Introduction
1. INTRODUCTION

1.1. PREFACE

Enzyme industry is one among the major industries of the world and there exists a great market for enzymes in general. World market for enzymes is about 500 million US dollars and the total market for food enzymes alone is estimated to be about Rs 300 crores, with India contributing to a mere 0.5%. Food industry is recognised as the largest consumer for commercial enzymes (Lonsane and Ramakrishna, 1989). Except papain which is produced in abundance, we depend on imports for majority of enzymes used in the food industry. Enzymes are in great demand for use in several industries, such as food, beverage, starch and confectioneries production as well as in the textile and leather processing, pharmaceuticals and waste treatment.

In industry, enzymes are frequently used for process improvement, for instance to enable the utilization of new types of raw materials or for improving the physical properties of a material so that it can be more easily processed. They are the focal point of biotechnological processes. The deliberate use of enzymes by man is central to the application of biotechnology, since enzymes are involved in all aspects of biochemical conversion from the simple enzyme or fermentation conversion to the complex techniques in genetic engineering.

Microbial enzymes are preferred over plant or animal sources due to their economic production, consistency, ease of process modification and optimization. They are relatively more stable than corresponding enzymes derived from plants or animals.
Further, they provide a greater diversity of catalytic activities. The majority of enzymes currently used in industry are of microbial origin, and the vast majority of these are produced from only about 25 species, including 12 species of fungi.

Indeed it has been estimated that only about 2% of the world's microorganisms have been tested as enzyme sources (Wiseman, 1978). Increased awareness of the use of biocatalytic capabilities of enzymes and microorganisms has made possible the creation of a new generation of rationally developed biologically based processes and products. Advances in the field of molecular biology of microorganisms have opened up new horizons in the applications of new enzymes for developing novel products and applications.

The marine biosphere is one of the richest of the earth's innumerable habitats, yet is one of the least well characterized. Because of the diversity and scale, it offers enormous current and future opportunities for non-destructive exploitation within the many facets of modern biotechnology.

Although the marine biosphere covers more than two-thirds of the world's surface, our knowledge of marine microorganisms, in particular fungi, is still very limited (Molitoris and Schumann, 1986). Further, as on date marine microorganisms remain as untapped sources of many metabolites with novel properties (Faulkner, 1986; Chandrasekaran, 1996). Marine microorganisms have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions with novel enzymes (Chandrasekaran, 1997). Thus there is enormous scope for the investigations exploring the probabilities of deriving new products of economic importance from potential marine microorganisms, especially fungi.
Cancer, particularly leukemia, is a global problem and in spite of sincere efforts paid in the past, search for efficient drugs to solve this problem is being continued worldwide. Although several kinds of treatments are available, enzyme therapy is equally effective. L-asparaginase and L-glutaminase (L-Glutamine amidohydrolase EC 3.5.1.2.) earned attention since the discovery of their antitumor properties (Broome, 1961; Roberts et al, 1970; Bauer et al, 1971; Abell and Uren, 1981; Raha et al, 1990; Pal and Maity, 1992). L-asparaginase, obtained from terrestrial bacterial sources, which is used currently for the treatment of leukemia is known to cause several side effects and hence there is a need for alternative enzyme drug that is compatible to human blood and immunologically induce less or no side effects in the patient. In this context, considering the fact that marine environment, particularly seawater, which is saline in nature and chemically closer to human blood plasma, it is anticipated that they could provide enzymes that are compatible and less toxic to human.

Ability of the L-glutaminase to bring about degradation of glutamine posses it as a possible candidate for enzyme therapy which may soon replace or combine with L-asparaginase in the treatment of acute lymphocytic leukemia. However, the large scale application of glutaminase in cancer chemotherapy is still under experimental condition and not much information is available.

Besides its therapeutical value, L-glutaminase is also useful in the food industry as it increases the glutamic acid content of the fermented food thereby imparting a unique flavor (Yokotsuka, 1985). Since the sources for L-glutaminases are limited, the search for potential microbial strains that hyper produce the enzyme with novel properties for their
industrial production is being pursued all over the world (Prabhu and Chandrasekaran, 1995)

In the case of fungi but for the report on terrestrial Aspergillus oryzae (Yano et al., 1988; Tomita et al., 1988) no information is available in the literature on extracellular L-glutaminase production by any marine fungi. Since the present source for this enzyme is limited to E. coli and Aspergillus oryzae alone, a search for potential strains that hyper produces this enzyme with novel properties under economically viable bioprocesses is pursued.

Marine bacteria produce extracellular enzymes, and are capable of colonizing barren surfaces (Austin, 1988; Chandrasekaran, 1996). The adsorption or attachment property has been well documented in the literature (ZoBell and Allen, 1935; Fletcher, 1980; Hermanson and Marshall, 1985). The unique property of marine bacteria to adsorb on to solid particle is a highly desirable feature for their use in the solid state fermentation process (Chandrasekaran, 1994, 1996). Marine fungi is also expected to have a similar kind of adsorption property which could make them ideal candidates for use in solid state fermentation similar to their counterparts from terrestrial environments.

Salt tolerant microbes and their products are extremely important in industries which require high salt concentrations such as the production of soy sauce, where the final salt concentrations are as high as 20-25%. Hence, there is an increasing interest in the salt tolerant marine microorganisms for their use in such industries (Moriguchi et al., 1994).

Traditionally, large scale production of useful metabolites from microorganisms is carried out by submerged fermentation (SmF) where the cost of production and
contamination problems are very low and it facilitates better process control. Solid state fermentation (SSF) is the culturing of microorganisms on moist solid substrates in the absence or near absence of free water (Cannel and Moo Young, 1980). It is also described as any fermentation process that takes place on solid or semisolid substrate or that occurs on a nutritionally inert solid support, which provides some advantages to the microorganisms with respect to access to nutrients (Aidoo et al., 1982). It has several advantages over SmF particularly for higher productivity, easy recovery, lower capital and recurring expenses, reduced energy requirement, simple and highly reproducible among others (Lonsane and Karanth, 1990). Recently there is a renewed interest all over the world on SSF, in spite of the fact that this technique is being practiced for centuries. Currently SSF is being used for the production of traditional fermented foods; mushroom cultivation; protein enrichment of animal feed; single cell protein; fuel generation; production of ethanol; organic acids; antibiotics; alkaloids; food flavors; enzymes such as amylase, glucoamylase, cellulase, protease etc., and in the disposal of solid wastes (Lonsane, 1994).

Filamentous fungi are of great importance to SSF because of their ability to penetrate and colonise the substrate by apical growth and can tolerate the low amount of water available (Lambert, 1983; Smith and Aidoo, 1988). Majority of the microorganisms used in SSF processes are native of terrestrial environments and reports on the use of marine microorganisms, especially fungi are not available.

The most widely exploited solid substrates for SSF are mainly materials of plant origin and includes food crops (grains, roots, tubers and legumes), agricultural and plant residues and lignocellulosic materials like wood, straw, hay and grasses (Smith and
Aidoo, 1988). An essential prerequisite of all potential substrates is that the microbial colonizer must be able to derive energy and cellular constituents from these compounds by oxidative metabolism. Several natural substrates are usually water insoluble and form a multi-faceted complex surface on which the microorganisms grow and the rate and direction of growth will be dependent on the nutrient availability and geometric configuration of the solid matrix (Moo Young et al., 1983). It is usual for the crude raw material to contain most, if not all, of the necessary nutrients for growth. Some degree of pre-treatment is normally necessary for successful colonization by the microorganisms. Pretreatment methods can be physical, chemical or biological. In most cases, some degree of particle size reduction will be necessary to ensure rapid fermentation (Smith and Aidoo, 1988).

Use of inert supports have been recommended for SSF in order to overcome its inherent problems and efforts are being made to search for newer and better materials to act as inert solid supports (Aidoo et al., 1982; Zhu et al., 1994). Polystyrene was recognized as an ideal inert support for L-glutaminase production by marine Vibrio costicol (Prabhu and Chandrasekaran, 1995)

Immobilization of cells can be defined as the attachment of cells or their inclusion in distinct solid phase that permits exchange of substrates, products, inhibitors etc., but at the same time separates the catalytic cell biomass from the bulk phase containing substrates and products. Immobilization is accomplished by entrapment in a high molecular hydrophilic polymeric gel such as alginate, carrageenan, agarose etc. Therefore it is expected that the micro-environment surrounding the immobilized cells is not necessarily the same experienced by their free cell counterparts. The application of
immobilized whole living cells/spores as biocatalysts represents a rapidly expanding trend in biotechnology.

The remarkable advantage of this new system is the freedom to determine the cell density prior to fermentation. It also facilitates to operate the microbial fermentation on continuous mode without cell washout. When traditional fermentation are compared with the microbial conversion using immobilized cells the productivity obtained in the latter is considerably higher, obviously partly due to high cell density and immobilization induced cellular or genetic modifications. The novel process of immobilisation technology eliminates many of the constraints faced with the free cells. The use of immobilized whole microbial cells and/or organelles eliminates the often tedious, time consuming and expensive steps involved in isolation and purification of intracellular enzymes. It also intends to enhance the stability of the enzyme by retaining its natural catalytic surroundings during immobilization and subsequent continuous operation. The ease of conversion of batch processes in to continuous mode and maintenance of high cell density without washout conditions, even at very high dilution rates are few of the many advantages of immobilized cell systems.

Hence, in the present study it was proposed to evaluate the potential of marine *Beauveria bassiana*, isolated from marine sediments (Suresh, 1996) for production of L-glutaminase as extracellular enzyme under different fermentation conditions including submerged fermentation (Smf), solid state fermentation (SSF) with polystyrene as inert support and Immobilization.
1.2. REVIEW OF LITERATURE

Marine Fungi as source of enzymes

Fungi are widely known in fermentation industry, for the production of α-amylase, protease and lipase (Lambert, 1983). Whereas, all the fungi known as potential enzyme producers are from terrestrial sources. Relatively information on marine fungi is very limited to occasional reports on the degradative processes involving the production of intra and extra cellular enzymes. Cellulolytic activity of the marine lignicolous fungi (Meyers et al. 1960; Meyers and Scott, 1968), and the degradative role of filamentous marine fungi in the marine environment (Meyers, 1968; Jones & Irvine, 1972) are reported. Production of cellulase applying the viscometric and agar plate method (Schumann, 1974), clearing of cellulose containing agar as a measure of cellulase and xylanase production (Henningsson, 1976), ability to degrade wood cell wall components by species belonging to the genera Cirrenallia, Halosphaeria, Humicola, Niaculcitina, and Zalerion and their production of cellulase, xylanase and mannase (Eaton, 1977), gelatinase activity (Pisano et al. 1964), dehydrogenase pattern (Rodrigues et al. 1970) in marine filamentous fungi, and cell- bound and extra-cellular laminarinase by Dendryphella salina (Grant and Rhodes, 1992) were reported.

Sources of L-Glutaminase

Glutaminase activity is widely distributed in plants, animal tissues and microorganisms including bacteria, yeast and fungi (Imada et al., 1973; Yokotsuka et
L-Glutaminase synthesis have been reported from many bacterial genera, particularly from terrestrial sources, like *E. coli* (Prusiner et al. 1976), *Pseudomonas* sp (Kabanova et al. 1986), *Acinetobacter* (Holcenberg et al. 1978), and *Bacillus* sp (Cook et al. 1981).

Although glutaminase have been detected in several bacterial strains, the best characterised were from members of Enterobacteriaceae family. Among them *E. coli* glutaminase have been studied in detail (Prusiner et al., 1976). However other members such as *Proteus morganni*, *P. vulgaris*, *Xanthomonas juglandis*, *Erwinia carotovora*, *E. aroideae*, *Serratia marcescens*, *Enterobacter cloacae*, *Klebsiella aerogenes* and *Aerobacter aerogenes* (Wade et al., 1971; Imada et al., 1973; Novak & Philips, 1974) were also reported to have glutaminase activity.

Among other groups of bacteria, species of *Pseudomonas*, especially, *P. aeruginosa* (Greenberg et al., 1964; Ohshima, 1976), *P. aureofaciens* (Imada et al., 1973), *P. aurantiaca* (Kabanova et al., 1986; Lebedeva et al., 1986), and *P. fluorescens* (Yokotsuka et al., 1987) are well recognised for the production of glutaminase. All these strains have been isolated from soil.

Among Yeasts, species of *Hansenula*, *Cryptococcus*, *Rhodotorula*, *Candida scottii* (Imada et al., 1973) especially *Cryptococcus albidus* (Imada et al., 1973; Yokotsuka et al., 1987; Fukushima, & Motai, 1990) *Cryptococcus laurentii*, *Candida utilis* and *Torulopsis candida* (Kakinuma et al., 1987) were observed to produce significant levels of glutaminase under submerged fermentation. Species of *Tilachlidium humicola*, *Verticillum malthosei* and fungi imperfecti were recorded to possess glutaminase activity (Imada et al., 1973). Glutaminase activity of soy sauce
fermenting *Aspergillus sojae* and *A. oryzae* were also reported (Furuya *et al.*, 1985; Yano *et al.*, 1988).

**Marine Microorganisms as source of L-glutaminase**

Reports on the synthesis of extracellular L-glutaminase by marine microorganisms are very limited to marine bacteria including *Pseudomonas fluorescens*, *Vibrio costicola* and *Vibrio cholerae* (Renu, 1991; Renu and Chandrasekaran 1992a,) and *Micrococcus luteus* (Moriguchi *et al* 1994), and marine fungi *Beauveria bassiana* (Keerthi *et al*., 1999) only.

**Beauveria bassiana**

*Beauveria bassiana*, which is known in general as an entamopathogenic organism (Steinhaus, 1967), is common in soil, and is known to be used for the large scale production of chitinase and other industrially important enzymes (Muzzarelli, 1977). This species is also known to produce several exocellular enzymes including proteinases, lipases and chitinase (Kucera and Samsinakova, 1968; Leopold and Samsinakova, 1970; Pekrul and Grula, 1979). Marine *Beauveria bassiana* was recently recognised to produce chitinase (Suresh and Chandrasekaran, 1998)

**Solid State Fermentation**

Extracellular L-glutaminase production employing solid state fermentation is reported with marine bacteria including *Pseudomonas fluorescens*, *Vibrio costicola* and *Vibrio cholerae* using wheat bran (Renu, 1991; Renu and Chandrasekaran 1992b,) and marine *Vibrio costicola* using polystyrene and different natural substrates (Prabhu and Chandrasekaran 1995, 1996,1997).
Literature on L-glutaminase production as extracellular enzyme under solid state fermentation by fungi is limited to the reports on *Aspergillus oryzae* using wheat bran (Tomita *et al* 1988; Yano *et al* 1988), *Aspergillus oryzae*, *Actinomucor elegans*, and *A. taiwanensis* using mixed substrate system (Chou *et al* 1993).

**Inert supports in SSF**

The use of nutritionally inert materials as supports for solid state fermentation facilitates accurate designing of media, monitoring of process parameters, scaling up strategies and various engineering aspects, which are either impossible or difficult with conventional SSF using organic substrates such as wheat bran. The inert material when impregnated with a suitable medium, would not only provide a homogenous aerobic condition in the fermenter, but also contribute to elimination of impurities to the fermentation product, besides facilitating maximal recovery of the leachate with low viscosity and high specific activity for the target product (Prabhu & Chandrasekaran, 1995).

Vermiculite, a synthetic inert solid material, was first used for the production of amylase by *Aspergillus oryzae* (Meyrath, 1966). It was found that the rate of enzyme production on vermiculite impregnated with 4% starch solution was as high as on wheat bran and the yield was almost double. Polyurethane foam was used for the production of glucoamylase by *Aspergillus oryzae* (Kobayashi *et al*, 1991), nuclease P1 from *Pencillium citrinum* (Zhu *et al*, 1994), and for higher yields of citric acid by *Aspergillus niger*, as compared to submerged or surface culture methods (Aidoo *et al*, 1982). Materials such as computer cards for β-glucosidase production by *Aspergillus niger* (Madamwar *et al*, 1989); ion-exchange resin. Amberlite IRA 900 for the growth
studies of *Aspergillus niger* (Auria *et al.*, 1990); and polystyrene, for producing L-glutaminase by marine *Vibrio costicola* (Prabhu & Chandrasekaran, 1995, 1997) and marine *Beauveria* sp (Sabu *et al.* 1999) have been tried as inert supports for SSF.

**Immobilization of Fungi**

Immobilization of whole cell is not a novel concept but rather a duplication and refinement of phenomena observed in nature—microbial activity in soil, leaching of mineral ores, and in certain industrial microbial processes, where microorganisms or cells are attached to solid surfaces or form films (Trickling filters, vinegar process, tissue culture). Use of immobilized microbial cell obviates, the often laborious and expensive steps involved in extracting, isolating, and purifying intracellular enzymes. Stability of the desired enzyme is normally improved by retracing its natural environment during immobilization as well as during subsequent operation.

Of the various methods available for the 'artificial' immobilization of cells, adsorption and entrapment have been most extensively used for filamentous fungi in ECTEOLA-Cellulose (Johnson and Ciegler, 1969), collagen (Venkatasubramanian, 1979), and calcium alginate (Lin Ko, 1981). The adsorption method is based on linking cells directly to water insoluble carriers. The adsorption effect is mainly due to electrostatic interactions between the microbial cell surface and the carrier material. The process is essentially mild and allows good retention of cell viability and enzymatic activity. However, desorption can occur rapidly under certain conditions. The strength of cell attachment appears to depend on a complex interactions of factors including, cell wall composition and cell age, various physicochemical surface properties of the carrier.
including surface area, and also pH and ionic strength of the solution in which the cells are suspended (Kolot, 1981).

Aspergillus and Penicillium sp. were immobilized by adsorption to several ion exchange resins, and ECTEOLA-Cellulose was observed as most suitable. Further immobilized spores were found to be more stable although less active than the vegetative mycelia (Johnson and Ciegler, 1969). *Penicillium chrysogenum* was immobilized on a variety of inorganic supports including fritted glass, cordierite and zirconia ceramic by adsorptive immobilization (Kolot, 1981). The latter material exhibited the highest biomass accumulation and the biocatalyst preparation formed using this carrier was found to be stable during long term continuous column operation.

Procedures, more extensively used than absorption for whole cell immobilization, involve entrapment using inert gels such as polyacrylamide and calcium alginate and these have been successfully applied to filamentous fungi (Linko, 1981). These methods are based on the inclusion of cells within polymeric matrices which allow diffusion of substrate and product but prevent cell loss. *Penicillium chrysogenum* was entrapped in calcium alginate and used in bubble column reactors with limited success (Mahmoud et al., 1987).

Fungal spores are capable of a wide range of substrate conversions, which could assign to them a real value in the fermentation industry (Durand and Navarro, 1978). The spores offer certain advantages, such that spores of various organisms can be stored, frozen for a long time without significant loss in activity and their removal is easy (Vezina et al., 1968). During transformation of substrates, even if they are maintained in the early pregermination stage, spores are 3 to 10 times more active than
mycelium on a dry weight basis. The field of 'spore process' has not thoroughly been explored and deserves further attention.

Entrapment of microbial cells within the polymeric matrices is preferred for its simplicity of the methodologies. Among them alginate gel has received major attention. There are several studies on the composition of alginate and their suitability for cell immobilization (Martinsen, et al.; 1989; 1992). Efforts are made in the recent years to study the diffusional characteristics of the immobilized system so as to enhance our understanding on the micro environment that prevail near the immobilized cells (Axelsson et al., 1994). Efforts are made towards optimization of immobilization protocols with a view to improve the stability of the gel beads by modifying the protocols (Ogbonna et al., 1989; Jamuna et al., 1992; Mohandass, 1992).

Immobilized spores of *Penicillium chrysogenum* are the most widely used system in the production of penicillin G. Fungal conidia entrapped in *k*-carrageenan were used for batch and continuous production of penicillin and compared with fungi adsorbed on celite (Jones et al., 1986; Kalogerakis et al., 1986).

Immobilized *Aspergillus niger* is widely used for the synthesis of organic acids and enzymes. The methods most widely used for immobilization of *A. niger* cells are the entrapment in alginate gels (Gupta and Sharma, 1994), agarose (Khare, et al., 1994), and polyacrylamide (Mittal et al., 1993).

The fungal fermentation have serious disadvantages of rising viscosity during growth, leading to poor oxygen supply to the cells. To compensate the same it is necessary to aerate the cultures with large volumes of sterile air. In case of Immobilized cells, since the growth is restricted, it is possible to operate the fermentation without
affecting the viscosity, facilitating good oxygen transfer rates with minimal cause (Honecker et al., 1989; Mittal et al., 1993; Gupta and Sharma, 1994).

Fungal fermentation for lactic acid production has also been studied using Rhizopus oryzae cells, immobilized with polymer supports prepared from polyethylene glycol (No.400) and dimethylacrylate as monomers by γ-ray induced polymerization (Tamada et al., 1992).

Aspergillus sp strains have been immobilized for the production of glucoamylase (Bon and Webb, 1989; Kuek, 1991; and Emili Abraham et al. 1991). Continuous production of glucoamylase by immobilizing mycelial fragments of A. niger was demonstrated and among the polymer matrices tried for immobilization, κ-carrageenan and alginate were found to be most effective (Emili Abraham et al. 1991). Tricoderma reesei was immobilized, for the continuous production of cellulase, on polyester cloth (Sheldon, 1988), nonwoven material (Tamada et al., 1989) and cellulosic fabric (Kumakura et al., 1989).

**Packed bed reactor**

Most bioreactor systems, now being studied, for immobilized cells are continuous columnar systems such as packed bed or fluidized bed systems (Scott, 1987). In fact, such systems demand that the organism be immobilized to prevent the washout at relatively high flow rates that are used. Packed bed reactors are tried for immobilized cellular processes more than any other bioreactor configuration (Scott, 1987). In general, such systems are appropriate when relatively long retention times are required and external biomass build up is minimal. There has been some innovation in the design and operation of such bioreactor concepts, including the use of
a horizontal packed bed reactor (Margaritis and Bajpai, 1983), a dry or gas phase system (de Bont and van Ginkel, 1983), and multiple columns in sequence (Tosa et al., 1984).

Properties of L-glutaminase

The pH and temperature tolerance of glutaminase from various microorganisms differed greatly. While optimal activities of glutaminase A and B of P. aeruginosa were at alkaline pH of 7.5-9.0 and 8.5 respectively (Soda et al., 1972), glutaminase from Pseudomonas sp was reported to be active over a broad range of pH (5-9) with an optimum near pH 7.0 (Roberts, 1976). Glutaminase of Pseudomonas acidovorans showed optimum activity at pH 9.5 and retained 70% activity at pH 7.4 (Davidson et al., 1977). An intracellular glutaminase from Cryptococcus albidus preferred an optimal pH of 5.5-8.5 (Yokotsuka et al., 1987). Whereas, glutaminase 1 and 11 isolated from marine Micrococcus luteus were active at alkaline pH values of 8.0 and 8.5 respectively (Moriguchi et al., 1994). Glutaminase from A. oryzae and A. sojae recorded pH optima of 9.0 and 8.0 respectively (Shikata et al., 1978). The intra and extracellular glutaminase from A. oryzae were most active and stable at pH 9.0 (Yano et al., 1988).

The temperature stability of glutaminases also showed wide variation. Glutaminase from Pseudomonas showed maximum activity at 37°C and were unstable at high temperatures (Ramadan et al., 1964), whereas, the enzyme from Clostridium welchii retained activity up to 60°C (Kozolov et al., 1972). Glutaminase from Cryptococcus albidus retained 77% of its activity at 70°C even after 30 minutes of incubation (Yokotsuka et al., 1987). Glutaminase 1&11 from Micrococcus luteus had a temperature optima of 50°C and the presence of NaCl (10%) increased the
thermostability (Moriguchi et al, 1994). The optimum temperature for activity of both intra and extracellular glutaminases from A. oryzae was 45°C while they became inactive at 55°C (Yano et al, 1988).

Sodium chloride was found to influence the activity of glutaminase from both fungi and bacteria of terrestrial origin. Glutaminase from E.coli, P.fluorescence, Cryptococcus albidus and A.sojae showed only 65, 75, 65 and 6% respectively of their original activity in presence of 18% NaCl (Yokotsuka, 1987). Similar results were obtained with glutaminase from Candida utilis, Torulopsis candida and A.oryzae (Kakinuma et al,1987; Yano et al, 1988). Salt tolerant glutaminase have been observed in Cryptococcus albidus and Bacillus subtilis (Iwasa et al, 1987; Shimazu et al, 1991). Glutaminase 1 and 11 with high salt tolerance was reported from Micrococcus luteus K-3 (Moriguchi et al, 1994).

Glutaminases also differed in their affinity towards L-glutamine. While the enzyme from Acinetobacter sp. recorded a Km of 5.8± 1.5 X 10-6 M, those from C. welchii had a Km of 10^3 M (Kozolov et al, 1972). The enzyme from Achromobacteraceae had a Km of 4.8 ± 1.4 X 10^{-6} M (Roberts et al, 1972). The average Km values for glutaminase- asparaginase from Pseudomonas 7A was 4.6± 0.4 X 10^{-6} M (Roberts, 1976). Whereas, that from P. acidovorans had 2.2X 10^{-5} M (Davidson et al, 1977). The glutaminase 1&11 from marine Micrococcus luteus had a Km of 4.4 mM respectively (Moriguchi et al, 1994).

The isoelectric point of glutaminase varied for different organisms. Thus, it was 5.5 for Clostridium welchii (Kozolov et al, 1972); 5.4 for E. coli (Prusiner et al 1976); 8.43 for Acinetobacter glutaminasificans (Roberts et al,1972); 5.8 for Pseudomonas
Glutaminase activity was found to be inhibited by various substances and heavy metals. Cetavlon, while accelerating glutaminase of Clostridium welchii, E.coli and Proteus moranii in crude extracts and intact cells, inhibited the purified enzyme (Hughes & Williamson, 1952). Glutaminase of E. coli was found to be sensitive to heavy metals (Hartman, 1968) and Acinetobacter glutaminase -asparaginase was inactivated by glutamine analogue 6-diazo 5-oxo L-norleucine even at very low concentration while unaffected by EDTA, NH₃, L-glutamate or L-aspartate (Roberts et al, 1972). Various investigations have shown that glutaminase from Pseudomonas was activated by certain divalent anions and cations while inhibited by monovalent anions and by certain competitive inhibitors like NH₃, D and L-glutamic acid and 6-diazo 5-oxo L-norleucine (Ramadan et al, 1964; Soda et al, 1972; Roberts, 1976). In the case of fungi both intra and extracellular glutaminase from Aspergillus oryzae were inhibited by Hg, Cr and Fe but were not affected by sulphydroxyl reagents (Yano et al, 1988). EDTA, Na₂SO₄, and ρ-chloromercuribenzoate strongly inhibited the Micrococcus luteus glutaminase I while glutaminase II was inhibited by EDTA, HgCl₂, Na₂SO₄, CuCl₂ and FeCl₃ (Moriguchi et al, 1994).

The bacterial amidohydrolases are reported to be homotetramers of identical subunits and the individual subunits are not catalytically active. The molecular weight ranges from 120,000 - 147,000 daltons (Ammon et al, 1988). The enzyme from Achromobacteraceae showed a molecular weight of 138,000 daltons with a subunit
molecular weight of 35,000, whereas that from \textit{P. acidovorans} had a larger molecular weight of approximately 156,000 and subunit weight of 39,000 daltons (Davidson \textit{et al}, 1977). The glutaminase-asparaginase from \textit{Erwinia chrysanthemi} had a subunit molecular weight of 35,100 and approximately 140,000 for the native protein (Tanaka \textit{et al}, 1988).

Enzyme with smaller molecular weight has also been reported (Prusiner \textit{et al}, 1976; Moriguchi \textit{et al}, 1994). Glutaminase B from \textit{E.coli} had a molecular weight of 90,000 daltons when estimated by gel filtration on sephadex G-200 and 100,000 daltons under electrophoresis (Prusiner \textit{et al}, 1976). Glutaminase 1 and 11 from \textit{Micrococcus sp} had a molecular weight of 86,000 daltons when measured by gel filtration on Supherose 12 column. Glutaminase 1 also showed a subunit molecular weight of 43,000 daltons upon SDS-PAGE (Moriguchi \textit{et al}, 1994).

Applications of Glutaminase in flavour industry

L-Glutaminase enhances the flavor of fermented foods by increasing their glutamic acid content thereby imparting a palatable taste (Yokotsuka, 1985, 1986). It is widely used in countries such as Japan where fermented foods like soy sauce is a highly valuable commodity. Of the many oriental fermented products, soy sauce is the one most widely consumed in China, Japan, Korea and other Asian countries as a condiment and coloring agent in the preparation of foods and for table use (Luh, 1995). In soy sauce fermentation it is important to increase the amount of glutamic acid for a delicious taste. Glutaminase is generally regarded as a key enzyme that controls the taste of soy sauce and other fermented foods (Yamamoto \& Hirooka, 1974; Tomita \textit{et al}}
Salt tolerant glutaminase from Cryptococcus albidus was used to increase the glutamic acid content of soy sauce (Nakadai and Nasuno, 1989).

Yokotsuka et al. (1987) isolated three strains of E. coli, Pseudomonas fluorescens Cryptococcus albidus as producers of heat stable and salt tolerant glutaminase. During enzymatic digestion of soyu koji especially when conducted with increased salt concentration and high temperature, enzyme was highly effective. Later he observed that the glutamic acid content of soyu was increased to 20% on addition of glutaminase (Yokotsuka, 1988).

Induced mutations were performed in Koji molds (Ushigima & Nakadai, 1983) and E. coli and Torulopsis tamata (Kakinuma, et al. 1987, Mugnetsyam and Stepanayan, 1987) to enhance glutaminase production. Glutaminase from Aspergillus oryzae is traditionally used for soy sauce fermentation in many countries. However, the enzyme from A. oryzae has been shown to be markedly inhibited by the high salt concentration in the fermentation process (Yano et al., 1988). Use of salt tolerant glutaminase from marine bacteria provides an interesting alternative in the soy sauce fermentation industry (Moriguchi et al., 1994).

A glutaminase with glutamyl transpeptidase activity was also isolated from A. oryzae with a view to improve the glutamic acid content of fermented foods (Tomita et al., 1988). Protoplast fusion among the species of A. sojae was employed to induce protease and glutaminase production (Ushijima and Nakadai, 1984). Cryptococcus albidus producing salt tolerant glutaminase was immobilized on silica gel and alginate-silica gel complex for obtaining a continuous production of glutamic acid from glutamine (Fukushima and Motai, 1990).
L- Glutaminase in cancer treatment

Tumors compete for nitrogen compounds. This produces in the host a negative nitrogen balance and a characteristic weight loss, and in the tumor a reciprocal nitrogen increase. Glutamine is an efficient vehicle for the transport of nitrogen and carbon skeletons between the different tissues in the living organism (Carrascosa, et al 1984; Argiles and Bieto, 1988). When a tumor develops, there is a net flux of amino acids from host tissues to the tumor and glutamine is the main source of nitrogen for tumor cells (OgMoreadith and Lehninger, 1984). Once glutamine has been incorporated into tumor cells, this amino acid is quickly metabolized (Marquez et al 1989). High rates of glutamine use is a characteristic of tumor cells. Both in vitro and in vivo (Lazarus. and Panasci, 1986) and experimental cancer therapies have been developed based on depriving tumor cells of glutamine (Roberts et al 1970; Rosenfeld.and Roberts, 1981).

Tumor inhibition is mediated by inhibition of both nucleic acid, and protein synthesis of tumor cells. As specific inhibition of tumor cell glutamine uptake could be one of the possible ways to check the growth, use of glutaminase enzyme as drug gains importance in this respect. An exciting breakthrough in the enzymatic treatment of cancer resulted from the discovery of metabolic difference between certain tumor and host cells (Sizer,1972). Only a limited number of microbial enzymes, that deplete nutritionally essential aminoacids, such as asparaginase (Roberts et al 1976, Sudha,1981); Glutaminases (Roberts et al ,1970, 1971, Chandrasekaran et al 1998) streptodornase (Nuzhina,1970), lysozyme(Oldham 1967), Serine dehydratases (Wade & Rutter,1970),
and carboxypeptidase (Bertino et al, 1971) have been suggested for the treatment of human leukemias and solid tumors.

The parenteral administration of enzymes which degrade amino acids required only for growth of neoplasms offers a potential cancer therapy with marked specificity for the tumor. In this context L-asparaginases and L-glutaminases have received greater attention with respect to their antitumor effect (Broome, 1971; Cooney and Rosenbluth, 1975; Abell & Uren, 1981; Flickinger, 1985).

L-glutaminase got the attention as a drug ever since microbial glutaminases exhibited antitumor activity (Greenberg et al., 1964; Roberts et al., 1970, 1971; Broome, 1971). Certain tumor cells grown in tissue culture required glutamine at a level which is ten fold or greater than any other amino acids (Eagle et al., 1956). Roberts et al., (1970) observed that glutaminase preparations, purified from a gram positive coccus and from three gram negative forms, with considerably lower Km values resulted in marked inhibition of an Ehrlich Ascites Carcinoma. A number of glutaminases with antitumour activity have been isolated from Acinetobacter glutaminasificans, Pseudomonas aureofaciens, P. aeruginosa, Pseudomonas 7 A and Achromobacter (Roberts, 1976; Spiers et al., 1976). Several of these enzymes reduced both asparagine and glutamine concentration in tissues and their therapeutic effect may depend on the combined depletion of both these aminoacids.

Roberts et al., (1972) described a glutaminase-asparaginase from Achromobacteriaceae with potent antineoplastic activity and established criteria for selection of a glutaminase for testing of antitumor activity which include optimal activity, stability under physiological conditions, low Km values, slow clearance from
blood and low endotoxic activity. *Achromobacter* glutaminase-asparaginase have also received attention with respect to human pharmacology, toxicology and activity in acute leukaemia (Spiers & Wade, 1979). Roberts and McGregor (1989) also reported that glutaminase had potent anti retroviral activity in vivo. They found that murine leukaemia virus required glutamine for replication and glutaminase mediated depletion of glutamine in animals resulted in potent inhibition of retrovirus replication, thereby increasing the median survival time of the animals.

Hambleton et al. (1980) studied clinical and biochemical aspects of microbial glutaminase toxicity in rabbit and rhesus monkey. According to them treatment with chemically modified glutaminases was lethal to rabbits and rhesus monkeys and lesions were produced in kidney, liver and intestine while treatment with unmodified glutaminase induced similar changes in rabbits but not in rhesus monkeys.
1.3. OBJECTIVES OF THE PRESENT STUDY

From the ongoing review of literature it is understood that information on L-glutaminase production by any marine fungi is not available. Further, use of different fermentation systems viz: submerged fermentation, solid state fermentation and immobilized system, for any extracellular enzyme production by marine fungi is also not reported.

Hence, in the present study it was proposed to evaluate Beauveria bassiana isolated from marine sediment, as a chitinolytic fungi, during an earlier investigation in our Department (Suresh, 1996), for production of L-glutaminase as an extra cellular enzyme, and to compare the three principal fermentation systems of submerged, solid state and immobilized conditions with a view to propose a suitable bioprocess technology for industrial production of L-glutaminase.

Specific objectives of the present study include

1. Production of L-glutaminase by marine Beauveria bassiana under submerged fermentation.
2. Production of L-glutaminase by marine B. bassiana under solid state fermentation using sea water based medium, employing polystyrene as inert support system
3. Production of L-glutaminase by terrestrial B. bassiana under solid state and submerged fermentation using distilled water and sea water based medium.
4. Production of L-glutaminase by marine \textit{B. bassiana} spores immobilized in calcium alginate beads in a packed bed reactor.

5. Comparison of L-glutaminase production by marine \textit{B. bassiana} under submerged, solid state and immobilized fermentation conditions.