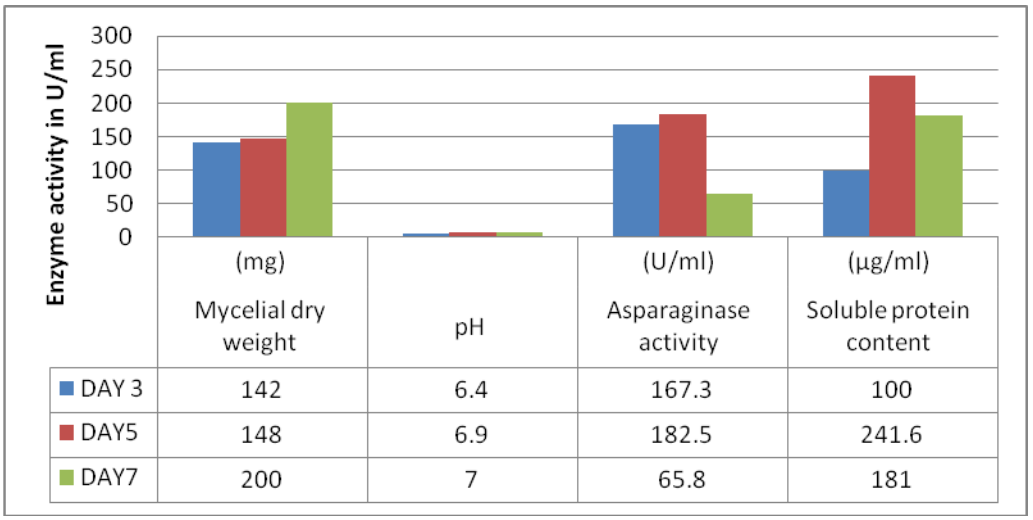
Effect of incubation period

(D) (E)



In this experiment, the production of asparaginase by *F. oxysporum* was studied along 7 days of incubation when cultivated on Czapek's Dox medium with 1% L-asparagine. The asparaginase activity was determined after 3, 5 and 7 days of incubation in order to determine the optimum incubation period for maximum production of asparaginase. Fig.2 reveals that the enzyme production was maximum at 5th day of incubation period (182.5 U/ml). A significant decrease of asparaginase activity was seen after 5th day of incubation. The maximum production of asparaginase was on 5th day of incubation period which is different when compared with other studies on different substrates. Similarly Radhika *et al.* (2012) have also reported the effect of incubation on asparaginase production by *Fusarium moniliforme* under solid state fermentation and suggested that maximum enzyme production depends on the nature of substrate, organism, additive nutrients and many other cultural conditions.

Fig 2: Effect of incubation period on L-asparaginase production by *F.oxysporum*



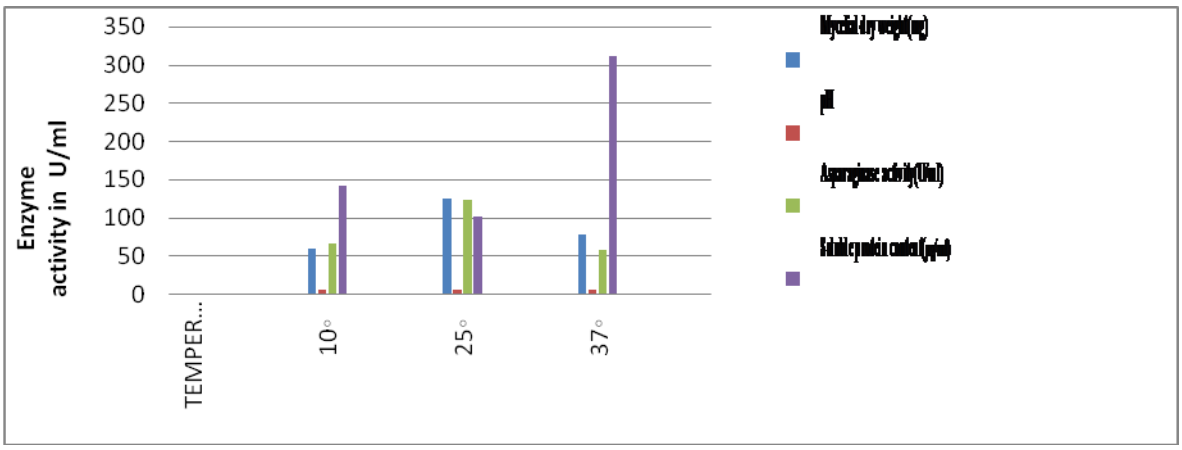
	(mg) Mycelial dry weight	pH	(U/ml) Asparaginase activity	(µg/ml) Soluble protein content
DAY 3	142	6.4	167.3	100
DAY5	148	6.9	182.5	241.6
DAY7	200	7	65.8	181

Effect of temperature

All forms of the life are greatly influenced by temperature. In fact the microorganisms are very sensitive to temperature since their temperature requirement varies with that of environment. Temperature influences the rate of the chemical reaction and protein structure integrity thus affecting rate of enzymatic activity. At low temperature enzymes are not denatured, therefore, every 10°C rise in temperature results in rise of metabolic activity and growth of microorganisms. However, the enzymes have a range of thermal stability and beyond it, their denaturation takes place. Thus, high temperature kills the microorganism. Each microorganism shows characteristic temperature dependence and possesses its own cardinal temperature i.e. minimal, maximum and optimal growth temperatures. The rate of enzymatic reaction is strongly influenced by temperature. As the temperature is raised, the reaction rate increases until the enzymes reach their maximum temperature, following these enzymes undergo conformational alteration resulting in a decrease or complete loss of activity caused by the increasing temperature. The production of asparaginase was maximum at temperature near to ambient temperature (25°C) with an activity of 123.2 U/ml and the mycelial weight was also the maximum (Fig 3).

A lower enzyme activity was obtained with cultivation temperatures lower and higher than the optimum temperature. At 10°C and 37°C activity of xylanase was 143.0 U/ml and 212.0U/ml respectively. The results obtained clearly indicate that the enzyme production corresponds closely to growth of the fungus and optimum temperature for asparaginase production is similar to the ambient temperature for the growth of the fungus. The result obtained was in agreement with Elizabeth *et al.* [20] who reported L-asparaginase production by *Fusarium solani*.

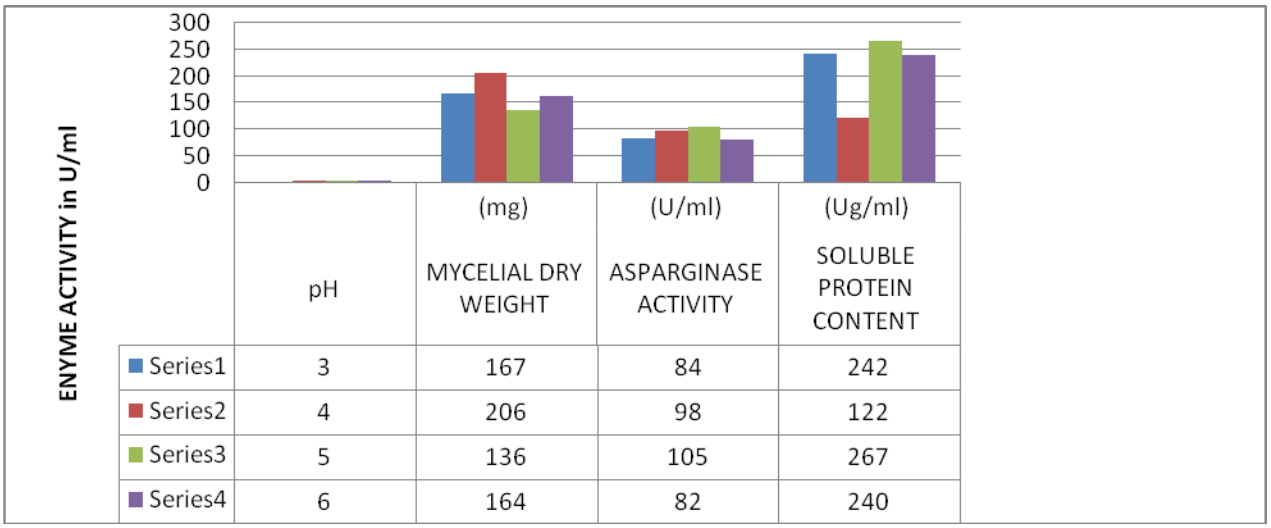
Fig 3: Effect of different temperatures on L-asparaginase production by *F.oxysporum*



Effect of pH

The activity of microbial enzymes depends on the change present on the surface of the amino acids. Any change in the environmental pH may either enhance the enzyme activity or inhibit its activity. Thus pH can dramatically affect the growth of microorganisms. Each species of microorganisms shows specific pH range for its growth. The initial pH influences enzymatic system and the transport of enzymes across cell membrane [21]. The charge of the amino acids varies with the pH value of the environment according to their dissociation constant. A change in pH value might affect the charges and configuration of the active site and thereby change the activity, structural stability or solubility of the enzymes. The highest enzyme activity was observed at pH 5.0 with an activity of 105.0 U/ml and the growth of the fungus was also maximum at the same pH value. However, asparaginase activity decreased at low pH (3 and 6) and high pH range (above pH 7-8).This might be due to the fact that acidic and alkaline pH has inhibitory effect on the growth of *F.oxysporum* and enzyme production (Fig 4). However, Thirunavukkarasu *et al*. [22] who worked on the effect of pH on asparaginase reported pH of 6.2 as optimum for asparaginase production by *Fusarium sp.*

Fig 4: Effect of pH on L -asparaginase activity

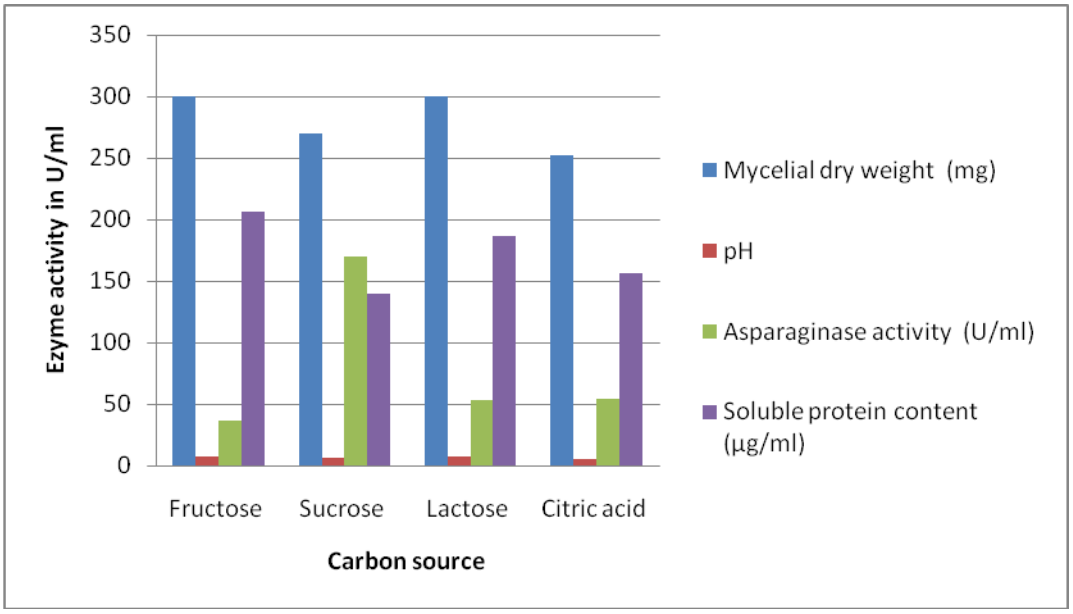


	pH	(mg) MYCELIAL DRY WEIGHT	(U/ml) ASPARGINASE ACTIVITY	(Ug/ml) SOLUBLE PROTEIN CONTENT
Series1	3	167	84	242
Series2	4	206	98	122
Series3	5	136	105	267
Series4	6	164	82	240

Effect of supplemented carbon source

Carbon is the major structural and functional component of microbial cells and plays an important role in the nutrition in fungi. Carbon source is required for all biosynthesis leading to reproduction, product formation and cell maintenance. Production of primary metabolite by microorganisms is highly influenced by their growth, which is determined by the availability of the nutrients in the substrates. Many fungal species are able to thrive on different kinds of aliphatic hydrocarbons. Thus fungi are capable of using wide variety of carbon compounds but most of the fungi prefer simple sugars.A perusal (Fig.5) indicates that *F. oxysporum* could produce maximum asparaginase when sucrose was used as the carbon source (170.0 U/ml), followed by glucose (70.9 U/ml) when compared to the control medium. Citric acid and lactose when supplemented as carbon sources supported only intermediate activity. Fructose was found to be poor substrate as it showed very less enzyme activity. Thus from the results obtained it is clear that there is no positive correlation between the vegetative growth and enzyme production. For instance fructose, sucrose and glucose were good in inducing the production of enzyme but showed less mycelial growth when compared to the other carbon sources understudy**.** Thus it is evident from the present study that Sucrose was an effective inducer and carbon source for asparaginase production by *Fusarium oxysporum.* In contrast to this observation Radhika *et al.* [19] reported highest amount of asparaginase production in the presence of glucose as carbon source while sucrose supported only minimum activity by *Fusarium sps*.

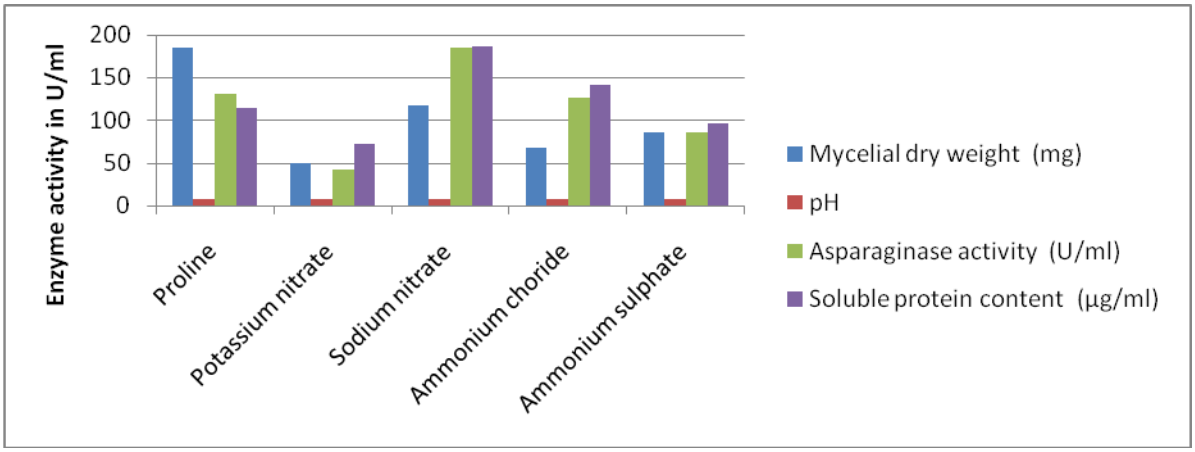
Fig 5: Effect of different carbon sources on L -asparaginase production by *F.oxysporum*



Effect of supplemented nitrogen source

The effect of supplementation of different inorganic and organic nitrogen sources on asparaginase production was evaluated. Fungi are reported to exhibit great specificity for nitrogen source present in the medium. Like carbon source, nitrogen is also used both for functional and structural purposes by fungi. The source of nitrogen has a profound influence on the metabolism of microorganisms. Literature is depleted with conflicting claims regarding the comparative superiority of a particular form or source of nitrogen over the other.The results (Fig.6) obtained reveals that the enzyme production was high in sodium nitrate (185.0 U/ml) containing broth when compared to the nitrogen sources studied. Potassium nitrate and ammonium sulphate were poor substrates as they showed very low enzyme activity but supported good mycelial growth of the fungus. Thus the results obtained also did not show any significant difference between organic and inorganic nitrogen source on the production of asparaginase by *F.oxyporum*. Radhika *et al.* [19] also reported sodium nitrate as the best nitrogen source for the production of asparaginase by *F. oxyporum*. However, Hosamani and Kaliwal [23] on the other hand reported ammonium sulphate as the best nitrogen source for inducing asparaginase production by *F.equiseti*.

Fig 6: Effect of different nitrogen sources on L - asparaginase production by *F.oxysporum*



IV. CONCLUSION:

The present investigation has revealed that carbon and nitrogen sources are very essential for producing maximum levels of L-asparaginase enzyme. Proline recorded highest mycelia weight as nitrogen source but it did not contribute much for the production of enzyme which suggests that it enhances the growth of fungus understudy. Based on the results obtained it is clear that mycelial dry weight did not contribute to the enzyme activity.The production of L-asparaginase, an enzyme widely used in cancer chemotherapy. L-asparaginase having anti-tumor activity can be employed for economic and industrial scale production. Modifications of the enzyme molecule such as PEG-L-asparaginase may prove to be more effective than the native enzyme. Therefore in detail study of the fungal asparaginase has to be carried for its use as an anti-tumor agent.

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