1. INTRODUCTION

Cancer is a group of diseases that impose a heavy burden on the public health and pose a challenge to science. While the century-long trend of increasing cancer mortality in this country was reversed in the mid-1990s, cancer remains the second leading cause of death, the toll on human suffering is profound, and its economic cost to society are substantial. Furthermore, cancer presents an intellectually complex set of problems because of multiple sites and causation, inadequately understood biology, and myriad intervention strategies (Robert and Breen, 2008). There were an estimated 12.7 million cancer cases around the world in 2008, out of these 6.6 million cases were in men and 6.0 million in women. This number is expected to increase to 21 million by 2030. The top three lungs, prostate and colorectal cancers, contributes about 40 % of all cancers (excluding non-melanoma skin cancer) where as breast cancer is the most common cancer worldwide in women contributing nearly 23 % of the total number of new cases diagnosed in 2008. WHO has estimated that 92 % of oral cancers in South-East Asia are directly attributed to the use of tobacco and this is the leading cause of oral cavity and lung cancer in India (IAEA Report, 2010). Other common cancers contributing more than 5 % are lung and stomach (GLOBOCAN 2008 database (version 1.2) http://globocan.iarc.fr). The American Cancer Society (2011) estimated that about 171,600 cancer deaths were caused by tobacco use alone. In addition, approximately one-third of the 572,210 cancer deaths occurred in 2011 are attributed to poor nutrition, physical inactivity, overweight, and obesity. The most important defining feature of cancer is the rapid growth of abnormal cells, which can invade adjoining parts of the body and spreads to other organs. Several factors operate to bring about carcinogenesis.
These are genetic, hormonal, metabolic, physical, chemical and other environmental factors (Cameron and Pool, 1981). Cancer rates are predicted to further increase if nothing changes, mainly due to steadily ageing populations in both developed and developing countries and current trends in smoking prevalence and the growing adoption of unhealthy lifestyles. It is estimated that almost half of cancer cases can be prevented by infection control, adoption of a healthy lifestyle (diet and exercise) and tobacco abstinence. Dietary causes amount 35% of annual cancer deaths (Bruce et al., 1997). Several viruses have been implicated in many cancers (Gross, 1970). Ionizing radiations, X-rays etc. are the physical agents that cause cancer (Beremblum, 1974). Tobacco is recognized as the most important human carcinogen, causing between 25 and 30 per cent of all cancers in developed countries (Peto et al., 1992). During cell division, malignant mutants may be formed which can proliferate to form tumors (Bermblum, 1974). Most of the tumors are arising from genetically altered cell (Hesten, 1974).

There are number of efforts to develop therapies for treating cancer. One approach is the prevention of formation of new blood vessels i.e., angiogenesis (Folkman and Engl, 1971). This has made to discover pro-and anti-angiogenic molecules, some of which are already in clinical trials. The mechanism of action of these molecules is now a beginning to be elucidated to treat cancer and other diseases (WHO Report, 2002). It has been reported that in the beginning many cells capable of forming undetectable tumors due to lack of nutrients and oxygen to the rapidly dividing tumor cells. These cellular foci continue to divide but simply replace the cells lost due to lack of nutrients, thus never increasing in size (Syn et al., 1997). Later these “microtumors” gain the ability to induce neovascularization in the surrounding tissue. The angiogenesis contributed significantly
to both the growth of tumors and their metastasis, inhibitors of this process have a significant clinical potential (Folonum and Ingher, 1992; Brem et al., 1993; Kim et al., 1993; Fiddler and Ellis, 1994; Auerbach and Auerbach, 1994). Therefore, inhibition of angiogenesis is the main area for the treatment of cancer and rheumatoid arthritis (Folkman, 1990). Hence much research has been focused on the design of novel chemical compounds or naturally occurring compounds that may inhibit angiogenesis (Griffith, et al., 1997; Sin et al., 1997; Eunyoung et al., 2005).

Documentation of the Ayurvedic system recorded in Sushruta and Charaka dates from about 1000 BC (Kappor, 1990). Over 2000 years ago ancient Chinese used to treat cancer from two derivatives of podophyllotoxin which are now being used in the treatment of human malignancies. The Greeks also contributed substantially to the rational development of the herbal drugs. In recent years surgery, radiotherapy and cytotoxic chemotherapy have been the mainstays of cancer treatment for many years and continue to be very important despite the introduction of novel approaches still chemotherapy is employed as the first line of treatment. The effectiveness of the drugs is directly proportional to the doubling time of the tumors is inversely proportional to the number of cancer cells (Robert, 1968). Cancer chemotherapy has been primarily directed at discovery of cytotoxic agents capable of inhibiting many aspects of mammalian cell division (Creasey, 1981). Drugs acting at different phases and actions may be advantageously combined (Capizzi et al., 1988). Almost 60 % of anticancer drugs are of natural origin, such as plants (i.e., vincristine, irinotecan, camptothecines) and microorganisms (i.e., doxorubicin, dactinomycines, mitomycin and bleomycin) (Grever, 2001). Since the discovery of novel anticancer agents doxorubicin and vincristine in 1969
(Altmann, 2007), natural products have been the cornerstone of cancer treatment. Plants and microorganisms have been the major sources of natural products throughout the centuries (Balunas and Kinghorn, 2005). The marine environment has also proven to be a very rich source of the potent compounds demonstrates significantly antitumor activity of the marine natural products that are accurately under clinical investigation as potential new anticancer drugs. The marine alkaloids eteinascidin-743 (ET-743) is the most advanced compound. The drug has broad spectrum antitumor activity and is especially effective against solid tumors such as sarcomas and breast cancer (Volotii et al., 1998). The search for improved cytotoxic agents (acting on ubiquitous targets such as DNA) continues to be an important line of modern anticancer drug discovery that will be critical for future advances in cancer therapy. As the major types of solid human tumors (breast, lung, prostate, and colon) are multi-causal in nature (in contrast to, e.g., CML, which is mostly driven by a specific genetic abnormality), there is a growing recognition that the treatment of solid tumors with “mechanism-based” agents alone is unlikely to be successful. In addition to chemotherapeutics, hormones have been employed in the management of hormone depended tumors, e.g., tumors of breast, prostate, ovaries, thyroid etc.

Despite the novel approaches still chemotherapy is employed as the first line of treatment. Unfortunately, commonly used cancer chemotherapy have presented unsatisfactory results, as the therapy is deleterious to patient health by making patients more susceptible to other diseases and often cause death by weakening the immune system of the patient body. Major challenges in cancer chemotherapy are related to toxicity on healthy proliferating cells and Multi-drug resistance (MDR) against anticancer
agents. The life threatening side-effects caused by nonspecific tissue distribution of the anticancer agents have restricted the systemic high dose strategy (Carelle, et al., 2002). Moreover, chemotherapeutic drugs also exert toxicity to normal cells which in turn causes the unpleasant side effects to the patients. For these reasons research and development for new class of anticancer agents which exhibit efficient and selective toxicity on tumor cells is attracting increased attention. This approach has led to the development of anticancer compounds produced by bacteria, fungi, plants, animals and mammalian cells in culture. There are number of reports, reviews and monographs on the discovery of the naturally occurring anticancer agents (Glasby, 1976; Aszalos and Berdy, 1978; Cassady and Duros, 1980; Aszalos, 1981; Dourans and Sufferess, 1981a). Among these, enzymes are used as anti tumor agents. With the development of science, a better understanding of the wide range of enzymes present in living cells and their mode of action was achieved (Reete et al., 2009). Many can be isolated without loss of catalytic function in vitro (Kiran et al., 2010). This unique ability of enzymes to perform their specific chemical transformations in isolation has led to an ever-increasing use of enzymes in industrial processes such as food technology, detergent, chemical industries and biomedical sciences (Jaeger et al., 1994; Ajay et al., 2010; Riaz et al., 2010). All living cells contain different types of enzymes and without which none of the living body survives. Depending upon the need of activity, enzymes are produced extracellular or intracellular (Joel et al., 1998). Enzymes can be obtained from plant (β-amylase, papain, bromelain, urease, ficin, polyphenol oxidase (tyrosinase), lipoxygenase, etc.), animal (pepsin, lipase, lysozyme, rennin, trypsin, phosphor-mannase, chymotrypsin, etc.) and microbial (α-amylase, pencillin acylase, protease, invertase, lactase, dextranase,
pectinase, pullulanase etc., sources (Zheng et al. 2011). In general, the enzymes from plant and animal sources are considered to be more important than those from microbial sources, but for both technical and economical reasons, microbial enzymes are considered to be more important (Sumitra et al., 2004). Therefore, increasing efforts are being pursued to produce enzymes by microbial fermentation (Arpana et al., 2011). Microbial enzymes are used in several industries, notably in detergent, food processing, brewing and pharmaceuticals (Vander et al., 2002; Simret et al., 2011). They are also used for diagnostic, scientific and analytical purposes (Biazus et al., 2007). In the present system enzymes became part of our daily life and used as commodities (Maria et al., 2005).

Most industrially important enzymes are extracellular and they have to be recovered by removal and separation from the cellular and other solid material (Masafumi et al., 2010). Therapeutic enzymes have a broad variety of specific use as oncolytics, thrombolytics or anticoagulants and as replacements for metabolic deficiencies. Additionally there is a growing group of miscellaneous enzymes of diverse function. Proteolytic enzymes have been widely used as anti-inflammatory drugs. It has been revealed that guinea pig serum inhibited a number of transplantable lymphomas in mice and rats as well as certain spontaneous and radiation-induced leukemias in mice. Broome (1963) has presented some evidence that the antitumor principle in guinea pig serum is L-asparaginase (Roberts, 1966).

The present investigation is one of the approaches that L-asparaginase may be used as anticancer agent. The L-asparaginase caused effective death of asparagine dependent tumor cells and gives aspartic acid and ammonia by the hydrolysis of asparagine (Campbell et al., 1967). Tsuji first reported deamidation of L-asparaginase by
extracts of *E. coli* in 1957 (Howard and Schwartz, 1968). Later, Mashburn and Wriston observed that L-asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) purified from cell extract of *E. coli* has an antitumor activity similar to that of guinea pig serum (Tosa *et al.*, 1961). A number of papers have been published on the production and the purification of the enzyme from *E. coli* (Arens *et al.*, 1970; Bilimoria, 1969; Campbell, 1967; Cedar and Schwartz, 1968; Ho *et al.*, 1970; Kristiansen *et al.*, 1970; Roberts *et al.*, 1968; Robinson and Berk, 1969; Schwartz *et al.*, 1966; Staerk *et al.*, 1970; Wagner *et al.*, 1969; Whelan and Wriston 1969). Erwinase®, Kidrolase®, Crisantipase®, Oncospar® and Elspar® are some of the formulations containing L-asparaginase from *Erwinia chrysanthemi* and *E. coli*. There are other bacteria also which are now found to produce L-asparaginase such as *Pseudomonas stutzeri* MB-405 (Manna *et al.*, 1995), *Thermus thermophilus* (Prista and Kyriakidis, 2001), *Pseudomonas aeruginosa* 5007 (El-Bessoumy *et al.*, 2001), *Staphylococcus* sp.-6A (Prakasham *et al.*, 2007), *Bacillus circulans* MTCC 8574 (Hymathi *et al.*, 2009) and *Serratia marcescens* SB08 (Venil and Lakshmanaperumalasamy, 2009), *Enterobacter aerogenes* (Mukherjee *et al.*, 2002), *Staphylococcus* species (Prakasham *et al.*, 2009), *Proteus vulgaris* (Tosa *et al.*, 1971), *Aerobacter*, *Bacillus*, *Erwinia*, *Pseudomonas*, *Serratia*, *Xanthomonas*, *Photobacterium* (Peterson, R.E. and A. Ciegler, 1969), *Vibrio* (Kafkewitz and Goodman, 1974), *Pseudomonas fluroscens* (Mardashen *et al.*, 1975), *Mycobacterium phlei* (Pastuszak *et al.*, 1976), *Corynebacterium glutamicum* (Mesas *et al.*, 1990) etc. However, L-asparaginase from bacterial origin can cause hypersensitivity in long term use leading to allergic reactions and anaphylaxis (Jayaram, 1968; Reynolds and Taylor, 1993). Therefore, the discovery of a new L-asparaginase immunologically different from that of
bacteria has been greatly desired. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi genera such as *Aspergillus*, *Penicillium* and *Fusarium* are commonly reported in scientific literature to produce L-asparaginase with less adverse effects (De-Angeli *et al*., 1970; Arima *et al*., 1972; Nakhama *et al*., 1973; Curran *et al*., 1985).

Most of the microbial L-asparaginase is intracellular in nature except few which are secreted outside the cell (Narayana *et al*., 2008). Extracellular L-asparaginase is more advantageous than intracellular since they could be produced abundantly in culture under normal condition and could be purified economically. L-asparaginase production throughout the world is carried out by submerged fermentation (SmF). However, this technique has many disadvantages. For instance, it is cost intensive and has low product concentration. In addition the extracellular excessive effluents and consequently needs handling and disposable of large volumes of waste water during downstream processing. Hence, solid-state fermentation (SSF) system has generated much interest in recent years because it offers several economical and practical advantages including high product concentration, improved product recovery, simple cultivation equipment and lower plant operational cost (Becerra and Siso, 1996; Pandey *et al*., 2001). The purification of the protein is an essential step in the study of its biological and physical properties. As enzymes are unstable molecules with physico-chemical organization, even slight change in this organization reduces the activity of the enzymes. A large number of stages are required to attain the necessary purity.
Since there are very less reports of the production of L-asparaginase from solid state fermentation by the filamentous fungi, an attempt has been made to produce the L-asparaginase through solid state fermentation employing *Fusarium equiseti* isolated from the rhizosphere soil of *Ipomea muricata* using soya bean meal as the substrate.

**OBJECTIVES OF THE STUDY**

The aim of the present study is to evaluate the production of L-asparaginase from the locally isolated filamentous fungi *Fusarium equiseti* using soya bean as the substrate. The soybean is high in protein, unsaturated fat, carbohydrates, and rich in vitamins and minerals and there has been some speculation that soy or isoflavones could be used in the treatment of existing tumors, either alone or in conjunction with conventional chemotherapeutic agents. Further, there are very few reports using soya bean for the production of L-asparaginase. Therefore the present investigation was carried out for the production of L-asparaginase from *Fusarium equiseti* under solid state fermentation. Further, the enzyme was purified and the properties were studied with the following objectives:

- Isolation of filamentous fungi from soil sample of various locations.
- Screening of the filamentous fungi for L-asparaginase production.
- Molecular identification of the high L-asparaginase producing isolate.
- Screening of the different substrates for the production of L-asparaginase under solid state fermentation.
• Optimization of various fermentation parameters for maximum L-asparaginase production.
• Large scale production of L-asparaginase using soya bean meal substrate.
• Purification and Properties of L-asparaginase.
PRESENTATION OF THE THESIS

The thesis is presented in six chapters as Introduction, Review of Literature, Materials and Methods, Results, Discussion, Summary and Conclusions.

At the end, the literature studied for the thesis is arranged in alphabetical order of the names of the authors, year of publication, article title, journal name, volume number and page numbers. References by the same author (s) are arranged chronologically and if more than one publication by the same author (s) published in the same year, then it is distinguished in the text and reference by the letters a, b, c, etc., after the year of the publication.

A list of publications made from the present work and papers presented at various symposia/conferences is given after the references. The list of Abbreviations, Tables, Figures and Photographs are given at the beginning of the thesis.