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Full Length Research Paper

Nutritional factors effecting the production of L-asparaginase by the *Fusarium* sp.

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The present work was directed to develop evaluation of nutritional requirements (carbon and nitrogen source) for the production of L-asparaginase by isolated *Fusarium* sp. The study was aimed at ascertaining optimal nutritional conditions for maximal enzyme production by submerged fermentation process. *Fusarium moniliforme, Fusarium oxysporum* and *Fusarium semitectum* were isolated from the soil samples of Warangal. *Fusarium* sp. used in the present investigation exhibited significant variations in their preference to carbon and nitrogen sources for their growth and L-asparaginase production. The highest amount of enzyme production by *F. semitectum* (328 IU/ml), *F. moniliforme* (300 IU/ml) and *F. oxysporum* (210 IU/ml) was obtained with glucose as carbon source, with lactose presenting the second best carbon source for *F. semitectum* (218 IU/ml) and *F. oxysporum* (178 IU/ml) and mannose for *F. moniliforme* (213 IU/ml). *Fusarium* sp. has shown great specificity for nitrogen source present in the medium while they failed to grow in the absence of nitrogen source. *F. moniliforme* (376 IU/ml) and *F. semitectum* (404 IU/ml) exhibited maximum growth and L-asparaginase production on proline. It can be seen that the enzyme production by *F. oxysporium* was higher in sodium nitrate as nitrogen source (360 IU/ml). Lysine was responsible for least production of L-asparaginase in all the three *Fuasrium* sp. The highest amount of biomass was obtained with proline as nitrogen source. With few exceptions, a positive correlation could be observed between growth and L-asparaginase production.

Key words: *Fusarium*, L-asparaginase, nutritional factors, submerged fermentation.

INTRODUCTION

L-Asparaginase (EC 3.5.1.1) is a tetrameric protein (David, 2005) belonging to oncolytic enzymes (Verma et al., 2007). It catalyses the hydrolytic deamination of asparagine to yield aspartic acid and ammonium ion (Dominika and Jaskolski, 2001). With the increasing misuse of L-asparaginase, the serious problem of hypersensitivity is arising very fast with the bacterial L-asparaginase. Therefore, intensive search for new L-asparaginase without side effects is going world wide (Sarquis et al., 2004). Eukaryotic microorganisms like yeast and filamentous fungi such as *Aspergillus, Penicillium* and *Fusarium*, have a potential for L-asparaginase production (Pinheiro et al., 2001) without side effects. L-asparaginases are known chemotherapeutic agents against cancer such as acute lymphoblastic leukemia and lymphosarcoma (Narta et al., 2007; Verma et al., 2007). L-asparaginase is used for cancer medication that interferes with the growth of cancer cells, slow their growth and spread in the body. L-asparaginase interferes with the protein synthesis (Stams et al., 2005) and also with DNA and RNA synthesis, and appears to be cell cycle specific for the G1 phase of cell division. L-asparaginase exhibits potent antineoplastic and antilymphomatic activity against tumors (Sahu et al., 2007). This enzyme causes selective death of asparagine dependent tumor cells and also induces apoptosis in tumor cells (Kelo et al., 2009). Although, L-asparaginase inhibits tumor cell growth, studies of this phenomenon have been limited, usually because sufficient quantity of the enzyme was not available. So, there is a need to

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make the production of L-asparaginase feasible, for this purpose, it is necessary to develop the optimum production conditions. Several researchers have contributed considerably in this field (Sarquis et al., 2004; Dhevagi and Poorani, 2005; Prakasham et al., 2005; Shah et al., 2010). The strains *Fusarium moniliforme*, *Fusarium oxysporum* and *Fusarium semitectum* isolated from the soil samples of Warangal, were found to produce L-asparaginase. In the research presented here, the main objective was to determine how carbon and nitrogen sources could be manipulated to enhance the L-asparaginase production by *Fusarium* sp.

MATERIALS AND METHODS

All the glass wares used are of borosil type. They were thoroughly cleaned and rinsed with distilled water before use. L-asparagine, and all other chemicals were of analytical grade and all the media ingredients were purchased from Himedia (Mumbai).

To find a more effective system for the production of L-asparaginase enzyme, soil samples from agricultural fields were brought into the laboratory and by using dilution plate method, fungi were isolated and screened for L-asparaginase production. A total of 16 strains of filamentous fungi, encompassing 13 different species, were tested for their ability to produce L-asparaginase. Among 16 species of *Fusarium,* three produced maximum amount of L-asparaginase in test condition. For the purpose of obtaining L-asparaginase in large quantities, we selected *F. moniliforme*, *F. oxysporum* and *F. semitectum* as the test organisms. By using these isolated organisms, we investigated the cell growth and enzyme production in the presence of different carbon and nitrogen sources at the Department of Biotechnology, Kakatiya University in the month of March, 2007.

Production of conidial suspension in submerged fermentation

F. moniliforme, F. oxysporum and *F. semitectum* fungi were grown in 50 ml of sterilized Asthana Hawkers medium taken in 250 ml of Erlenmeyer conical flask and incubated at selected temperature (27±2°C) in orbital shaking incubator set at 120 rpm for 16 days. Influence of carbon and nitrogen source on growth and production of L-asparaginase was investigated by substituting glucose and KNO_3 of the basal medium by different carbon (lactose, sucrose, fructose, glucose, sorbitol, mannitol, galactose, mannose and citric acid) and nitrogen (potassium nitrate, sodium nitrate, urea, thiourea, lysine, L-alanine, L-asparagine and proline) source so as to supply same amount of carbon or nitrogen.

L-Asparaginase assay

L-asparaginase activity was assayed by the method of Gulati et al. (1997). At the end of incubation period, the flasks were filtered through filter paper and culture filtrate was used for the L-asparaginase assay. To 0.1 ml of enzyme (culture filtrate), 0.9 ml of sodium borate buffer (pH 8.5) and 1 ml of L-asparagine (0.04 M) were added and incubated for 10 min at 37°C. To this, 0.5 ml of 15% tricarboxylic acid (TCA) was added and centrifuged. Following centrifugation, 0.1 ml of the supernatant was taken and to this, 8 ml of distilled water, 1.0 ml of Nessler's reagent and 1.0 ml of 2 M NaOH were added and allowed to stand for 15 min. Absorbance was read at 500 nm. The amount of L-asparaginase was calculated from a standard graph. One unit of the L-asparaginase (IU) is defined as the amount of enzyme that is capable of producing 1 µmol of ammonia per minute at 37°C.

RESULTS

The main objective of this work was to evaluate the effect of different carbon and nitrogen sources on the biosynthesis of L-asparaginase by *Fusarium* sp. The studies on nutrient requirements of *Fusarium* sp. conducted in our laboratory have shown that carbon and nitrogen were essential for cell growth as well as L-asparaginase biosynthesis. Though, all the three *Fusarium* strains under study secreted L-asparaginase, degree of production varied with the organism and the carbon and nitrogen sources present in the medium. Nine different carbon sources and eight different nitrogen sources were added to the production media at a concentration of 3.5% (w/v). There was a high degree of variation in the level of L-asparaginase activity when different carbon and nitrogen sources were tested in the medium. The L-asparaginase production of *Fusarium* sp. was greatly influenced by addition of glucose, reaching the highest enzyme activity, followed by lactose, citric acid, mannose, sucrose and fructose, whereas sorbital had repressed the production of the L-asparaginase (Figure 1). The highest amount of enzyme production by *F. semitectum* was obtained with glucose (328 IU/ml) as carbon source, while lactose (218 IU/ml) was the second best carbon source. When citric acid, mannose, galactose, mannitol, sucrose, fructose and sorbitol were used as carbon sources for *F. semitectum*, corresponding yields of L-asparaginase were in descending order. The L-asparaginase produced by *F. oxysporum* was highly induced by glucose (210 IU/ml), while lactose, citric acid, mannose, fructose, sucrose, galactose, mannitol and sorbital induced L-asparaginase in a descending order. *F. moniliforme* produced highest (300 IU/ml) amount of L-asparaginase with glucose, while mannose, lactose, citric acid, sucrose, galactose, fructose, mannitol and sorbitol were induced in a descending order (Figure 1). The highest yields of enzyme were obtained with simple sugars as carbon sources. Polysaccharides or oligosaccharides are found to be the next better carbon sources for L-asparaginase production. All the three strains attained more growth on glucose and lactose than other sugars. Sucrose, mannitol and sorbitol, though supported growth of fungus, failed to produce increased amounts of L-asparaginase. Fructose, citric acid and mannitol could support the biosynthesis of L-asparaginase by *Fusarium* sp. to a significant degree. Sucrose and mannose were responsible for limited production of L-asparaginase. The rest of the carbon sources were responsible for intermediate degree of L-asparaginase production. On the other hand, sorbitol was a poor carbon source for the growth and production of L-asparaginase by *Fusarium* sp.

The effect of changing the nitrogen source on L-asparaginase production by *Fusarium* sp. is summarized

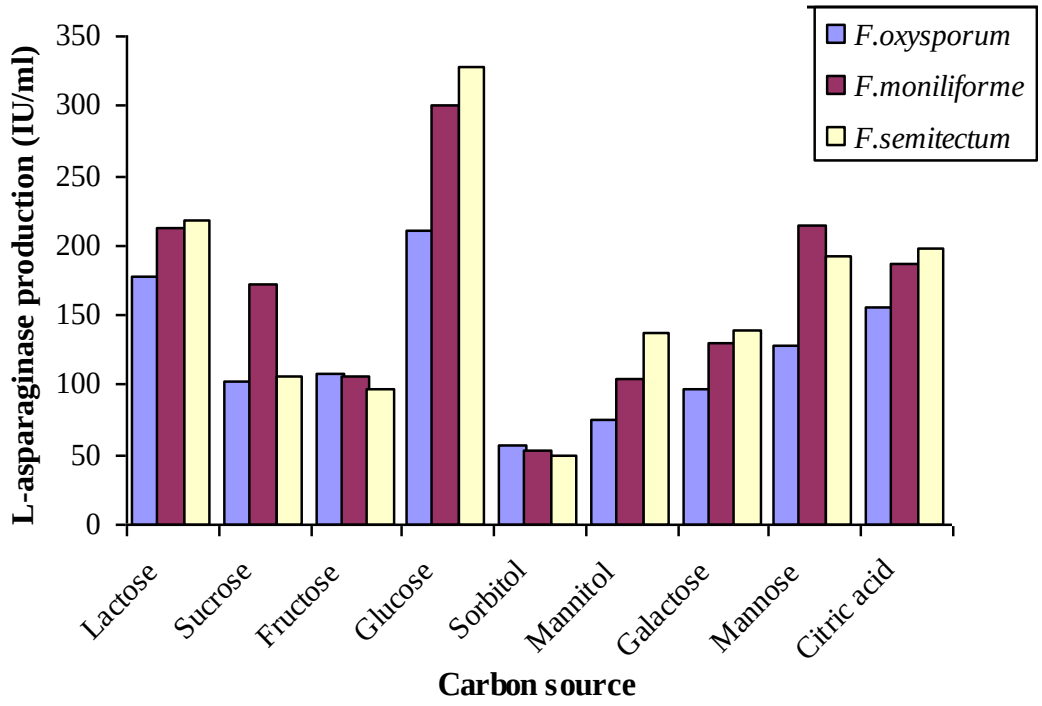

Figure 1. Effect of various carbon sources on L-asparaginase production by *Fusarium* sp.

in Figure 2. It is evident that the type of nitrogen source present in the medium exerted significant influence on biosynthesis of L-asparaginase by *Fusarium* sp. It can be seen that the enzyme production by *F. semitectum* (404 IU/ml) and *F. moniliforme* (376 IU/ml) was higher with proline as nitrogen source. Sodium nitrate as the next best nitrogen source had maximum production of L-asparaginase by *F. oxysporum* (360 IU/ml). *Fusarium* sp. has shown great specificity for nitrogen sources. *Fusarium* sp. failed to grow in the absence of nitrogen source. Lysine was responsible for least production of L-asparaginase. Higher biomass production occurred upon supplementation with a nitrogen source in the proline form. Biomass accumulation was lower only in the presence of the thiourea. Higher growth and higher enzyme activities were detected in the media supplemented with proline, sodium nitrate and L-asparagine. Urea and thiourea induced a lower production of biomass, and a different level of L-asparaginase enzyme secretion: high in urea and low in thiourea was observed in *F. oxysporum* and *F. moniliforme*, but this was opposite in *F. semitectum*. The results described in this work indicate that the metabolism of fungi is regulated by the level of structural complexity of the nitrogen source in correlation to the carbon source.

DISCUSSION

The production of L-asparaginase, an enzyme widely used in cancer chemotherapy, is mainly regulated by carbon and nitrogen sources. This study was carried out to understand how different carbon and nitrogen sources affect the production of this enzyme in *Fusarium* sp. All the three strains grown with various carbon and nitrogen sources showed a distinct level of the enzyme production.

An objective of the present investigation was to avoid the potential side effects of an enzyme L-asparaginase, obtained from bacterial origin. Another objective of the present investigation is to provide an improved process for getting good yield of L-asparaginase having anti-tumor activity, which can be employed for an economic and industrial scale production. Thus, the present investigation has revealed that carbon and nitrogen sources are very essential for producing maximum levels of L-asparaginase enzyme.

The development of nutritive conditions which would support unusually high populations of *Fusarium* cells may be important for obtaining maximal quantities of L-asparaginase. In this study, the enzyme content was expressed as IU/ml of the medium rather than a dry weight basis for the following reasons - The aim of the study was to determine the effect of changes in the carbon and nitrogen source of media on the total enzyme production and the production of the enzyme was not always directly proportional to the biomass.

The L-asparaginase production of fungi has been described in the literature (Elizabeth et al., 1991; Sarquis et al., 2004). The experiments were carried out with diffe-

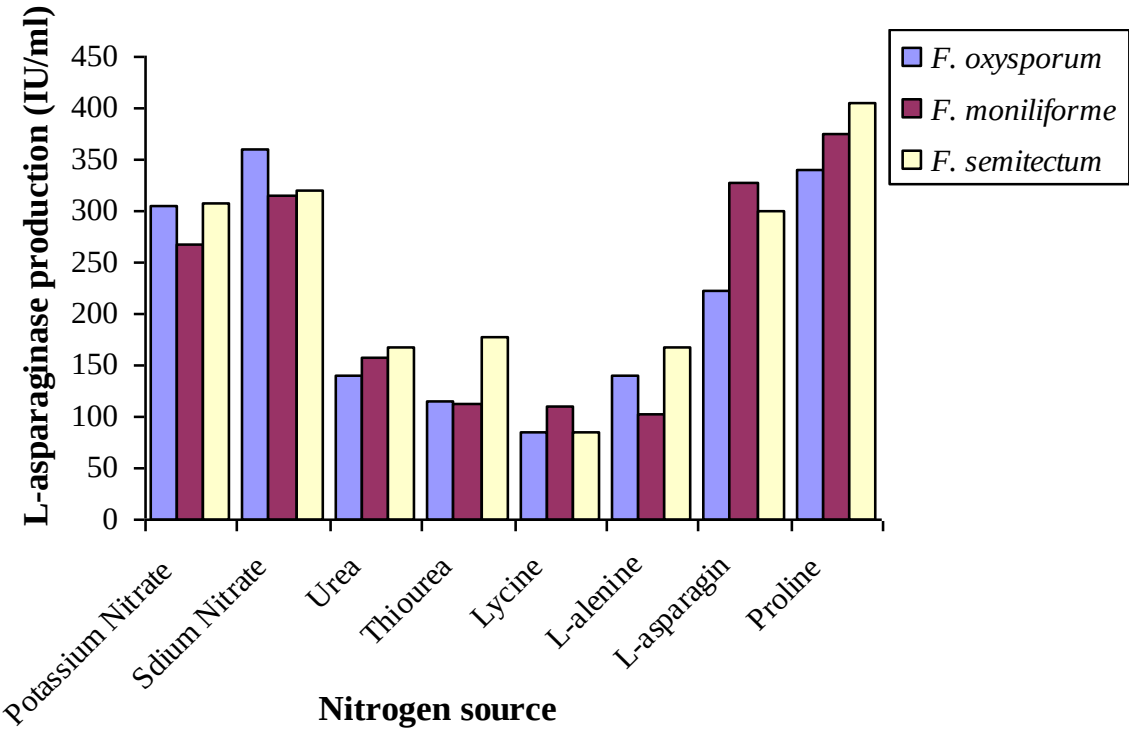

Figure 2. Effect of various nitrogen sources on L-asparaginase production by *Fusarium* sp.

rent carbon and nitrogen sources and corresponding data are shown in Figures 1 and 2. The results suggest that L-asparaginase production is regulated by carbon and nitrogen sources. For effective utilization of any microbial system at bioprocess level, it is essential to screen and evaluate various nutritional requirements for microbial growth and subsequent biocatalyst production (Sreenivas et al., 2004; Prakasham et al., 2005), as culture conditions that promote optimum enzyme production differ significantly with the molecular nature of micro-organism (Prakasham et al., 2007).

Glucose, which is a primary choice of carbon source of many microorganisms, was also favourable for bio-synthesis of L-asparaginase by *Fusarium* sp. It was observed that the enzyme synthesis was stimulated by the addition of glucose in the medium. Sukumaran et al. (1979) reported the same results in *Serratia marcescens* mutants. According to Sukumaran et al. (1979), glucose and sucrose were the best carbon sources for L-asparaginase production by two mutants. The results in the present study confirm their findings. Dhevagi and Poorani, (2005) found that simple sugar glucose as carbon source was useful for the production of good yield of L-asparaginase. It is clear that out of nine carbon sources used, glucose was the best followed by sucrose, fructose and citric acid with a few exceptions. A positive correlation could be observed between growth and L-asparaginase production. Heinemann and Howard (1969) reported in *S. marcescens*, that addition of glucose to the medium resulted in substantial reduction in the quantity of L-asparaginase produced. In contrast to the above findings, we found maximum L-asparaginase production by addition of glucose to the medium. The present study reveals that sorbitol was a poor carbon source for the growth and production of L-asparaginase by *Fusarium* sp. The same was observed by Sukumaran et al. (1979), using *S. marcescens* mutant 933. Sarquis et al. (2004) reported that *Aspergillus terreu* strain in proline medium showed the highest enzymatic activity. Similarly, in our study by using proline as nitrogen source, maximum production of L-asparaginase by *Fusarium* sp. was recorded. On the other hand, lysine was responsible for least formation of L-asparaginase.

In conclusion, our results reveal that glucose and proline were the best carbon and nitrogen sources for L-asparaginase production by *Fusarium* sp.

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