L-GLUTAMINASE PRODUCTION BY MARINE FUNGI

Thesis submitted

under the Faculty of Science to the

Cochin University of Science and Technology

in partial fulfilment of the requirements

for the Degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

BY

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DECEMBER, 1999

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CERTIFICATE

This is to certify that the work presented in the thesis entitled " **L-glutaminase production by marine fungi** " is based on the original research done by Mr. Sabu. A, under my guidance and supervision at the Department of Biotechnology and no part there of has been included in any other thesis for the award of any degree.

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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 worlds 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 third 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 MooYoung, 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 faced 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, or 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 costicola* (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 counter parts. 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, Niaculcitlna*, 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*

al., 1987). 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*, *Xanthmonas juglandis*, *Erwnia 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 malthoasei* 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. taiwanenesis* 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 P 1 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).

Glitaminases also differed in their affinity towards L-glutamine. While the enzyme from *Acinetobacter sp.* recorded a Km of 5.8$\pm$ 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$\pm$ 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*

(Holcenberg *et al*, 1976); 7.6 for another species of *Pseudomonas* (Katsumata *et al*,1972); 3.94-4.09 for *Cryptococcus albidus* (Yokotsuka, 1987) and *Pseudomonas acidovorans* (Davidson *et al*, 1977).

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, NH_3, 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 NH_3, 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, Na_2SO_4, and ρ-chloromercuribenzoate strongly inhibited the *Micrococcus luteus* glutaminase 1 while glutaminase 11 was inhibited by EDTA, $HgCl_2$, Na_2SO_4, $CuCl_2$ and $FeCl_3$ (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 *P. acidovorans* had a larger molecular weight of approximately 156,000 and subunit weight of 39,000 daltons (Davidson *et al,* 1977). The glutaminase-asparaginase from *Erwinia chrysanthemi* had a subunit molecular weight of 35,100 and approximately 140,000 for the native protein (Tanaka *et al*,1988)

Enzyme with smaller molecular weight has also been reported (Prusiner *et al,* 1976; Moriguchi *et al,* 1994). Glutaminase B from *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 *et al,* 1976). Glutaminase 1 and 11 from *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 *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 *et*

al, 1988; Yano *et al,* 1988). 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 fluoroscens 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 aminoacids 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 aminoacids (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 aureofaciems, 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 *B. bassiana* spores immobilized in caclium alginate beads in a packed bed reactor

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

Materials and Methods

2. MATERIALS AND METHODS

2.1. Microorganism

Beauveria bassiana BTMF S10 was used throughout the course of study.

2.1.1. Source of strain

The selected fungal strain was isolated from marine sediment of coastal environments of Cochin (Suresh, 1996) and is available as a stock culture in the culture collection of the Department of Biotechnology, Cochin University of Science and Technology, Cochin, India.

2.1.2 Maintenance of culture

The culture was maintained on Bennet's agar (HIMEDIA) slants and sub cultured once in a month. One set was maintained as stock culture preserved under sterile mineral oil. Another set was used as the working culture for routine experiments.

2.2 L-glutaminase production by marine *Beauveria bassiana* under Submerged fermentation (SmF).

2.2.1 Medium

Mineral salt glutamine medium with the composition given below was used as a basal medium (unless otherwise mentioned) for L-glutaminase production by *B. bassiana* under submerged fermentation.

The composition of the mineral salt glutamine medium (Renu and Chandrasekaran,1992a) is as follows.

Components	g/L
K_2HPO_4	10
KH_2PO_4	5
$MgSO_4.7H_2O$	10
L-Glutamine	10
Sodium Chloride	10
Distilled water	1000 ml
pH	8.0

The prepared medium was autoclaved at 121°C for 15 minutes and used.

2.2.2 Preparation of inoculum

2.2.2.1 Spore inoculum

1. *Beauveria bassiana*, was raised as agar slope culture on Bennet's agar, prepared in aged sea water, (in 50 ml capacity capacity test tubes),
2. To fully sporulated (two weeks old) agar slope culture, 20 ml of sterile physiological saline (0. 85 % NaCl) containing 0.1 % Tween 80 was added by means of a sterile pipette.
3. Then, the spores were scrapped using an inoculation needle, under strict aseptic conditions,
4. The spore suspension obtained was adjusted to a concentration of 12 x 10^8 spores / ml using sterile physiological saline.
5. The prepared spore suspension was used as the inoculum.

2.2.2.2 Vegetative inoculum

Vegetative mycelial inocula was prepared in 250 ml of GPYS medium(Glucose-1gm, peptone-0.5gm, Yeast extract-0.1gm in one liter sea water, pH-7.6). Prepared medium taken in a one litre flask was inoculated with 10 ml of spore suspension (prepared as described under section 2.2.2.1). The inoculated broth was incubated at room temperature (28 $\pm$ 2°C) on a rotary shaker at 150 rpm for 48 hours. The mycelia was collected asceptically by centrifugation at 10,000 rpm for 10 minutes, and washed repeatedly with sterile physiological saline. The separated mycelial pellets were broken down by vigorous agitation with sterile glass beads (3mm), using a vortex mixer, and suspended in 100ml of the same saline. The concentration of the prepared suspension was approximately 25 µg dry weight equivalent of mycelia per ml. The prepared suspension was used as vegetative mycelial inoculum.

2.2.3 Inoculation and Incubation

The prepared inoculum was used at 4% (v/v) level, (arbitrarily selected before optimisation) and incubated at room temperature (28 $\pm$ 2°C), on a rotary shaker at 150 rpm, for 48 hours (unless otherwise specified).

2.2.4 Measurement of growth

Growth was estimated in terms of total protein content of the biomass (Herberts *et al.*, 1971) using Folin's ciocalteu reagent (Lowry *et al.*, 1951), as detailed below.

1. After incubation for the desired period the mycelia were harvested by centrifugation (at 10,000 rpm for 20 minutes, at 4° C),
2. Washed repeatedly (by consecutive centrifugation) with sterile distilled water to remove the residual medium constituents and the metabolites, homogenized with a tissue homogenizer,
3. Suspended in sterile distilled water.
4. 2 ml of the prepared mycelial suspension was taken in a test tube,
5. 2 ml of 1 N NaOH was added
6. The tube with contents was placed in a boiling water bath for 5 minutes.
7. Cooled to room temperature,
8. The undissolved residue was removed by centrifugation at 5000 rpm for 10 minutes.
9. One ml of the supernatant was mixed with freshly prepared 2.5 ml of alkaline reagent (50 ml of 5% Na_2CO_3 + 2 ml of 0.5% $CuSO_4$. $5H_2O$ in 1% sodium potassium tartrate),
10. Allowed to stand for 10 minutes,
11. 0.5 ml of Folin-Ciocalteu reagent was rapidly added
12. Allowed to stand for thirty minutes,
13. The blue colour developed was measured by taking the absorbance at 750 nm in a UV-visible spectrophotometer (Spectronic Genesis, Milton Roy. USA), against the reagent blank.
14. Bovine Serum Albumin was used as standard for computation of protein content and expressed as mg/ml.
15. Protein content was expressed as mg/ml.

2.2.5 Enzyme assay

The fermented broth, after incubation for the desired period, was centrifuged at 10,000 rpm for 20 minutes, at 4°C, in a refrigerated centrifuge (Kubota 6900. Japan) and the cell free supernatant was collected and used for enzyme assay.

L-Glutaminase was assayed according to Imada *et al* (1973) with slight modifications, as given below.

1. An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of phosphate buffer (0.1M, pH 8.0)
2. The mixture was incubated at 37°C for 15 min. and the reaction was arrested by the addition of 0.5 ml of 1.5 M Trichloro Acetic Acid.
3. To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added.
4. The absorbance was measured at 450nm using a UV-Visible spectrophotometer (Spectronic Genesys5, Milton Roy USA)
5. A standard graph was plotted using ammonium chloride as the standard for computation of the concentration of ammonia, liberated due to enzyme activity
6. One international unit of L-glutaminase was defined as the amount of enzyme that liberates one μ mol. of ammonia under optimum conditions. The enzyme yield was expressed as Units / ml (U/ml)
7. Appropriate controls were included in the experiment.

2.2.6 Determination of Enzyme Protein

The enzyme protein was determined following the method of Lowry *et al* (1951), as detailed below.

1. To 1 ml of the enzyme, 5ml of alkaline reagent was added
2. Contents were mixed thoroughly and left for ten minutes.
3. 0.5 ml of Folin' s reagent diluted with an equal volume of water was added to each tube.
4. After 40 minutes, absorbance was measured at 750 nm in a UV-Visible Spectrophotometer. (Spectronic Genesys5, Milton Roy USA)
5. Bovine Serum Albumin was used as the standard.
6. Protein was expressed in mg/ml.

2.3 Optimisation of Process parameters for L- glutaminase production under Submerged fermentation (SmF) by marine *Beauveria bassiana.*

Optimum conditions required for maximum L-glutaminase production under SmF was determined for incubation temperature, pH of the medium, substrate concentration, sodium chloride concentration, additional nitrogen sources, aminoacids, and inoculum concentration, by varying the variables and evaluating the rate of L-glutaminase production.

The protocol adopted for optimization of various process parameters influencing glutaminase production was to evaluate the effect of an individual parameter and to incorporate it at the standardized level in the experiment before optimizing the next

parameter. All the experiments were carried out in triplicate and the mean values are reported.

2.3.1 Temperature

The optimum temperature required for maximal L-glutaminase production by marine *Beauveria bassiana* under SmF was estimated by incubating the inoculated medium at various temperatures (22, 27, 32, 35, and 42°C) for a period of 48 hours as mentioned under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected and enzyme was assayed as described under section 2.2.5.

2.3.2. pH

Optimal pH required for enhanced level of L- glutaminase production by marine *Beauveria bassiana* under SmF was determined at various levels of pH (6-13) adjusted in the medium using 1N HCl / NaOH. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.3.3. Additional Nitrogen sources

Requirement for additional nitrogen sources, besides L- glutamine, in the medium for enhanced enzyme production under SmF was determined by incorporating various nitrogen sources (Peptone, Yeast extract, Malt extract, Beef extract, Ammonium sulphate, Ammonium Nitrate, Calcium nitrate and Potassium Nitrate, individually at 1%

(w/v) level in the medium. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the culture broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

Since, yeast extract and Potassium nitrate were found to promote enhanced levels of L-glutaminase production, as additional nitrogen source, optimal concentrations of the same were determined further by incorporating these compounds at different concentrations, (1-5 % w/v) in the medium. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5

2.3.4. Additional Carbon Sources

Need for additional carbon sources, along with glutamine, for enhanced enzyme production by marine *Beauveria bassiana* under SmF was tested by incorporating maltose, glucose, mannitol, mannose, sucrose and sorbitol, in the medium, individually at 1% (w/v) level. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the culture broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5

Since sorbitol, as an additional carbon source, was found to promote enhanced level of enzyme production under SmF, optimal concentration of the same required for the purpose, was determined by incorporating the same at different concentrations (1-7% w/v) in the medium, and evaluating the level of enzyme production. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation,

the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.3.5 Amino acids

Impact of amino acids, as inducer substances in the medium, on enzyme production by marine *Beauveria bassiana* under SmF was tested with glutamic acid, asparagine, arginine, methionine, proline and lysine in the medium, at 1 % (w/v) level. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

Since, methionine was found to promote enhanced level of enzyme production, under SmF, optimal concentration of methionine required was determined by evaluating the level of enzyme production at different concentrations of the same (0.1 -1 % w/v) in the medium. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.3.6 Sodium Chloride concentration

Impact of sodium chloride on enzyme production by marine *Beauveria bassiana* was determined by subjecting the strain to various levels of NaCl concentration (0-15 % w/v) adjusted in the medium. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.3.7 Time Course Study.

After optimising the various process parameters a time course study was carried out at the optimised conditions. Inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.4 L-glutaminase production by marine *Beauveria bassiana* under solid state fermentation (SSF) using inert solid support.

2.4.1 Inert solid support

Expanded polystyrene (poly (1-phenylethylene)), a commercially available insulating and packaging material, was used as inert solid support for solid state fermentation production of L-glutaminase by marine *Beauveria bassiana.* It is odourless, nontoxic, tasteless, low in weight, less brittle, and non biodegradable. Its maximum water absorbancy is 2.0g/100cm^2 (Brydson, 1982). Although it is nutritionally inert, it could act as a support for attachment of microorganisms during fermentation (Prabhu and Chandrasekaran, 1995). Moreover, marine microorganisms have capacity to adsorb or anchor onto solid particles. It facilitates the growth of organism. Polystyrene being nutritionally inert, do not support production of undesirable protein in the presence of specific substrate. Whereas, usual SSF media such as wheat bran support synthesis of several undesirable proteins along with the

desired protein. Hence, in the present study polystyrene was used as support for L-glutaminase production by marine *Beuaveria bassiana* under SSF.

2.4.2 Media

L- glutaminase production by *Beauveria bassiana.* under solid state fermentation using polystyrene was optimised using a basal medium containing L-glutamine (10g/L), and D-Glucose(10/g/L) dissolved in aged sea water (35.0 ppt salinity) with pH.8.0.

2.4.3 Preparation of solid substrate medium

Polystyrene beads of 2-3 mm diameter were pretreated by autoclaving at 121°C for 15 min. during which the beads collapsed and reduced to about one third of their original size (Brydson, 1982). The reduced beads of uniform size (1-1.5mm) were used for fermentation studies (Prabhu and Chandrasekaran,1995)

Ten grams of pretreated polystyrene beads were taken in 250 ml Erlenmeyer flasks, moistened with the prepared medium (as mentioned under section 2.4.2 unless otherwise mentioned), autoclaved for 1 hour and cooled to room temperature before inoculation.

2.4.4. Preparation of Inoculum

Inoculum was prepared as described under section 2.2.2.1

2.4.5 Inoculation and incubation procedures

The sterilized solid substrate media was inoculated with the prepared inoculum (12 x 10^8 spores/ ml - arbitrarily selected before optimization of inoculum concentration). Care was taken such that no free water was present after inoculation. The contents were mixed thoroughly and incubated in a slanting position at 27 $\pm$ 2°C for 5 days, under 80% relative humidity (Suresh, 1996).

2.4.6 Enzyme recovery

Enzyme, after solid state fermentation using polystyrene, was extracted employing simple contact method using phosphate buffer (0.1M, pH 8.0 (Prabhu, 1996). After mixing the solid fermented substrates with 50 ml of phosphate buffer, the flasks were kept on a rotary shaker (150 rpm) for 30 minutes, and the contents were pressed in a dampened cheese cloth to recover leachate. The process was repeated twice, the extracts were pooled, and centrifuged at 10,000 rpm for 20 minutes at 4°C in a refrigerated centrifuge. The supernatant was used for the enzyme assay.

2.4.7 Assays

2.4.7.1. L-Glutaminase

L-Glutaminase was assayed according to Imada *et al* (1973), with slight modifications, as described under section 2.2.5.

2.4.7.2 Protein

Total cell protein and enzyme protein were estimated using Lowry's method with bovine serum albumin as the standard (Lowry *et al*, 1951), as described under sections 2.2.4 and 2.2.6. respectively. The values were expressed as mg/ml.

2.5. Optimization of process parameters for L- glutaminase production by marine *Beauveria bassiana* under solid state fermentation using polystyrene as inert support.

The impact of various process parameters on L-glutaminase production by marine *B. bassiana* under SSF using polystyrene as inert support was evaluated. The various process parameters studied include incubation temperature, initial pH of the medium, initial moisture content of the medium, inoculum concentration (both spore and vegetative), sea water concentration, substrate (L-glutamine) concentration, additional NaCl concentration, additional carbon source, additional nitrogen source, and amino acids. Finally a time course study was carried out incorporating all the optimised parameters.

The protocol adopted for optimisation of various process parameters influencing glutaminase production was to evaluate the effect of an individual parameter and to incorporate the same at the standardized level in the subsequent experiment before optimizing the next parameter (Sandya and Lonsane, 1994). All the experiments were carried out in triplicate and the mean values are reported.

2.5.1. Initial pH of the medium

The effect of initial pH of the sea water based medium on enzyme production by *B.bassiana* during SSF was studied at various pH levels (pH 4-11) adjusted in the enzyme production medium using 1N NaOH / 1N HCl. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.2. Incubation temperature

Optimal temperature required for maximal enzyme production by *B.bassiana* under SSF was determined by incubating the inoculated flasks at different temperatures (22-47°C) under 75-80% relative humidity. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5respectively.

2.5.3. Additional carbon sources

Requirement for additional carbon sources other than L-glutamine, in the medium, for enhanced enzyme production by *B. bassiana* under SSF was tested by the addition of different carbon sources (glucose, maltose, lactose, Sucrose, sorbitol and mannitol) at 1% (w/v)level in the SSF medium. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.3.1. D- glucose

Since D-glucose was found to promote enhanced enzyme production (2.4.8.3) as additional carbon source optimal concentration of the same, was determined by conducting the solid state fermentation at different concentrations of the same (0.25-4.0 % w/v) incorporated in to the SSF medium. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.4. Additional nitrogen sources

Requirement for additional nitrogen sources, other than L-glutamine, for enhanced enzyme production by *B.bassiana* under SSF was tested by the addition of different nitrogen sources(Beef extract, malt extract, yeast extract, peptone, ammonium sulphate, ammonium nitrate, calcium nitrate and potassium nitrate) at 1% (w/v)level in the SSF medium. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.5. Amino acids

Requirement for amino acids in addition to L-glutamine for enhanced enzyme production by *B.bassiana* was tested by the addition of different amino acids(glutamic acid, asparagine, arginine, methionine, proline and lysine,) at 1% (w/v)level in the SSF medium. Preparation of solid substrate, preparation of inoculum, inoculation and

incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5.respectively.

2.5.6. L-glutamine concentration.

The influence of L-glutamine concentration on glutaminase production by *Beauveria bassiana* during SSF was evaluated by supplementing the fermentation medium with different concentrations of glutamine (0.1-5.0% w/v). Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5. respectively.

2. 5.7. Initial moisture content.

Optimal level of initial moisture content of the solid support medium required for maximal enzyme production under SSF was determined by preparing the solid substrates with varying levels of moisture content in the range of 60-100%, using different quantities of the basal media prepared in sea water (section 2.4.2). Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.8. Inoculum concentration

2.5.8.1. Spore inoculum

Optimal concentration of spore inoculum that enhance maximal enzyme production under SSF by *B.bassiana* was determined at different spore concentrations ranging from

6.0 - 48 x 10^8 spores/ml. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.2.2.1, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.8.1. Vegetative inoculum

Optimal concentration of vegetative mycelial inoculum that enhance maximal enzyme production under SSF by *B.bassiana* was determined at different inoculum concentrations varying from 0.125 - 0.750 mg/ ml (w/v). Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.2.2.2, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.9. Sea water concentration

The optimal concentration of aged sea water required for enhanced glutaminase production by *B.bassiana* during solid state fermentation was evaluated by diluting with distilled water to various levels of concentration (0, 25, 50, 75 and 100 % v/v) and preparing the solid state fermentation media. Aged sea water as such was considered as 100% for comparison purposes. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.10. Additional NaCl Concentration

Effect of NaCl, added to aged sea water, on enzyme production of *B. bassiana* under solid state fermentation was evaluated by incorporating NaCl at different concentrations (0.0 to 4.0% w/v) in the medium, prepared with aged sea water. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively.

2.5.11. Time course study

After optimising the various process parameters, a time course study was carried out by providing optimised bioprocess conditions. Preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.4.4, 2.4.5, 2.4.6 and 2.2.5 respectively. Total cell protein and Enzyme protein were also determined as described under 2.2.4 and 2.2.6. respectively.

2.6. L-glutaminase production by terrestrial *Beauveria bassiana* under solid state fermentation and submerged fermentation.

L - glutaminase production by terrestrial *B. bassiana* NCIM 1216 (obtained from the culture collection of National Chemical Laboratory, Pune, India) was determined under solid state fermentation and submerged fermentation conditions using media based on both sea water and distilled water at enzyme production conditions optimised for the marine *Beauveria bassiana* strain.

For solid state fermentation preparation of solid substrate, preparation of inoculum, inoculation and incubation, extraction and assay of enzyme were carried out as described earlier under sections 2.4.3, 2.2.2.1, 2.4.5, 2.4.6 and 2.2.5respectively.

For submerged fermentation, inoculation and incubation were done as mentioned earlier under section 2.2.3. After incubation, the fermented broth was centrifuged, supernatant was collected, and enzyme was assayed as described under section 2.2.5.

2.7. L-glutaminase production by immobilized marine *Beauveria bassiana.*

Although several methods are available to effect immobilization such as entrapment, cross linking, covalent binding and physical adsorption, in the present study gel entrapment method using calcium alginate was adopted for the preparation of immobilized spores of marine *B. bassiana.*

2.7.1. Media

Enzyme production media used for L- glutaminase production by immobilized spores of marine *B. bassiana* included

L- glutamine	0.25 %
D- glucose	0.5 %
pH	9.0
Aged sea water	100 ml.

The prepared medium was autoclaved at 121°C for 1hour and used.

2.7.2 Preparation of spore suspension

Spore suspension was prepared as described under section 2.2.2.1

2.7.3 Preparation of support material for immobilization

Initially 6g of sodium alginate was slowly added to 150 ml of distilled water (4%) while being continuously stirred (Mohandass, 1992). The stirring was continued for a further period of 1-2 hours in a magnetic stirrer until complete dissolution of the sodium alginate was effected. The prepared sodium alginate solution was autoclaved at 121 ° C for 20 minutes and used for preparation of beads.

2.7.4 Preparation of beads

Beads with immobilized fungal spores were prepared as suggested by Mohandass, (1992). Under sterile conditions the prepared spore slurry was mixed with the sodium alginate solution, at a concentration of 12 x 10^8 spores/ ml, at a ratio of 1:2 and mixed thoroughly using a sterile glass rod to get a spore - alginate slurry. This was then extruded drop wise in to a solution of 0.2 M $CaCl_2$ from a height of about 10 cm using a syringe with a pore size of 2 mm. The entrapped calcium alginate beads were then maintained in a solution of $CaCl_2$ (0.2 M) for 2 hours for curing. After this the beads were thoroughly washed with physiological saline 3-4 times and maintained at 4°C until use.

2.7.5. Activation of immobilized spores.

The prepared beads with immobilized fungal spores were suspended in a solution of 1 % glutamine in aged sea water, pH 9.0, in a conical flask and incubated on a rotary shaker (90 rpm), at 28 $\pm$ 2 ° C, for 14 hours (prior to optimisation of activation time). The activated beads were then removed, washed twice with fresh enzyme production medium and used for further studies.

2.7.6. Incubation procedures

Twenty grams of beads with immobilized spores were weighed, transferred to 50 ml of enzyme production media taken in a 250 ml conical flask, kept on a rotary shaker (90 rpm), and incubated for 24 hours at room temperature (28 $\pm$ 2 ° C).

2.7.7. Enzyme assay

After incubation for the desired period, the enzyme production media was decanted from the flask, centrifuged at 10,000 rpm for 10 minutes at 4 ° C in order to remove leached out spores if any. Supernatant obtained was assayed for L-glutaminase following the method of Imada *et al* (1973), with slight modifications as described under section 2.2.5.

2.8. Optimisation of immobilization process conditions.

Optimal concentration of immobilizing support material, concentration of fungal spore in the beads, $CaCl_2$ concentration, curing time of beads, activation time and

retention time, incubation temperature and pH of the media, that could promote maximal production of L - glutaminase by immobilized *B. bassiana* were determined as detailed below.

2.8.1 Support concentration

The optimal support concentration required for the preparation of active and stable beads with maximum enzyme production was determined using sodium alginate at different concentrations (1.5-5 % w/v). Immobilized spore in beads were prepared as mentioned under section 2.7.4.

2.8.2. Spore concentration in the beads

Beads with immobilized spore were prepared using spores at different concentrations (2-16x10^8 spores/gm beads) and effect of the same on L-glutaminase production was determined (section 2.7.7).

2.8.3. Calcium Chloride concentration.

The optimal concentration of calcium chloride required for the preparation of active and stable immobilized viable spore beads with maximum enzyme production was determined by using calcium chloride at different molar concentrations (0.05M - 0.4 M).

2.8.4. Curing time

Optimum curing time for preparing stable immobilized viable spore beads were determined by allowing the beads formed in 0.2 M $CaCl_2$, to remain as such for varying periods of curing time (1-5 hours). Later, the beads were washed with physiological

saline and the optimal curing time was assessed by estimating L-glutaminase (section 2.7.7) production in the medium.

2.8.5 Activation Time

Optimal activation time for enhanced L-glutaminase production by immobilized fungal spores was determined by incubating the beads with immobilized spores in a solution of 1 % glutamine in sea water, pH 9, on a rotary shaker at 90 rpm for varying time intervals (5-25 hours) at room temperature (28 ± 2^{0} C), followed by incubation in enzyme production medium as described under section 2.7.6. The optimal activation time was assessed by quantifying the L-glutaminase (section 2.7.7) in the media.

2.8.6. Retention time

Optimal retention time required for maximal L-glutaminase production was determined by incubating the beads with immobilized fungal spores in the production medium for varying periods and estimating L-glutaminase production as mentioned under section 2.2.5.

2.8.7. Incubation Temperature.

Optimal temperature that support the maximum enzyme production by immobilized spores of *B. bassiana* was found out by incubating the beads at different

temperatures 22, 27,32, and 37°C and then estimating the L-glutaminase production as mentioned under section 2.7.7

2.8.8. pH of the media

Optimal pH of the media that support maximal enzyme production by beads with immobilized spores was determined by subjecting the beads to media with different pH, ranging from 5-10, and then estimating the L- glutaminase production as mentioned under 2.7.7

2.9. Continuous production of L-glutaminase by immobilized spores of marine *B. bassiana.*

Continuous production of L-glutaminase by gel entrapped immobilized spores of marine *B. bassiana* was monitored in a Packed Bed Reactor. The effect of flow rate of the media in to the reactor, aeration rate and bed height, were determined. The cumulative production of glutamine in packed bed reactor was also determined.

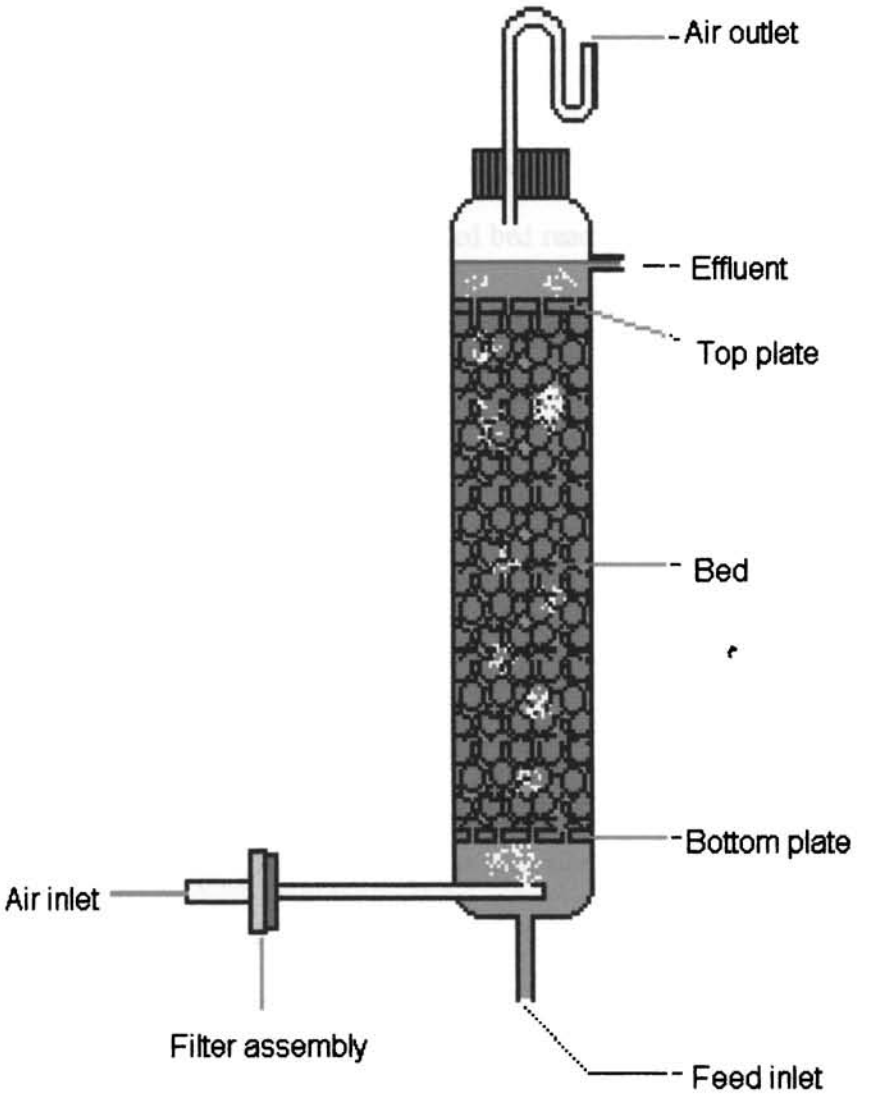
2.9.1. Packed Bed Reactor.

The cylindrical glass column of 5 cm diameter, 30 cm length was used as the reactor (Fig.1). The bottom of the column which was hemispherical in shape with a 5 mm diameter in let, was packed with small amount of glass wool and glass beads of 4 mm diameter up to 20 mm height. A sieve plate with 1 mm perforations were placed over the glass beads and immobilized spore beads were packed up to different heights. A sieve plate was placed over the beads and fixed to the top of the reactor. The column

Fig.1

Continuous production of glutaminase in a packed bed reactor

Experimental set up for packed bed reactor studies



was provided with a side tube of 5 mm diameter at the top portion, through which the sample broth was withdrawn. The reactor was fed from the bottom using a peristaltic pump (Murhopye. India Ltd.) and the effluent was removed from the top. Sterile air was admitted in to the reactor through a bacterial filter (Millipore) from an air pump with flow meter (Eyela, Japan)

2.9.2 Activation of the packed bed reactor

The immobilized bed in packed bed reactor was activated using 1 % glutamine solution in sea water (pH 9) for a period of 15hours, before commencing the experiment.

2.9.3. Estimation of void volume

The voidage of the packed bed reactor was determined by measuring the volume of the liquid actually occupied in the reactor. The reactor was packed with spherical gel beads to the desired level and bed depth was noted. Physiological saline was filled from the bottom with the help of the peristaltic pump till the liquid layer reached to the top layer of the gel beads. The liquid was then slowly withdrawn from the column till its level reached the bottom layer of the bead and the quantity of saline thus collected was measured. The procedure was repeated thrice and the average value was taken.

2.9.4. Enzyme Recovery

Effluent medium, from the packed bed reactor, was recovered from the upper outlet of the reactor at one hour intervals and those along with the accumulated effluent

from the container, was centrifuged at 10,000 rpm, at 4^0 C for 20 minutes and the supernatant was used for enzyme assay.

2.9.5. Enzyme assay

L-Glutaminase in the effluent medium collected from the out let and container were assayed separately following the method of Imada *et al* (1973) with slight modifications, as described under section 2.2.5.

2.9.6. Flow rate

Effect of flow rate of the media on the continuous production of L- glutaminase by the immobilised spores of marine *Beauveria bassiana* and the performance of the immobilised reactor was evaluated by monitoring the rate of L-glutaminase production under different flow rates(20-60 ml/hour). For each flow rate samples were withdrawn after one hour interval for up to five hours and the rate of enzyme production was determined.

2.9.7. Substrate concentration

Optimal substrate concentration required for maximal glutaminase production by immobilized spores of *B.bassiana* under continuous production in a packed bed reactor was determined as detailed below. Prepared beads of immobilized spores were packed to the required height in the reactor and the substrate solution having different concentrations (0.125 - 1.0 %) were passed through the reactor one after another separate cycles. Enzyme production was assayed as described under section 2.2.5.

2.9.8. Aeration rate

The effect of aeration on L-glutaminase production by immobilized *B. bassiana* was determined by supplying sterile air through a filter at rates ranging from 0.4 - 1.7 vvm.

2.9.9. Bed Height

Optimal bed height required for maximal glutaminase production by immobilized *Beauveria bassiana* under continuous production in a packed bed reactor was determined by raising the bed height from 5- 15 cms. at a flow rate of 20 ml/ hr. at $28 \pm 1^{\circ}C$. Enzyme production was assayed as described under section 2.2.5.

2.10. Statistical analysis.

All the experiments and analysis were carried out in triplicate and mean values alone are taken. Statistical Analysis (Standard Deviation) was done using SIGMA STAT- version 2.01

Results

3.RESULTS

3.1. L-Glutaminase production by marine *Beauveria bassiana* under Submerged Fermentation(SmF).

Extracellular L-glutaminase production by marine *B. bassiana* under submerged fermentation was evaluated in distilled water based medium. Initially the various process parameters that influence the rate of enzyme production were optimized towards maximal enzyme production.

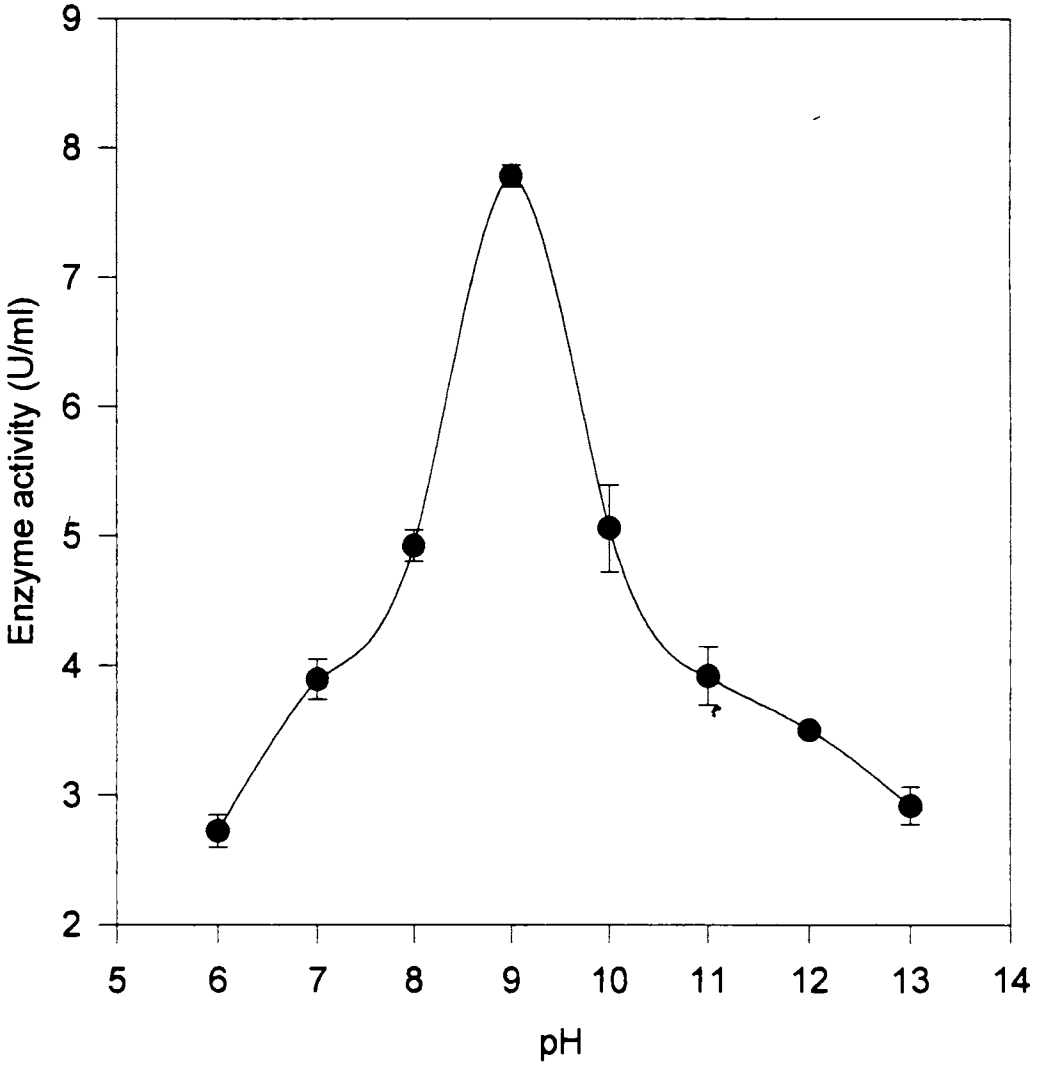
3.1.1. pH .

The results presented in Fig.2. indicate that pH of the fermentation media influence enzyme production by marine *B. bassiana*. Thus maximal enzyme production was observed at pH 9.0 (7.78 U/ml), and pH above or below pH 9.0 led to a decrease in L-glutaminase production by this fungus. Results also suggest that this marine fungus is alkalophilic in nature.

3.1.2. Incubation temperature.

Incubation temperature influenced the rate of L-glutaminase production by marine *B. bassiana*. Thus maximal L-glutaminase production was observed at 27° C (14.11 U/ml), and further increase in temperatures adversely affected enzyme

Fig.2
Effect of pH on enzyme production by *Beauveria* sp.under SmF.



production (Fig.3). Nevertheless, considerable level of L-glutaminase production was recorded at 22°C.

3.1.3. Additional Nitrogen Sources.

Incorporation of additional nitrogen sources, along with L-glutamine in the enzyme production medium influenced the rate of L-glutaminase production by marine *B. bassiana*. From the results presented in Fig.4. it is inferred that among the organic nitrogen sources tested yeast extract supported maximum enzyme production (14.59 U/ml) followed by beef extract (12.85 U/ml) and peptone (12.64 U/ml).

Further studies on optimization of concentration of yeast extract indicated that 2 % (w/v) yeast extract concentration is optimal for maximal enzyme production (16.38 U/ml) and further increase in the concentration of yeast extract resulted in a decrease in enzyme production (Fig.5).

Among the inorganic nitrogen sources tested Potassium nitrate promoted maximal enzyme production (13.96 U/ml) followed by Calcium nitrate (9.17 U/ml)(Fig.6). Whereas, both Ammonium sulphate and Ammonium nitrate inhibited enzyme production.

Since Potassium nitrate supported enhanced level of enzyme production optimal concentration of the same was standardized. Among the different concentrations of Potassium nitrate tested 1 % (w/v) concentration promoted maximal enzyme production (Fig.7). Further increase in potassium nitrate concentration resulted in a mild decrease in enzyme production.

Fig.3
Effect of temperature on enzyme production by *B.bassiana* under SmF.

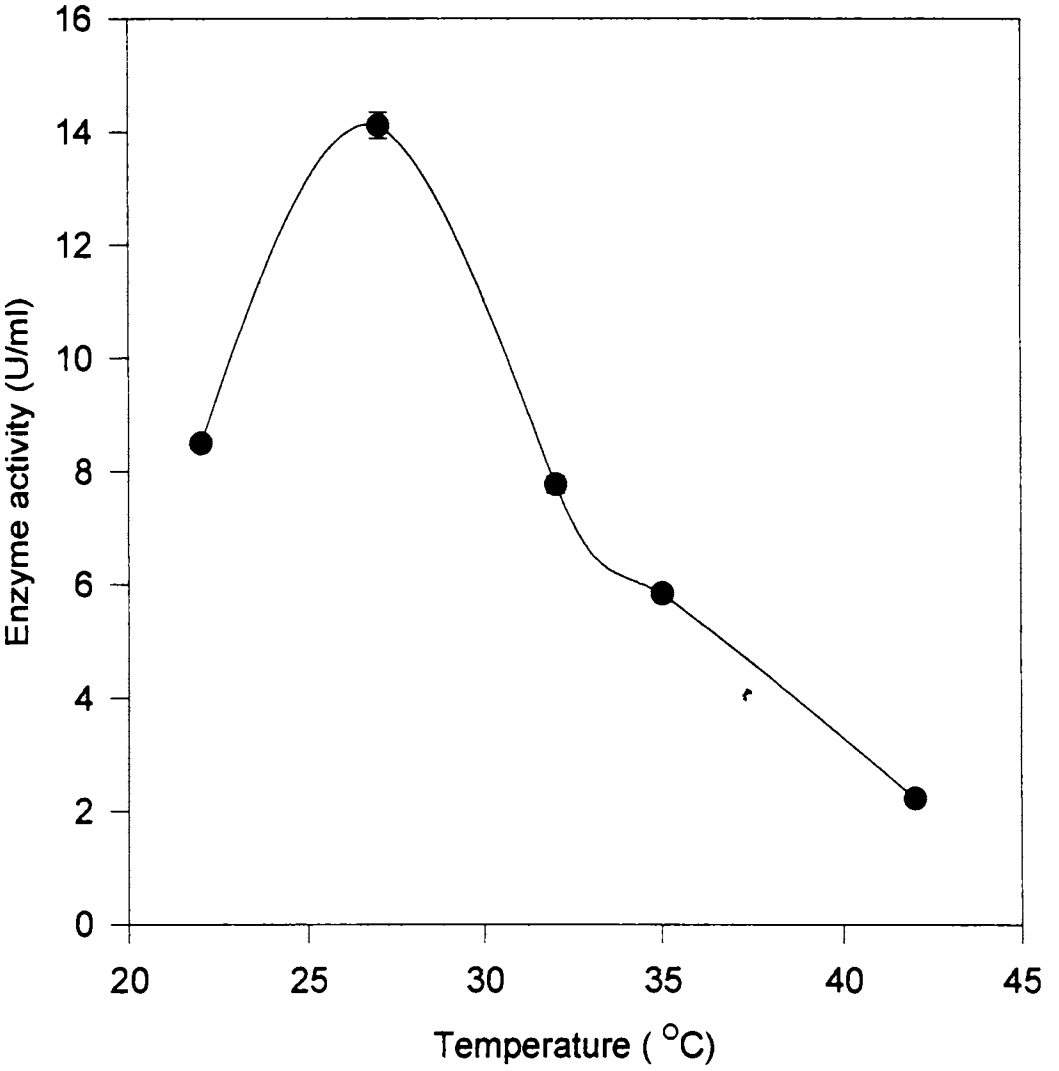


Fig.4
Effect of organic nitrogen sources on enzyme production by *B.bassiana* under SmF.

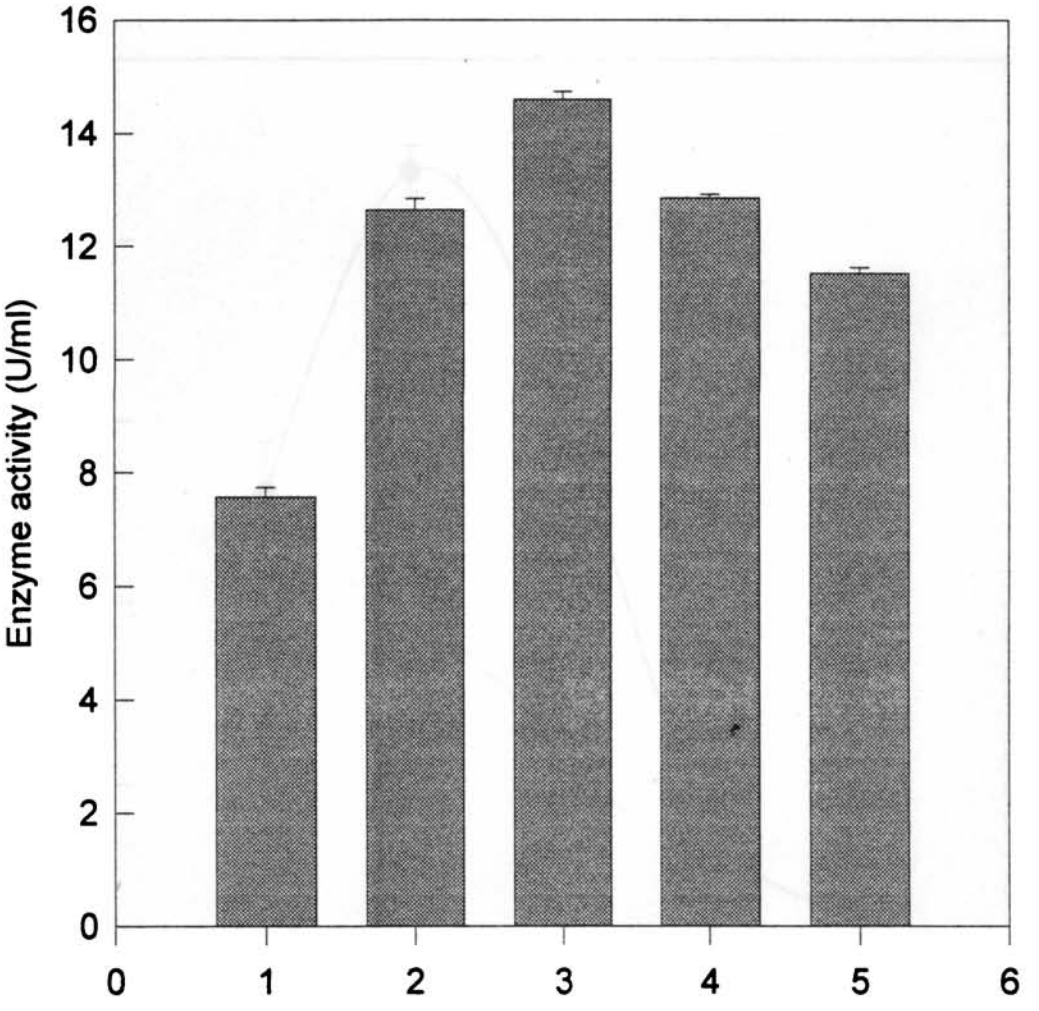


Fig. 5
Optimization of Yeast extract concentration for enzyme production by *B.bassiana* under SmF

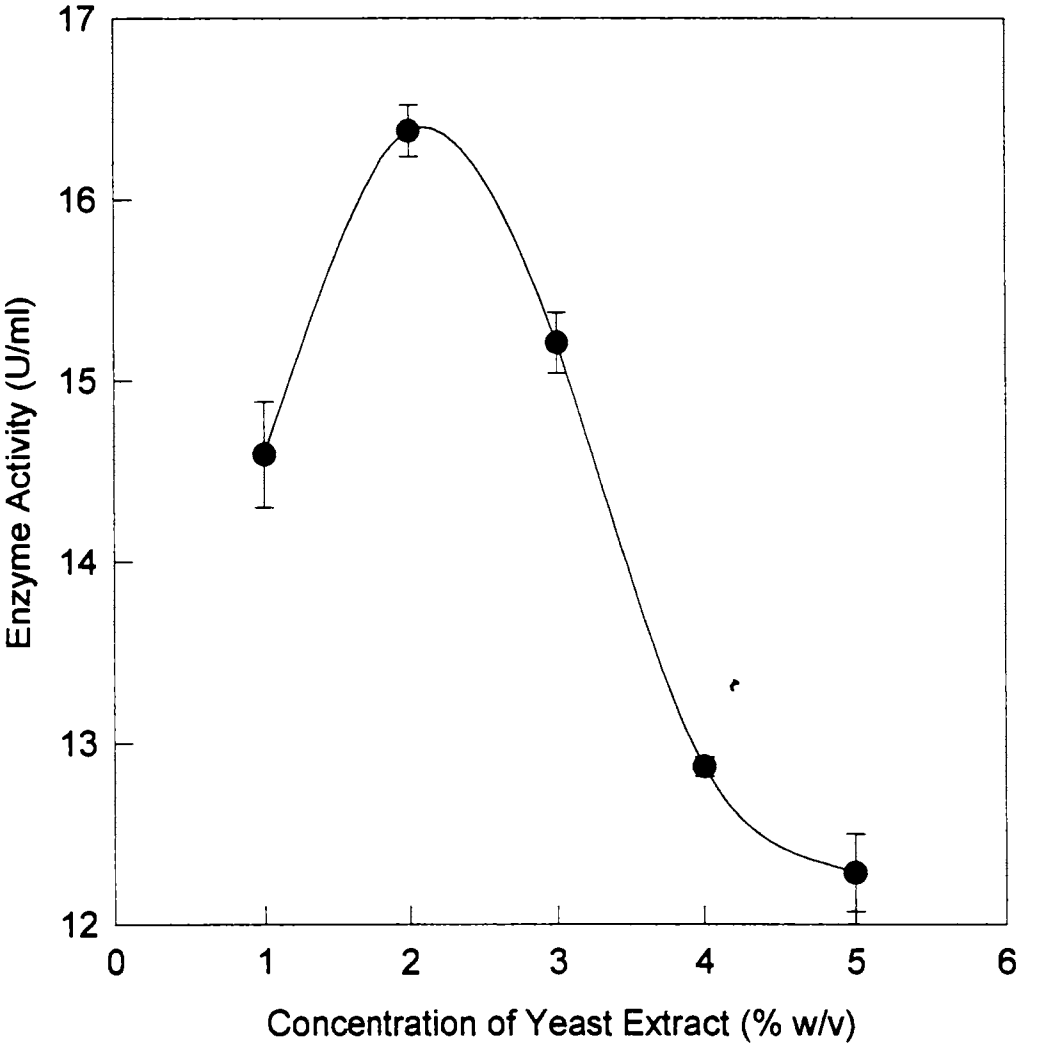


Fig. 6
Effect of inorganic Nitrogen sources on enzyme production by *B.bassiana* under SmF

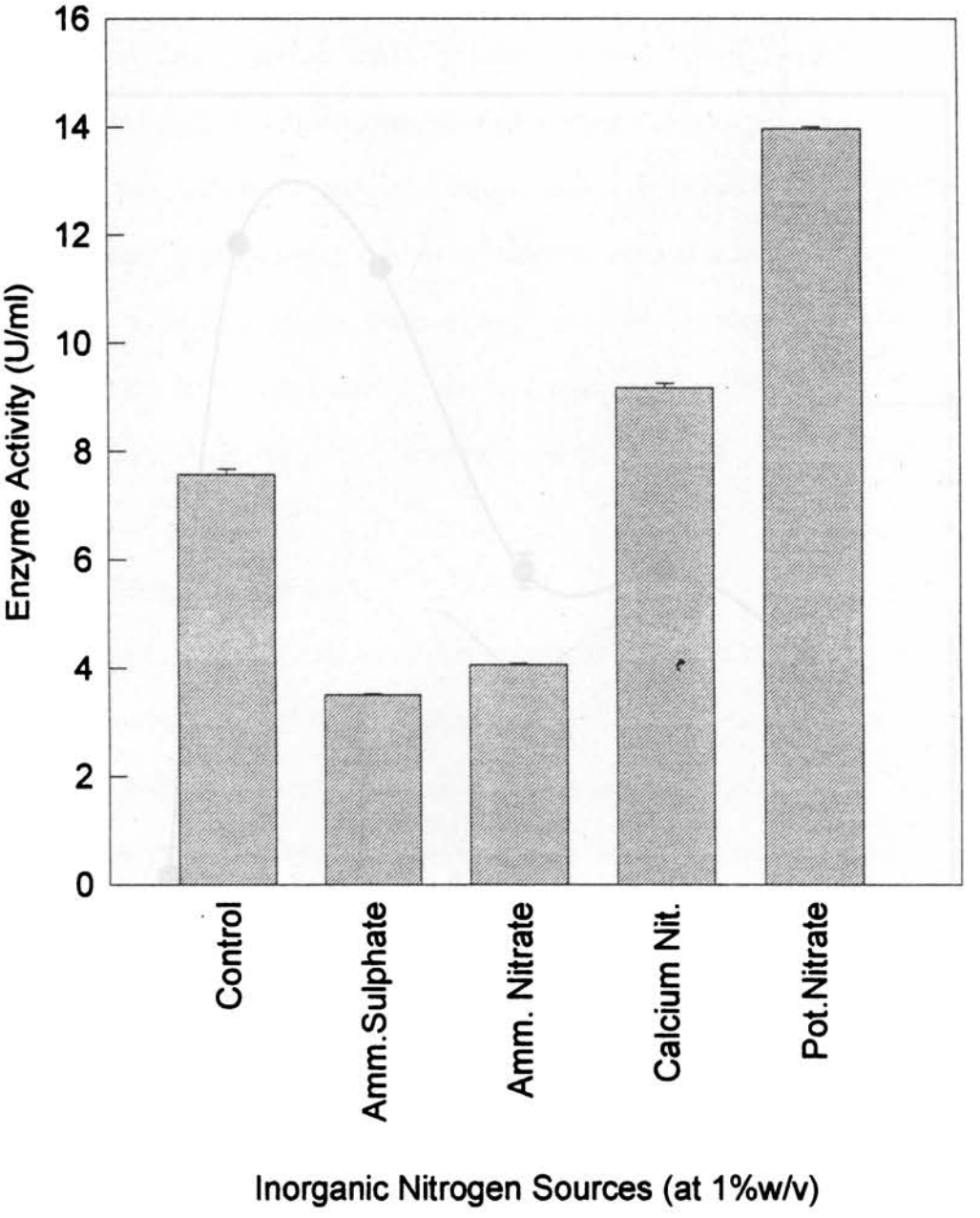
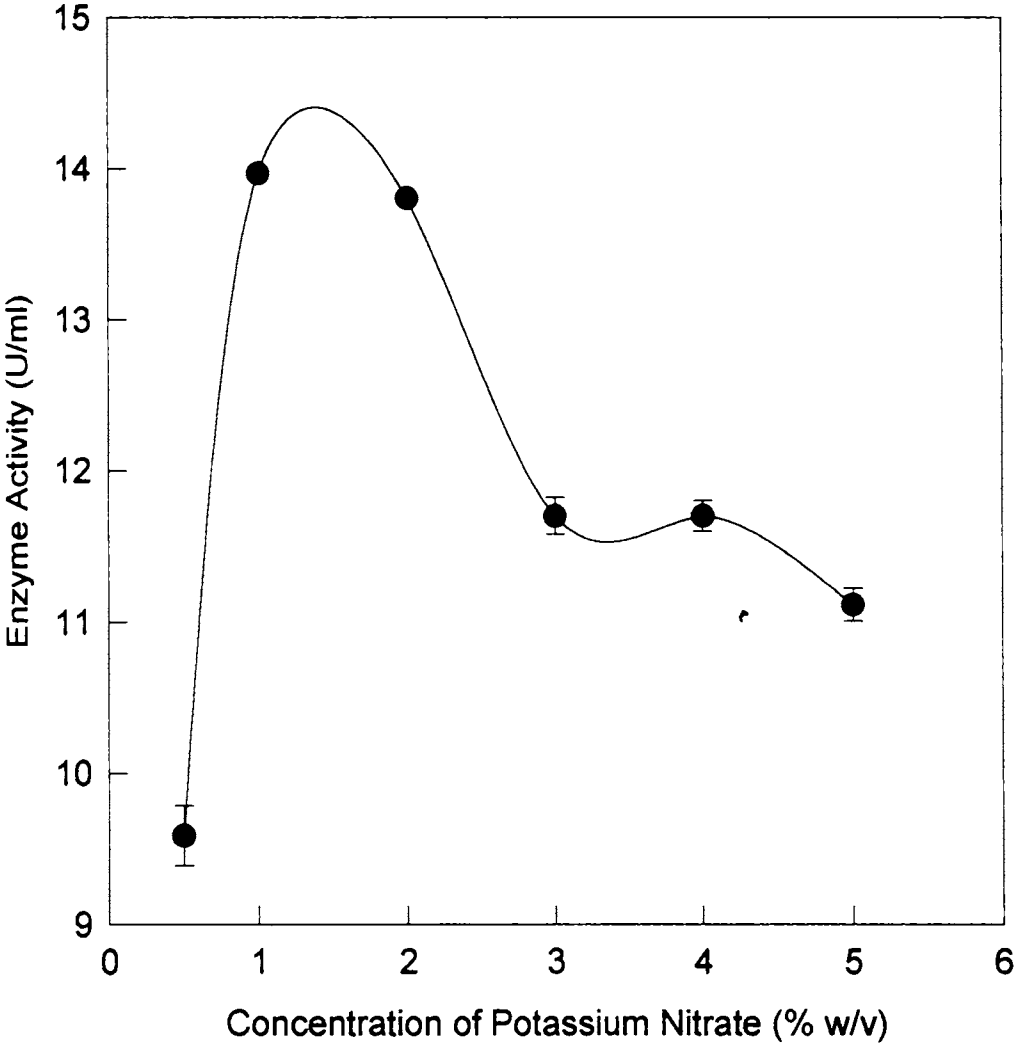


Fig.7
Optimization of Potassium Nitrate concentration for enzyme production by *B.bassiana* under SmF



3.1.4 Additional Carbon Sources

Presence of additional carbon sources, along with L-glutamine in the medium promoted enzyme production by marine *B. bassiana*. All the carbon sources tested enhanced L-glutaminase production compared to control experiment (Fig.8). Among the various carbon sources tested, Sorbitol promoted maximal enzyme production (15.03 U/ml), followed by Sucrose, Mannitol, Maltose and Glucose.

Since Sorbitol, as additional carbon source, promoted enhanced level of L-glutaminase, in the enzyme production medium, optimal concentration of Sorbitol required in the medium was standardized. A two fold enzyme yield was obtained (15.03 U/ml) at 1% (w/v) Sorbitol concentration (Fig.9). However, further increase in Sorbitol level resulted in a rapid decrease in enzyme production.

3.1.5 Effect of Amino acids

Need for amino acids as inducer compound for enhanced enzyme production was evaluated by incorporating different amino acids along with the carbon source (D-glucose) in the enzyme production medium. The results presented in Fig. 10. showed that all the amino acids tested, except Asparagine, supported enhanced level of enzyme production, compared to glutamine. Particularly Methionine was observed to promote maximal L-glutaminase synthesis (15.82 U/ml), followed by Proline (14.97 U/ml) and Arginine(14.27 U/ml). Glutamic acid, Lysine, and L-asparagine inhibited L-glutaminase synthesis, since the level of enzyme produced was very less compared to glutamine.

Fig. 8
Effect of additional Carbon Sources on enzyme production by *B.bassiana* under SmF

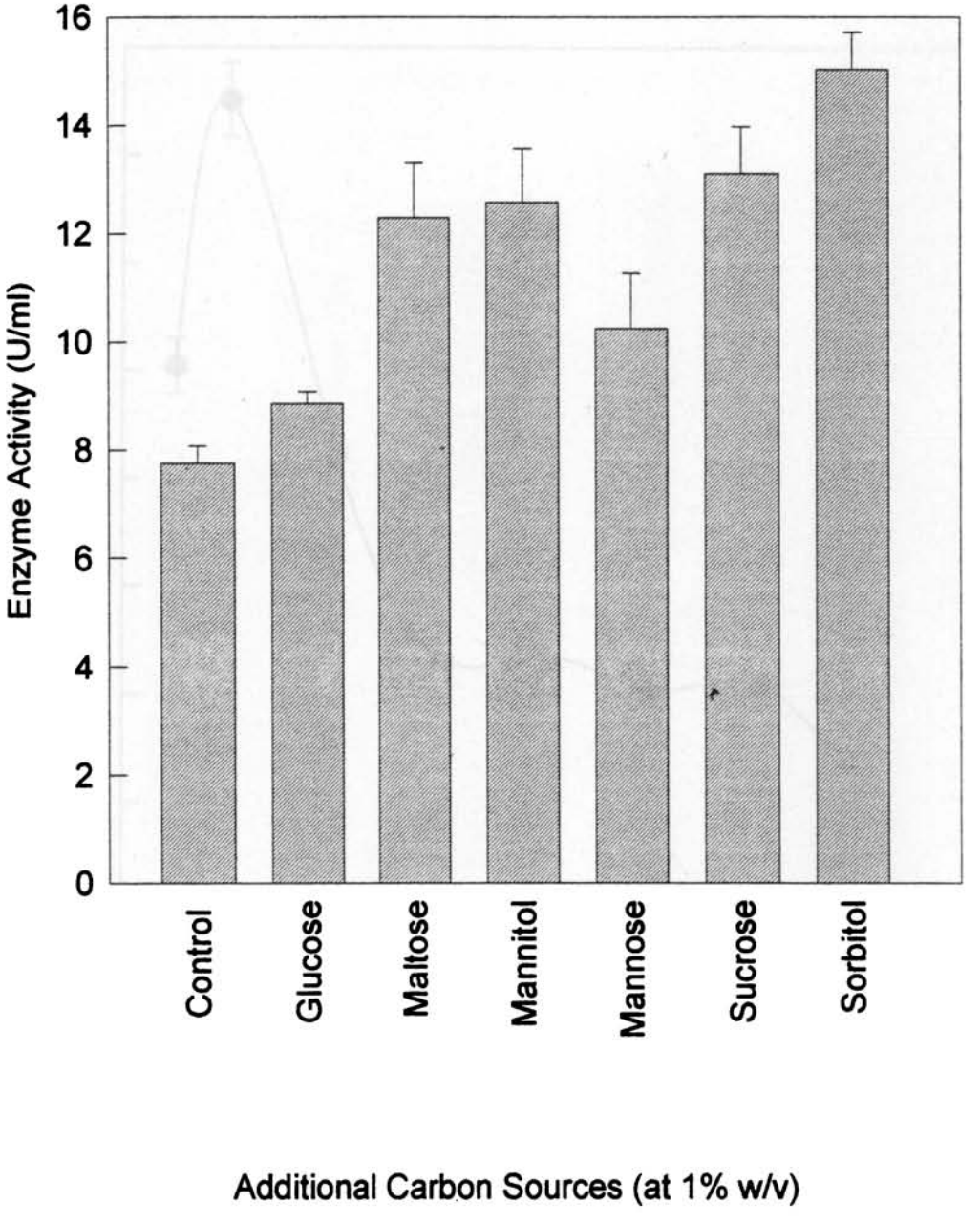


Fig. 9
Optimization of Sorbitol concentration for enzyme production by *B.bassiana* under SmF

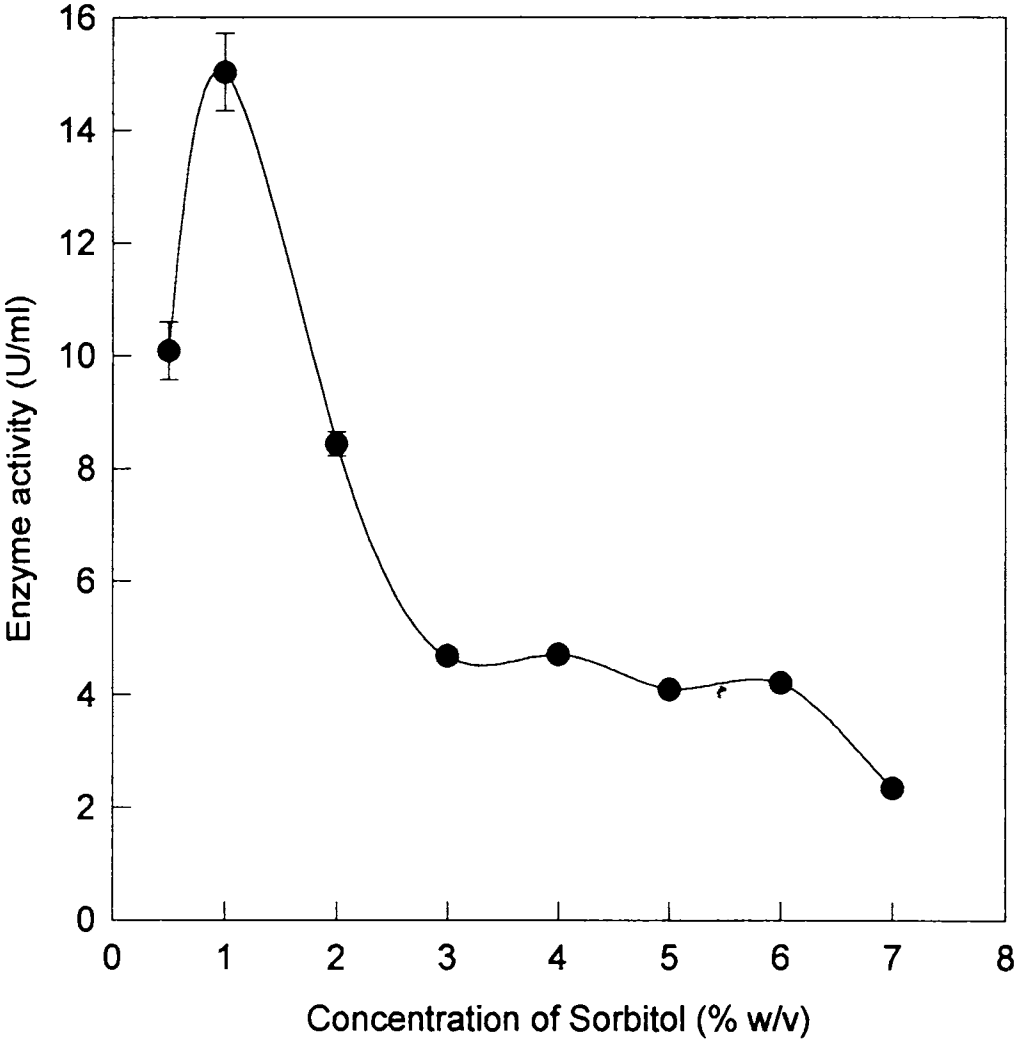
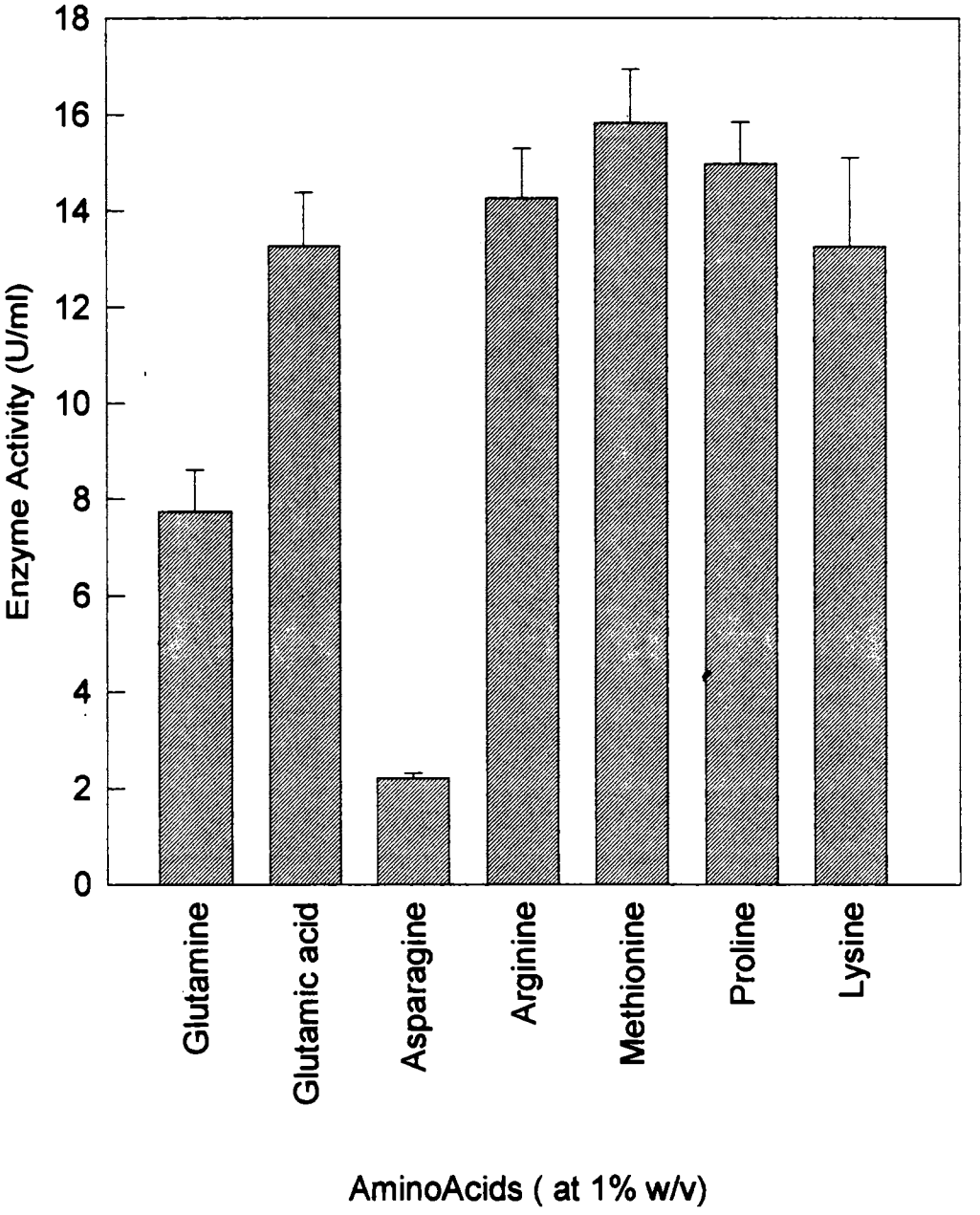


Fig.10
Effect of Aminoacids on enzyme production by *B.bassiana* under SmF



3.1. Methionine concentration.

Since Methionine promoted maximal enzyme production compared to L-glutamine, optimal concentration of the same as enzyme substrate was evaluated. From the results obtained (Fig.11)for the different Methionine concentrations tested, it was evident that 0.2 %(w/v) concentration promoted maximum production of L-glutaminase (17.33 U/ml). Further, in general, concentrations up to 1 % were observed to support enhanced enzyme production.

3.1.7. NaCl concentration.

Presence of sodium chloride in the basal media led to increased enzyme synthesis. Among the concentrations tested 9% (w/v) NaCl supported maximal enzyme production (Fig.12). With further increase in sodium chloride concentration enzyme production decreased rapidly. It was observed that enzyme synthesis was very less at sodium chloride concentrations between 0.0 and 6 %(w/v). Further, these results also indicated a halophilic nature of the strain used in the present study.

3.1.8. Time course experiment.

After optimization of various bioprocess conditions, a time course experiment was carried out to monitor the rate of L-glutaminase production by marine *B. bassiana* at optimized conditions. Results presented in Fig.13 evidence that L-glutaminase production increased progressively along with increase in incubation time until 108 hours, when maximal enzyme production was recorded (47 U/ml).

Fig.11
Effect of Methionine Concentration on enzyme production by *B.bassiana* under SmF

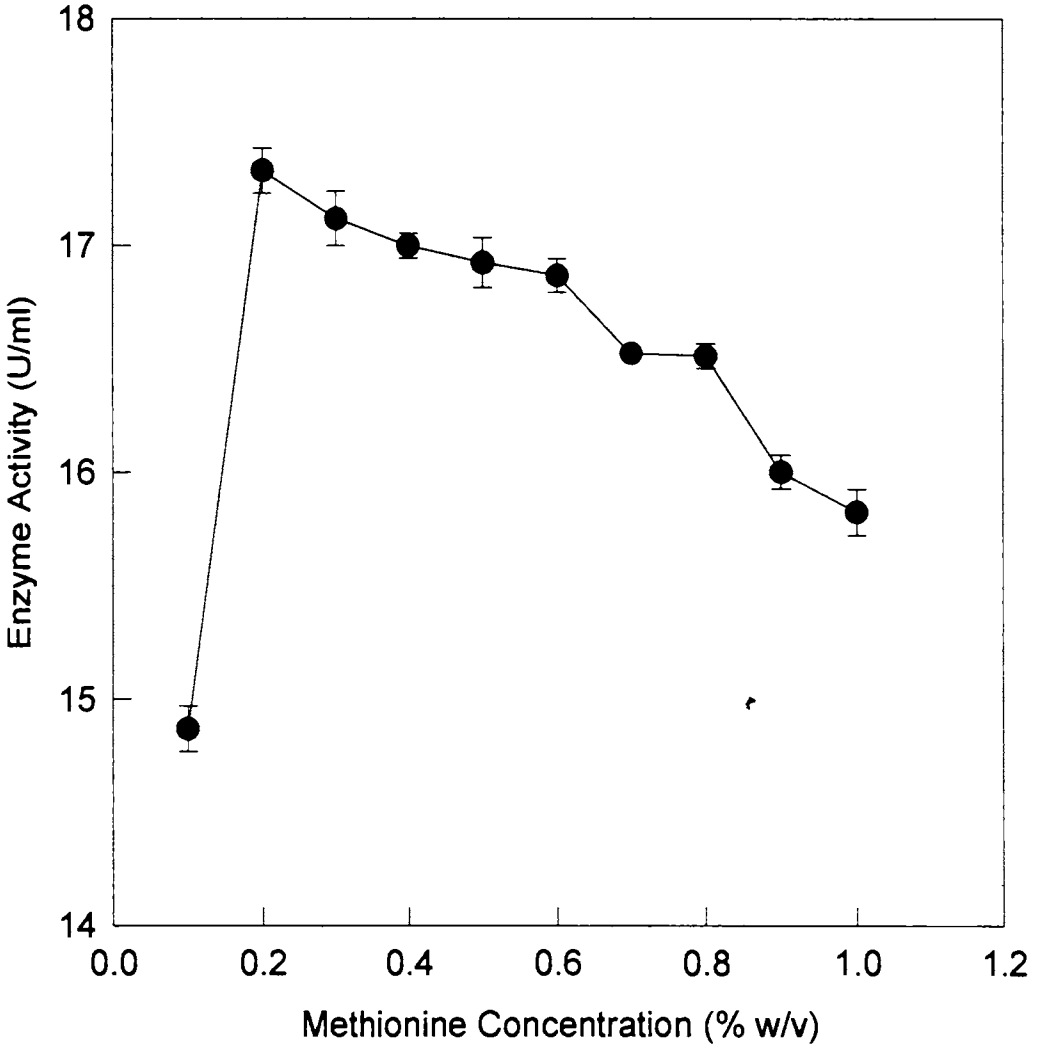


Fig. 12 .
Effect of NaCl concentration on enzyme production by *B.bassiana* under Smf

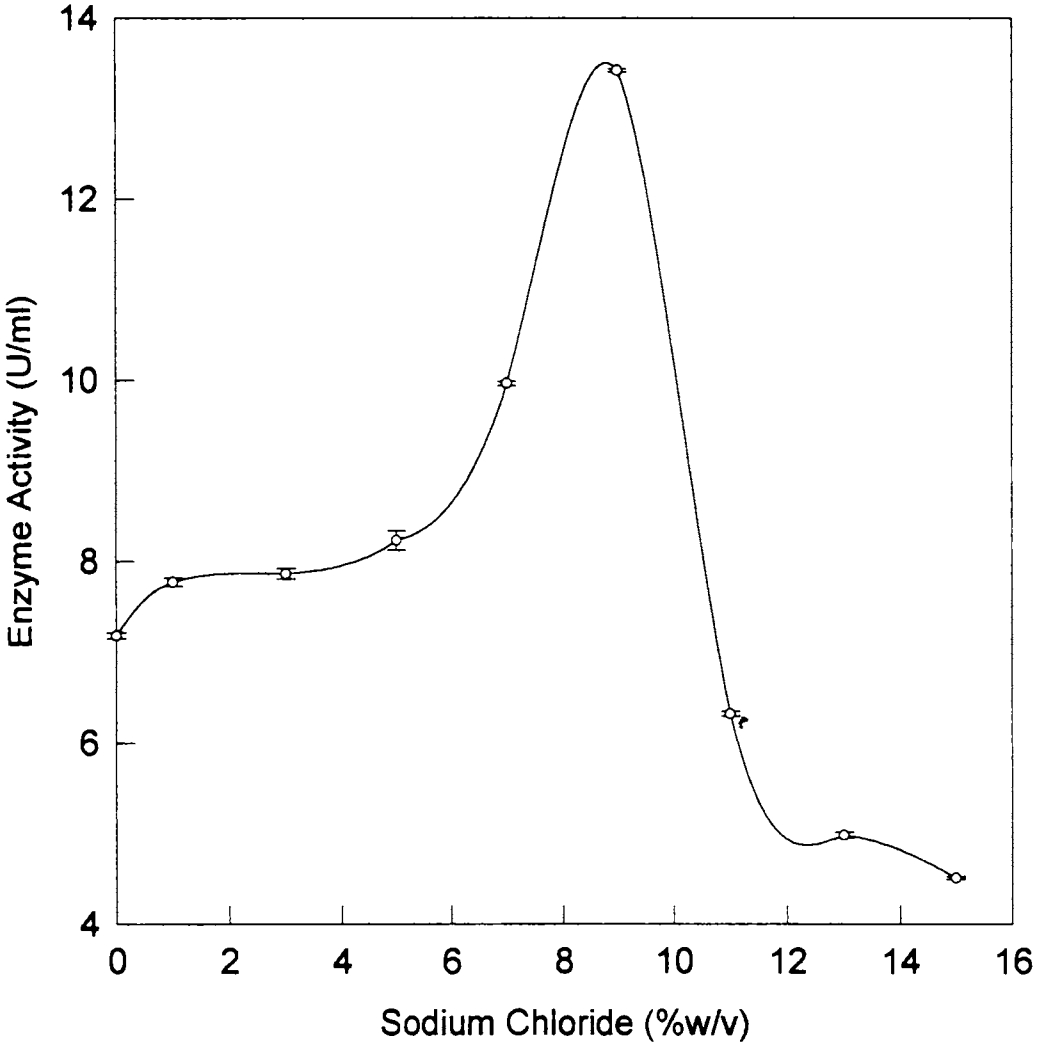
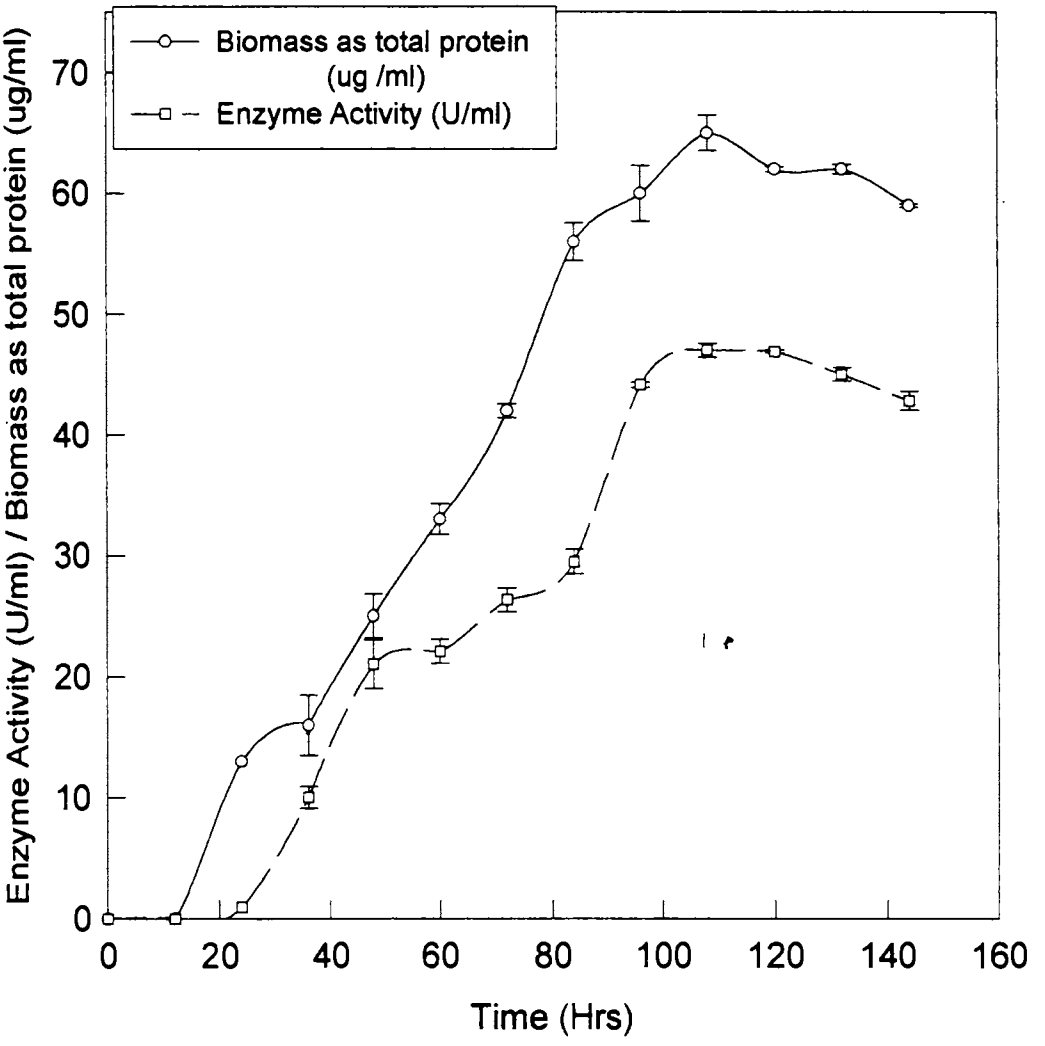


Fig.13.
Time course of growth and enzyme production by *B.bassiana* under SmF



During the time course study it was observed that this strain produced L-glutaminase along with rapid growth, suggesting that the enzyme production is growth associated. However, enzyme production was observed only after 24 hours of incubation. Rapid and enhanced enzyme production was recorded during 24 - 48 hours and between 84 - 96 hours, while there was a lag during the rest of the period between 48 - 84 hours. Results also indicated that enzyme production was associated with actively growing log phase culture and not during stationary phase, as the level of enzyme production remained stagnant during stationary phase of the culture. The biomass values also remained same for some time after 108 hours, which later declined.

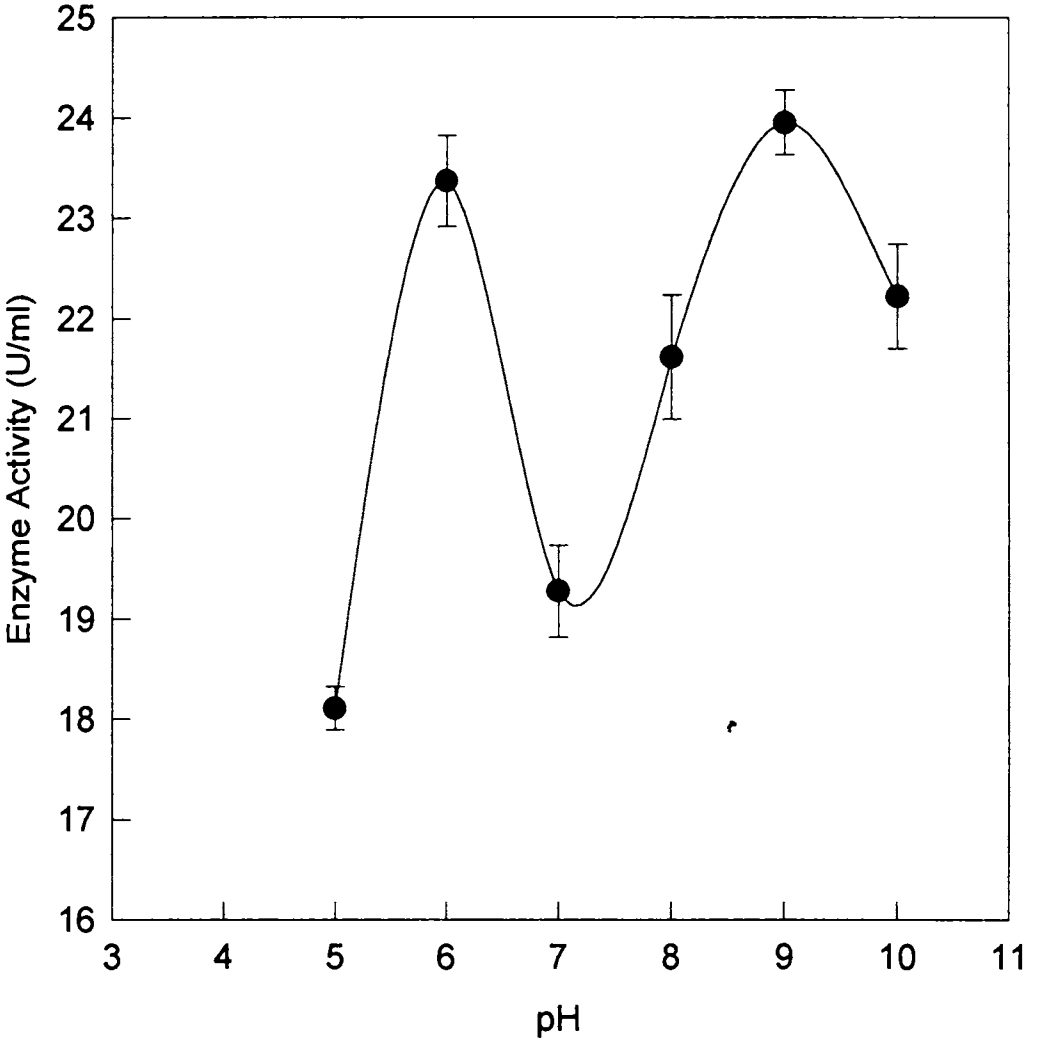
3.2. L-Glutaminase production by marine *Beauveria bassiana* under Solid state fermentation using polystyrene in sea water based medium.

Extracellular L-glutaminase production by marine *B. bassiana* under Solid state fermentation using polystyrene as inert solid support was evaluated in sea water based medium. Initially the various process parameters which influence the enzyme production were optimized towards enhanced enzyme production.

3.2.1. pH .

The results presented in Fig.14 indicated that pH does not seriously influence L-glutaminase production by marine *B. bassiana* under solid state fermentation using polystyrene. It was observed that this marine fungus has two pH optima that could support maximal enzyme production, one at pH 9.0 (23.96 U/ml), and another at pH 6.0 (23.37 U/ml), although it could produce invariably considerable level of enzyme

Fig.14
Effect of pH on enzyme production by *B.bassiana* under SSF



over a wide range of pH between 5.0 (18.11 U/ml) and pH 10.0 (22.2 U /ml). These observations also suggest that this fungal strain is tolerant to a wide range of pH.

3.2.2. Incubation temperature.

Data presented in Fig.15 suggested that marine *B. bassiana* could produce L-glutaminase at relatively higher levels over a wide range of incubation temperature varying from 22°C (29.42 U/ml) to 47°C (21.41 U/ml) with a maximum at 27°C (32.1 U/ml). However, it was observed that temperatures above 27°C did not enhance enzyme production compared to lower temperatures.

3.2.3. Additional Carbon Sources.

Requirement for additional carbon sources, in the fermentation medium, for enhanced enzyme production was evaluated by incorporating different carbon sources along with L-glutamine. The results presented in Fig.16, indicated that, among the various carbon sources tested, D- glucose and maltose alone enhanced L-glutaminase compared to control in the presence of L-glutamine. While glucose at 1% (w/v) level enhanced L-glutaminase production to 32.17 U/ml compared to 25.52 U/ml recorded with L-glutamine alone, maltose effected only a marginal increase in enzyme yield. All the other carbon sources tested resulted in a decrease in enzyme yield when compared to control.

Since, D-glucose, as additional carbon source, enhanced enzyme production, in the presence of L-glutamine, optimal concentration of D-glucose required for enhanced glutaminase production was optimized. A gradual increase in enzyme yield along with

Fig. 15
Effect of temperature on enzyme production by *B.bassiana* under SSF

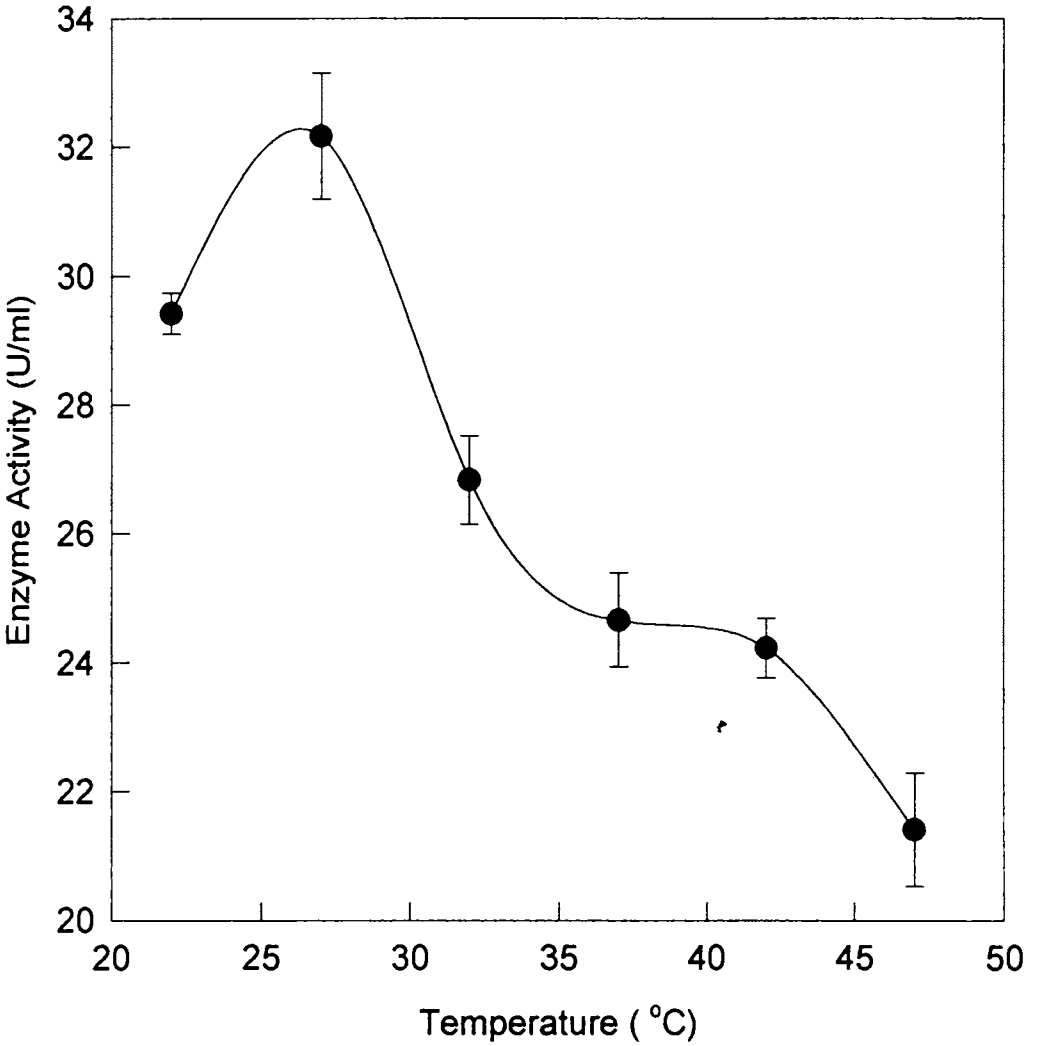
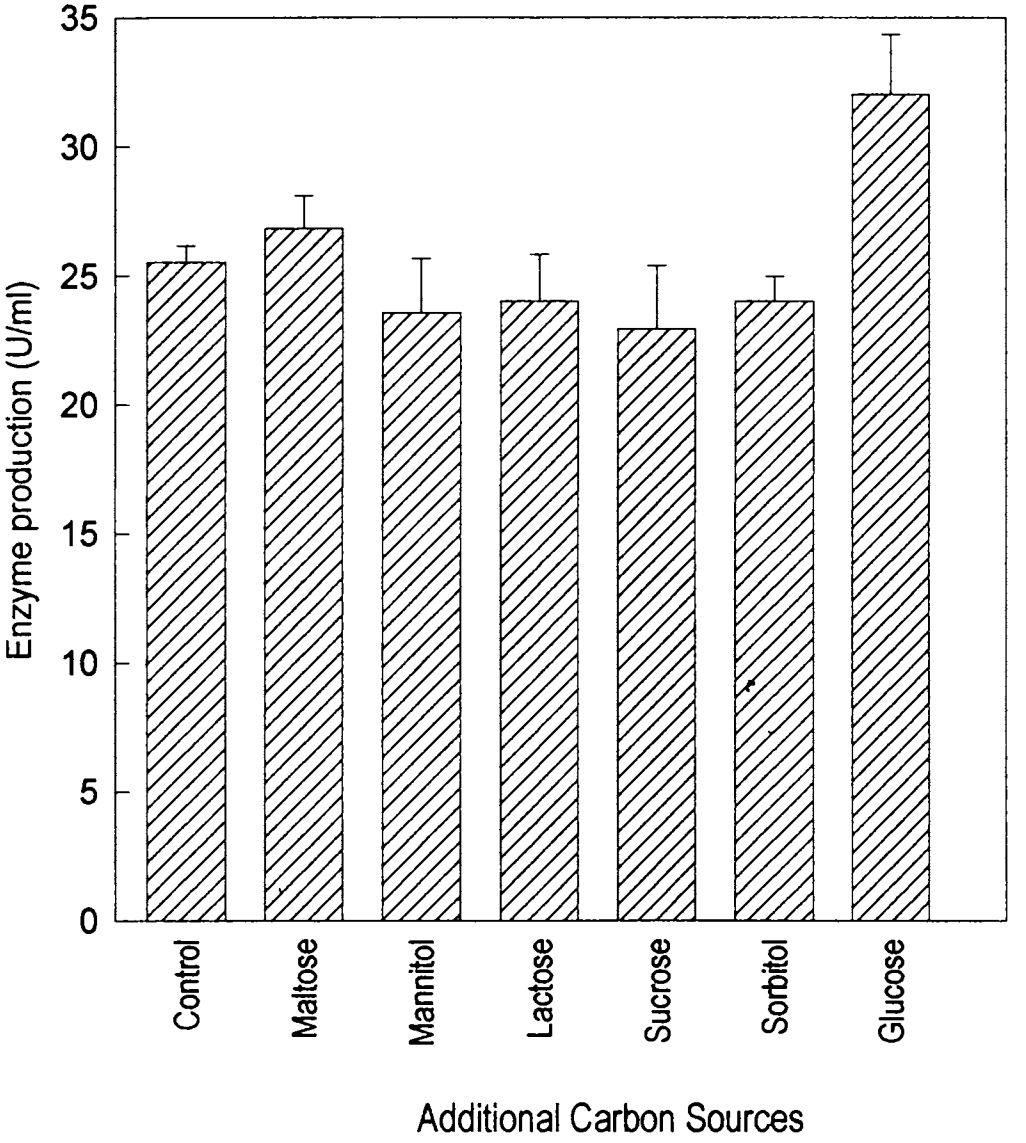


Fig.16
Effect of additional Carbon Sources on enzyme production by *B.bassiana* under SSF



increase in D- glucose concentration was recorded for the concentrations varying from 0.25% (23.4 U/ml) to 0.5% (w/v), where the maximum enzyme yield (42.12 U/ml) was recorded (Fig. 17). However, increase in glucose concentrations above 0.5 % resulted in a gradual decrease in enzyme yield. Nevertheless, the enzyme yield was comparatively at appreciable levels (26.91 U /ml) even at a concentration of 4.0% (w/v).

3.2.4. Additional Nitrogen Sources.

Impact of additional nitrogen sources, on enzyme production was evaluated by incorporating various organic and inorganic nitrogen sources in the medium along with L-glutamine. The results presented in Fig.18 and 19 indicated very clearly that addition of other nitrogen sources in the presence of L-glutamine did not enhance enzyme production compared to the maximum of 32.02 U/ml which was recorded with L-glutamine alone. Instead these additional nitrogen sources inhibited L-glutaminase synthesis by marine *B. bassiana*.

3.2.5 Effect of Amino acids.

Whether different amino acids could induce L-glutaminase production or not, was evaluated by incorporating different amino acids along with D-glucose in the fermentation medium, prepared with sea water. The results presented in Fig.20 indicated that among the different amino acids tested, L-glutamine was observed to enhance L-glutaminase synthesis (32.17 U/ml) compared to control experiment (without any amino acid in the medium). Interestingly, there was considerable level of L - glutaminase production (23.4 U/ml) even in the absence of addition of any amino

Fig.17
Effect of D-glucose concentration on enzyme production by *B.bassiana* under SSF

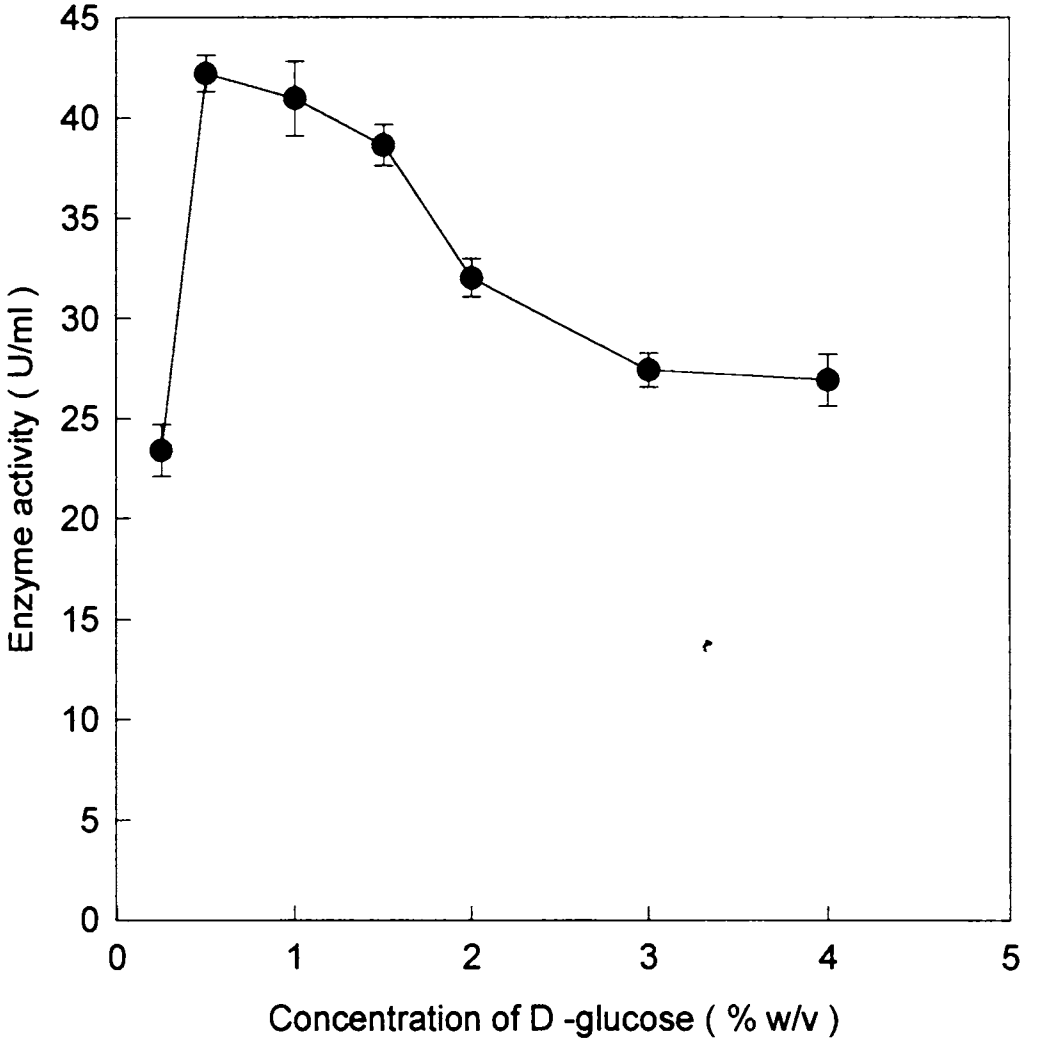


Fig.18
Effect of Organic Nitrogen Sources on enzyme production by *B.bassiana* under SSF

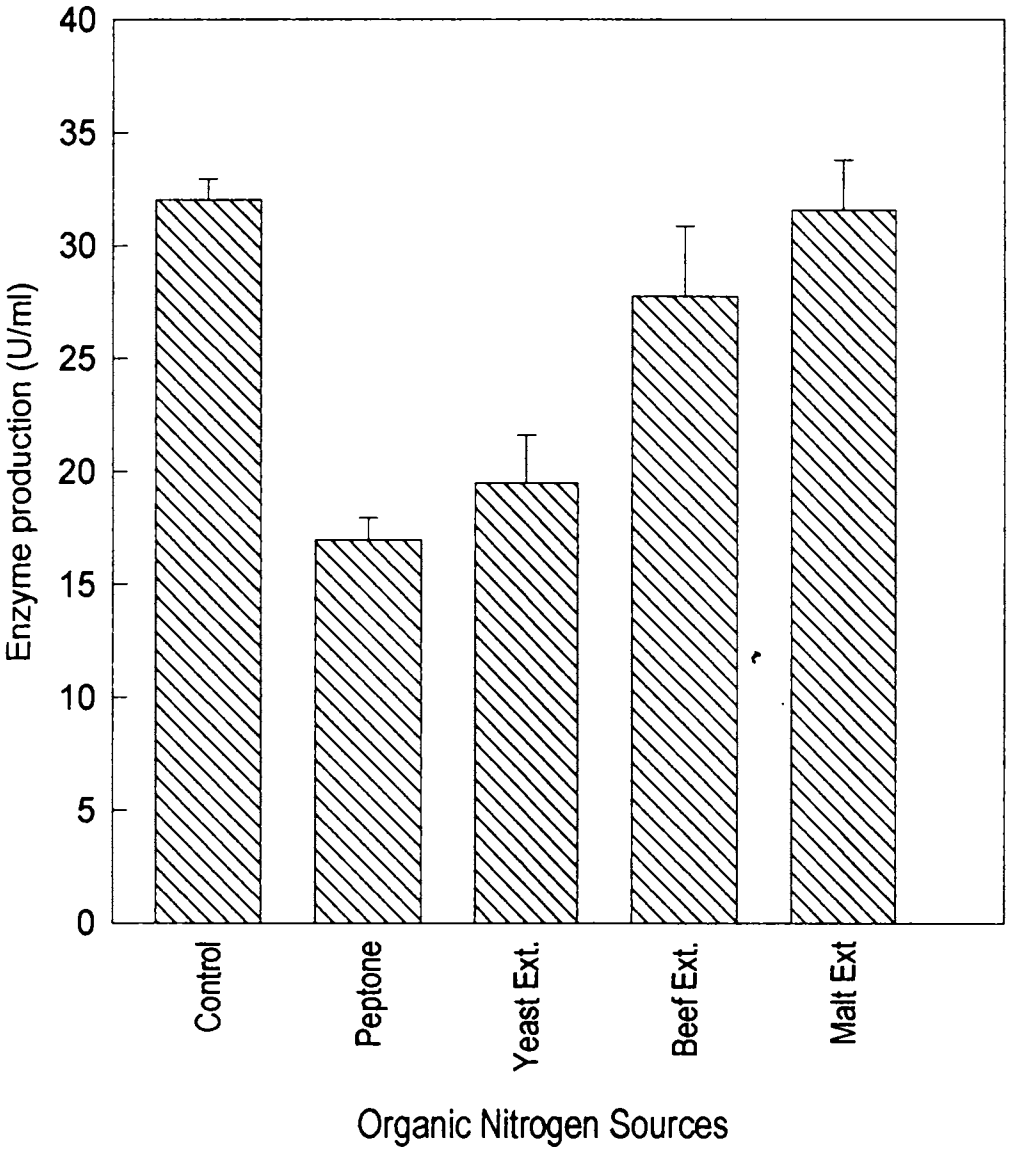


Fig.19
Effect of Inorganic Nitrogen Sources on enzyme production by *B.bassiana* under SSF

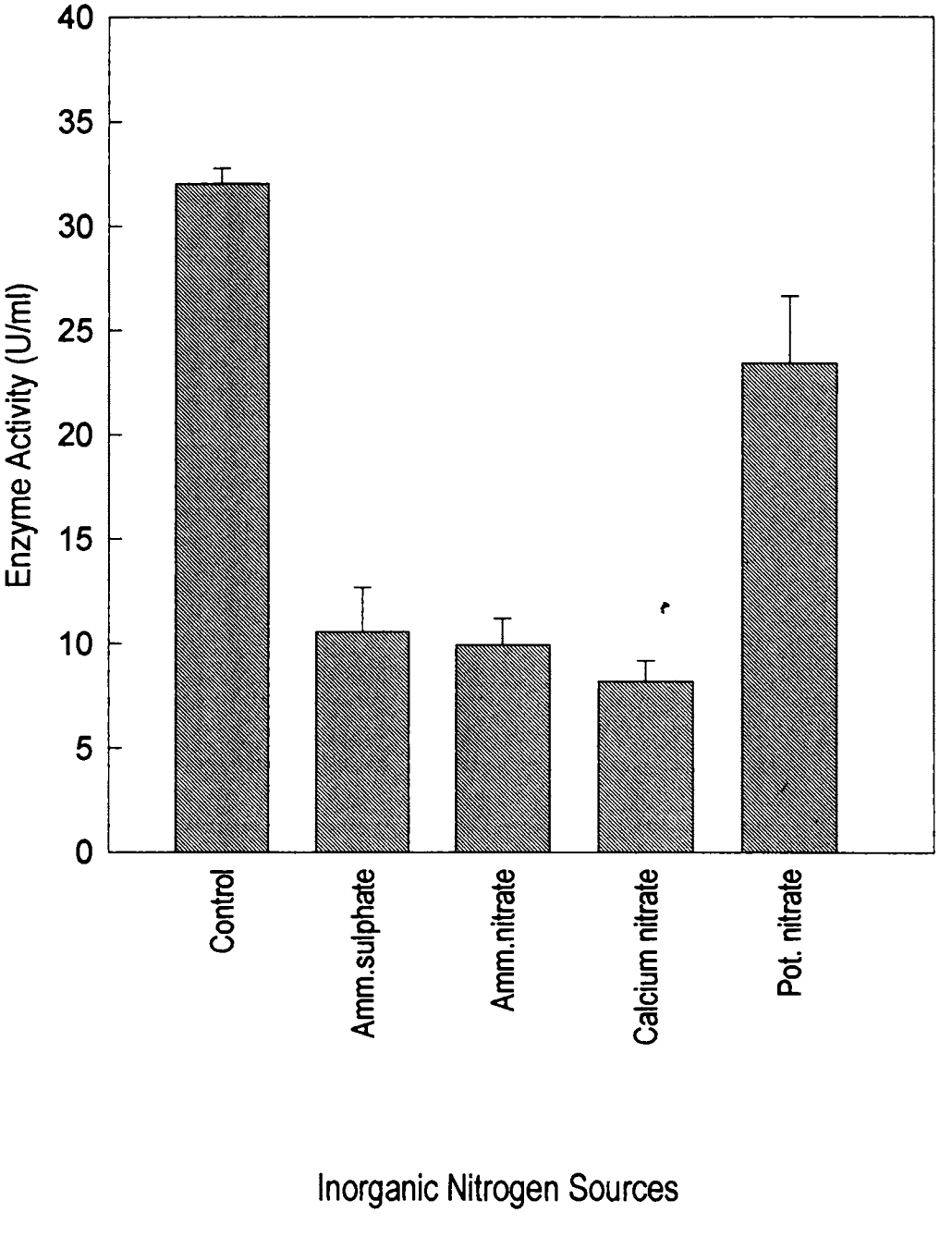
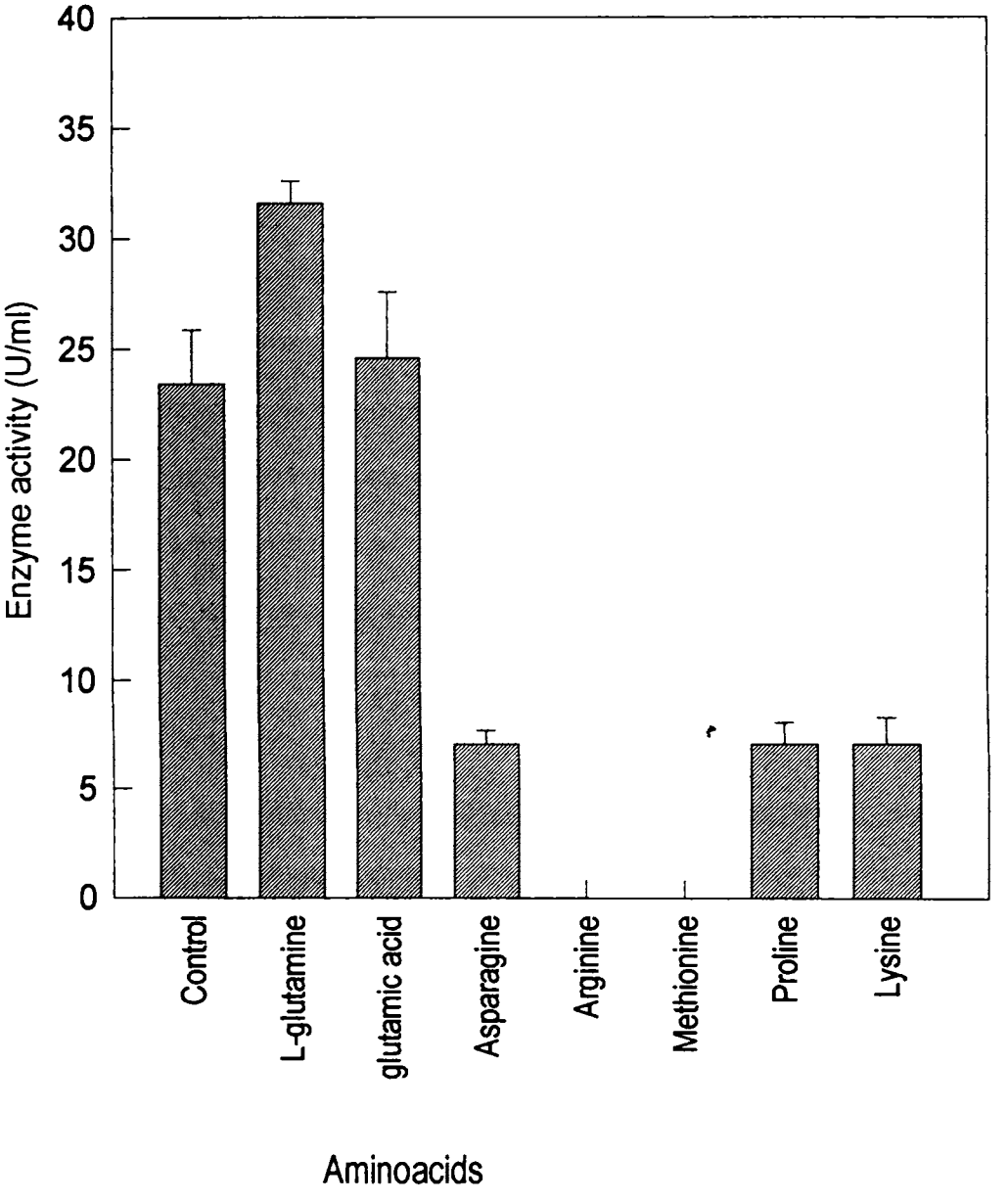


Fig.20
Effect of different amino acids on enzyme production by *B.bassiana* under SSF



acid to the basal seawater medium. Glutamic acid effected a marginal increase in L-glutaminase production compared to control. Both arginine and methionine showed complete inhibition of L- glutaminase synthesis, compared to asparagine, proline and lysine, which showed support for minimal level of L-glutaminase synthesis.

3.2.6 Substrate concentration (L- Glutamine).

Since L-glutamine was observed to enhance enzyme synthesis, .optimal concentration of L-glutamine was standardized. Among the various concentrations of L-glutamine tested towards determining the optimal concentration for enhanced enzyme production it was observed that 0.25% substrate concentration promoted maximum enzyme production (40.95 U/ml) compared to other concentrations tested (Fig.21). Further, it was also observed that concentrations above 1%, inhibited glutaminase synthesis.

3.2.7 Impact of L-glutamine concentration on enzyme production in the absence of additional carbon source.

Whether L-glutamine, in the absence of any other carbon source such as glucose, in the sea water based enzyme production medium, can promote glutaminase synthesis was evaluated. From the results presented in Fig.22 it is inferred that L-glutamine promoted L-glutaminase production of 25.52 U/ml at 1 %(w/v) concentration compared to control experiment, (23.4U/ml) in sea water based media without any additives. Further increase in L-glutamine concentration resulted in gradual decrease in the production of L-glutaminase.

Fig. 21
Effect of L-glutamine concentration on enzyme production by *B.bassiana* under SSF

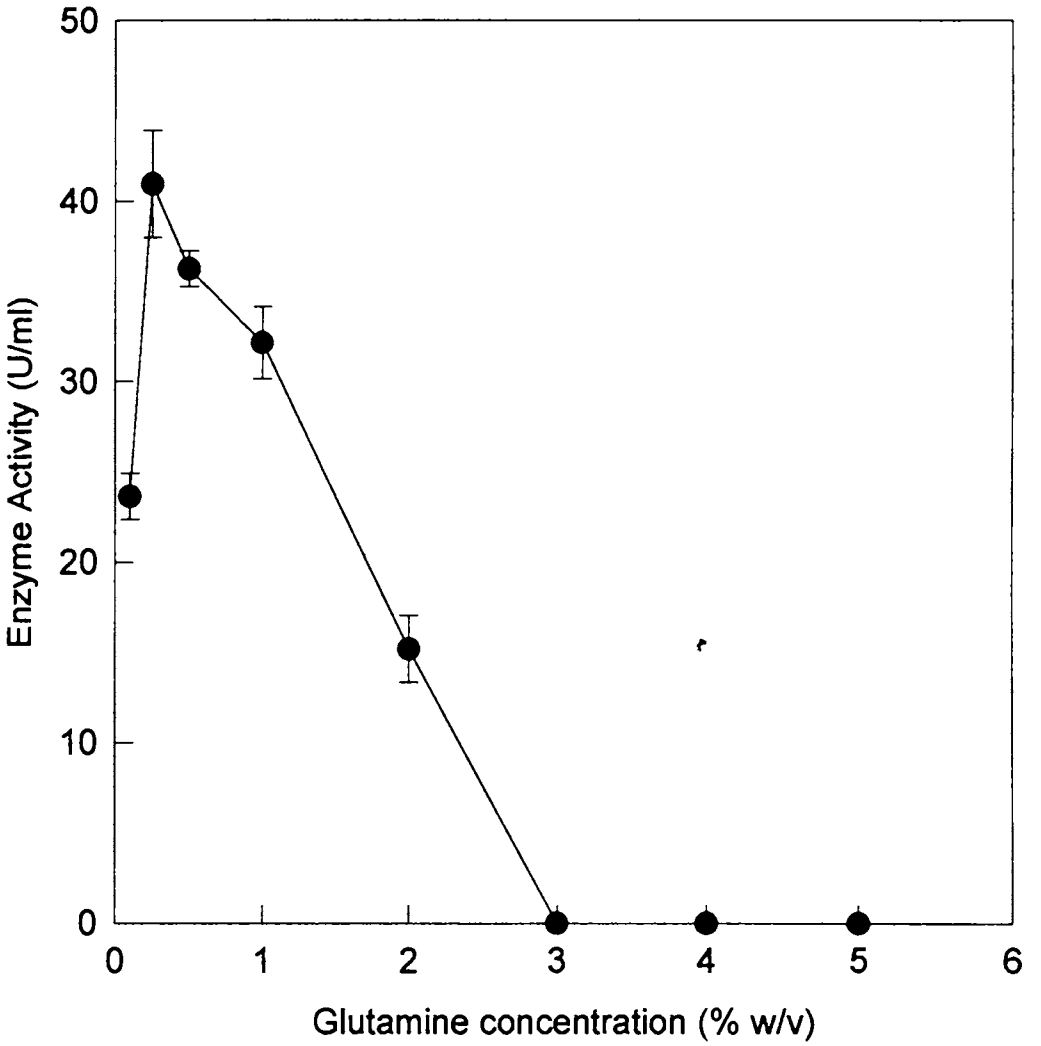
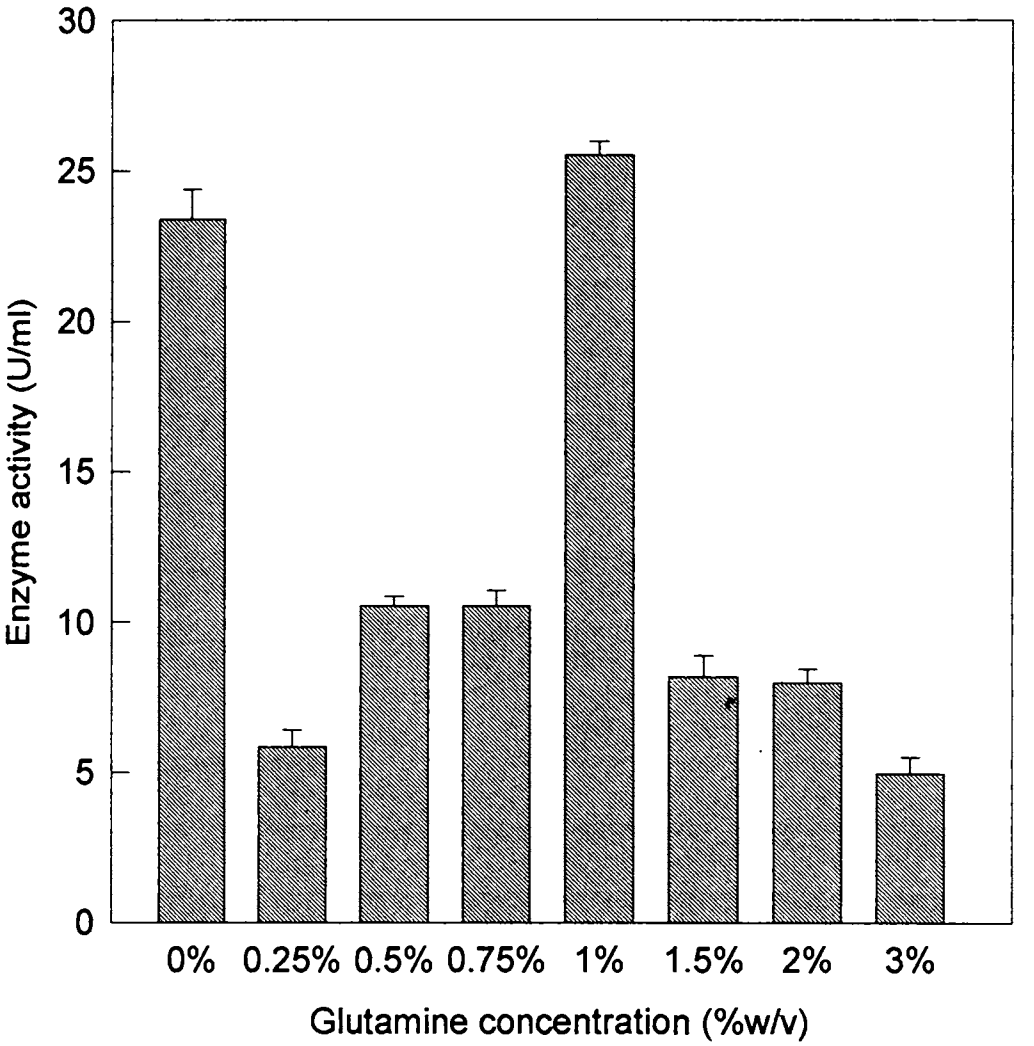


Fig.22
Effect of L-glutamine concentration on enzyme production in the absence of additional carbon source by *B.bassiana* under SSF



3.2.8 Initial moisture content.

Optimal level of initial moisture content in the solid support system required for enzyme production during solid state fermentation was determined by altering the volume of moistening medium added to polystyrene, such that different moisture levels were achieved. Maximal enzyme production (32.17 U/ml) was recorded with 80% moisture content, after 5 days of fermentation (Fig.23). Whereas, at reduced moisture content (60%) the enzyme production declined and moisture content levels above 80%, inhibited enzyme production by marine *B. bassiana*.

3.2.9 Inoculum concentration.

Optimal inoculum concentration required for maximal L-glutaminase production was evaluated using both spore and vegetative inoculum.

3.2.9.1. Spore Inoculum

Inoculum concentration influenced the rate of L-glutaminase synthesis by marine *B. bassiana*. It was observed that there was a gradual increase in the synthesis of enzyme along with increase in the concentrations of spore inoculum up to $3.7x10^8$ spores/ml (Fig. 24). Thus a maximum of 44.46 U/ml was recorded with $3.7x10^8$ spores/ml. However, spore concentration above this level resulted in a marginal decline in enzyme production. Nevertheless, considerable levels of enzyme production was observed at other spore inoculum concentrations tested during the study.

Fig.23
Effect of initial moisture content on enzyme production by *B.bassiana* under SSF

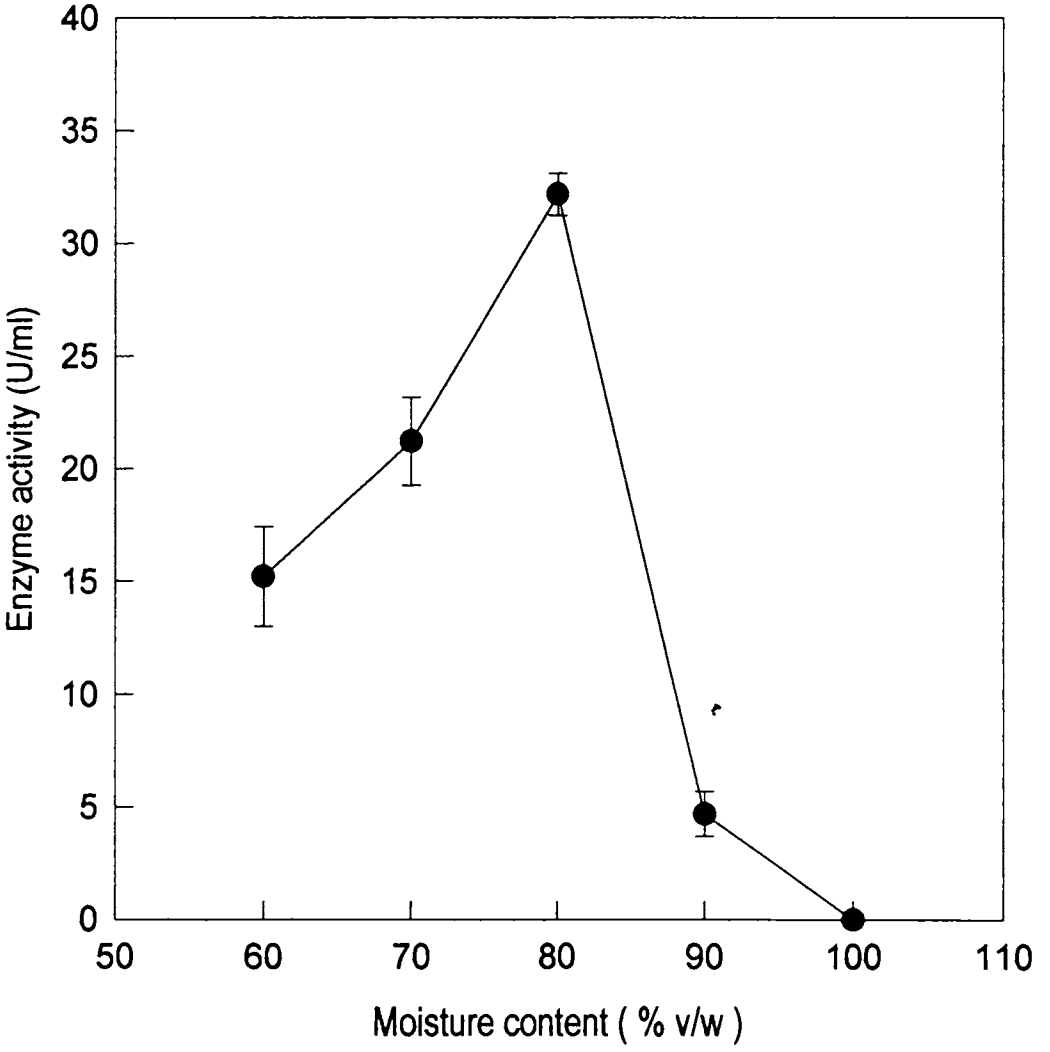
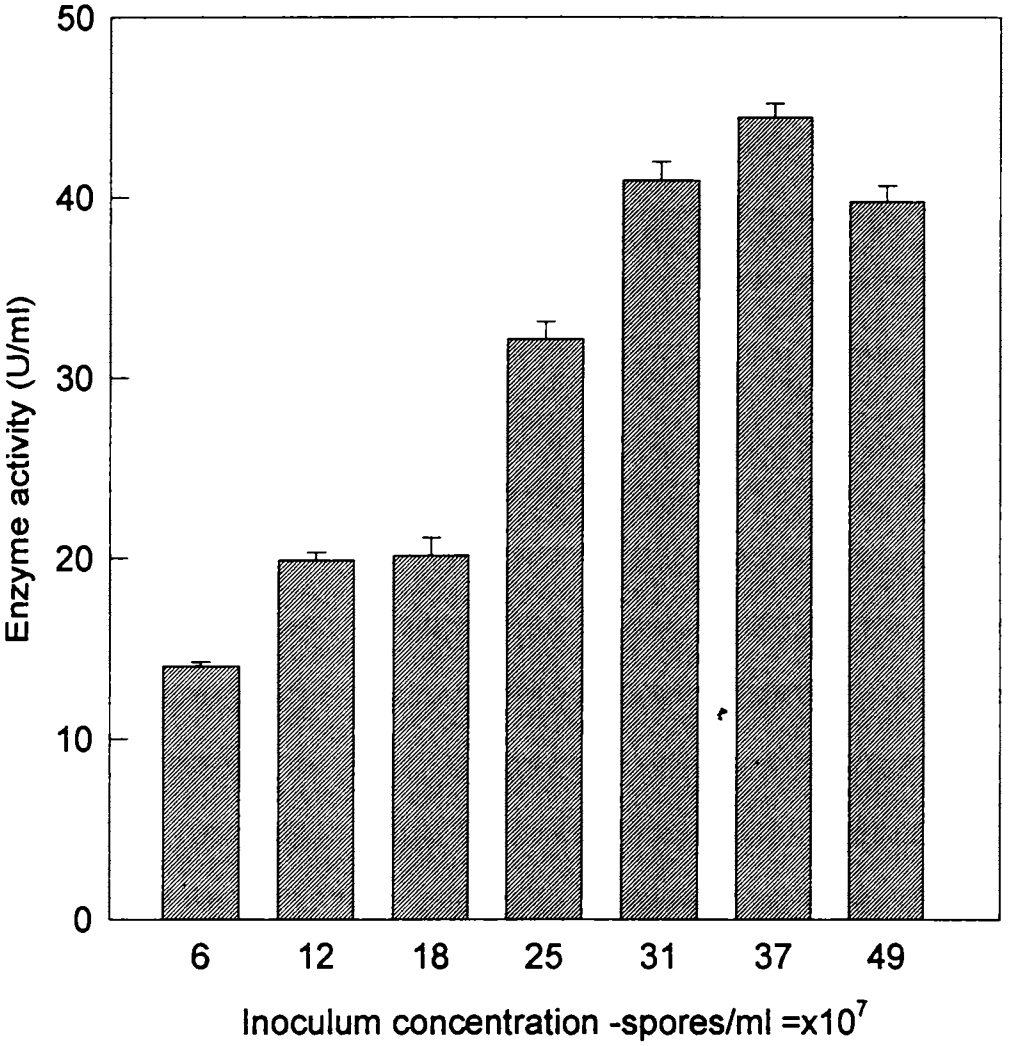


Fig.24
Effect of spore inoculum concentration on enzyme production by *B.bassiana*. under SSF



3.2.9.2 Vegetative inoculum

In a similar fashion observed with the spore inoculum, concentration of vegetative inocula also resulted in an increase in the rate of synthesis of L- glutaminase (Fig. 25). Thus, maximal enzyme production (26.32 U/ml) was obtained at 50 μg dry weight of inocula per gram of polystyrene beads. However, further increase in vegetative inocula concentration resulted in a rapid decrease in the synthesis of enzyme. It was also observed that 28μg dry weight of inocula per gram of polystyrene beads also supported enhanced levels of L-glutaminase.

3.2.10 Impact of sea water concentration

Concentration of sea water, which was used as the basal media component has significant impact on enzyme synthesis. Results presented in Fig.26 showed that with increase in concentration of sea water there was a gradual increase in the production of L-glutaminase and the maximal production was obtained at full strength sea water (44.46 U/ml). Interestingly, even at 0% seawater concentration (distilled water) *B. bassiana* could produce 25.74 U/ml of enzyme, indicating an euryhaline nature of the strain used in the study.

3.2.11. NaCl concentration.

Impact of additional sodium chloride concentration in sea water, contributing to high salt concentration in the fermentation media and consequent influence on rate of enzyme synthesis was evaluated. It was noted that addition of sodium chloride to the sea water based medium did not enhance enzyme production (Fig.27) and instead led to

Fig.25
Effect of vegetative inoculum concentration on enzyme production by *B.bassiana* under SSF

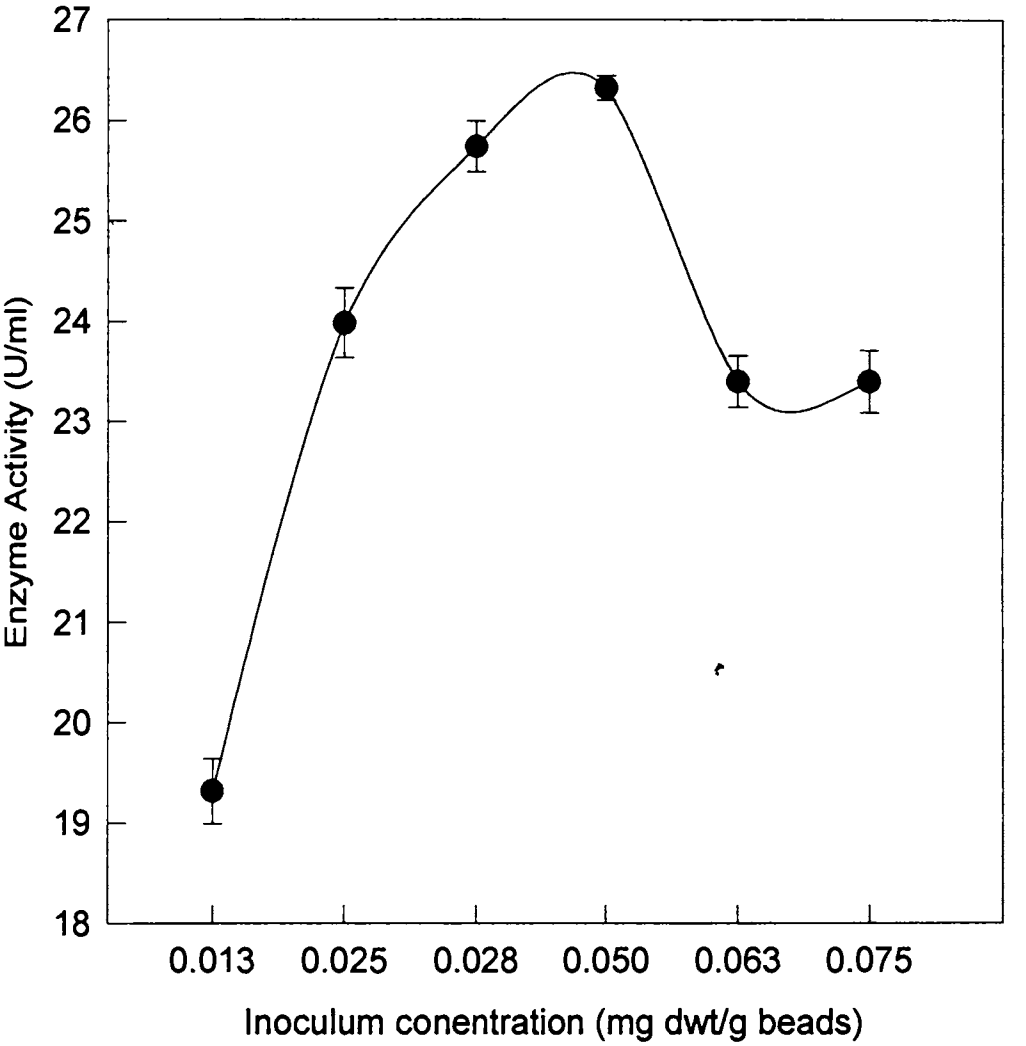


Fig.26
Effect of sea water concentration on enzyme production by *B.bassiana* under SSF

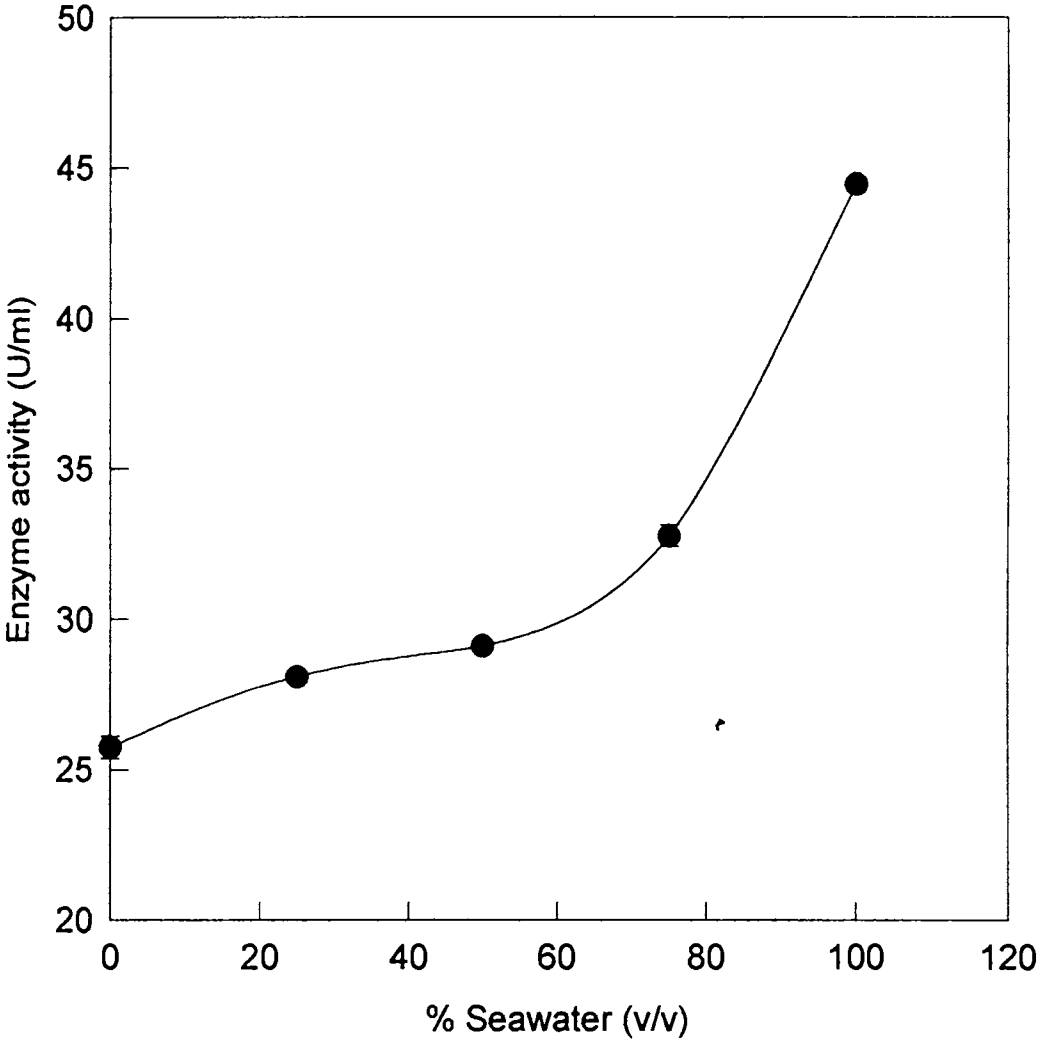
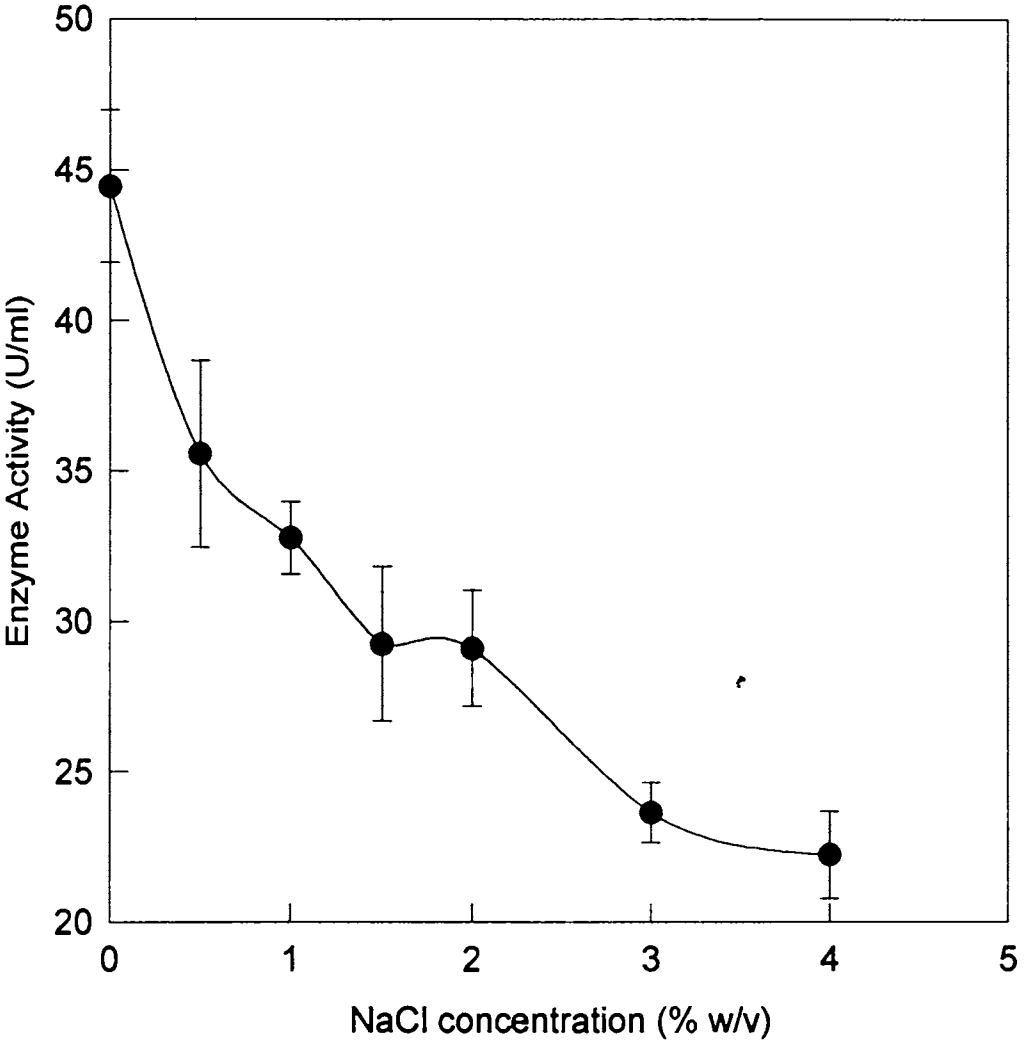


Fig.27
Effect of Additional NaCl concentration on enzyme production by *B.bassiana* under SSF



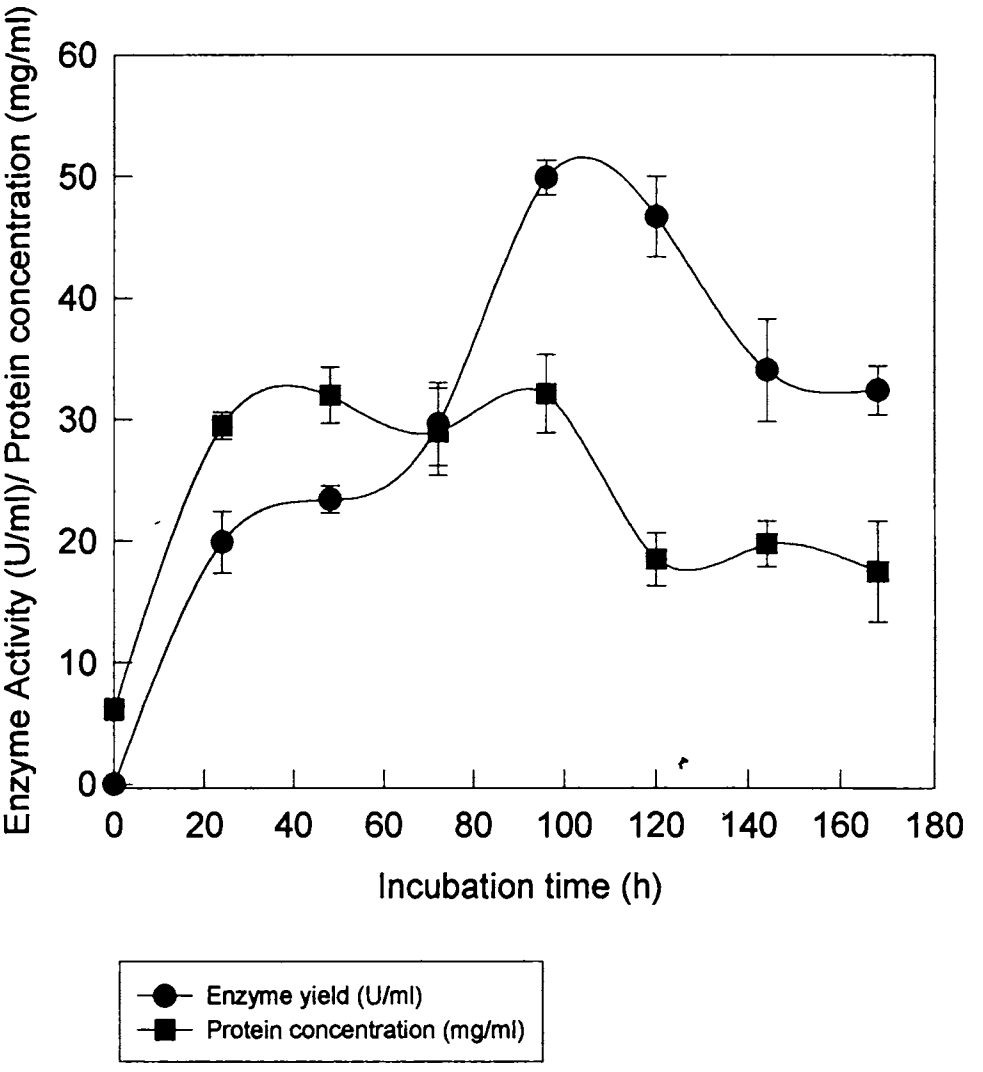
a sharp decrease in enzyme activity along with increase in sodium chloride concentration from 0 % to 4.0 %.

3.2.12 Time course experiment.

After optimisation of various bioprocess conditions, a time course experiment was carried out to monitor the rate of L-glutaminase production by *B. bassiana* at optimized conditions. Results presented in Fig.28 suggest that L-glutaminase production increased progressively along with increase in incubation time until 96 hours, when maximal enzyme production was recorded (49.89 U/ml). However, the enzyme yield declined during further incubation beyond 96 hours. It was also observed that enzyme synthesis was rapid during early 24 hours compared to the period 24 - 72 hours.

Determination of the total cell protein of the organism during the time course experiment revealed that the fungal strain quickly passed into stationary phase during solid state fermentation after 48 hours. A comparison of biomass and enzyme activity curves indicated that maximal enzyme synthesis occurred during stationary phase. Nevertheless, considerable level of enzyme synthesis was recorded during logarithmic phase also. In general the results suggest that the enzyme synthesis is growth associated.

Fig .28
Time cource of L-glutaminase production by *B.bassiana* under SSF on polystyrene as inert support



3.3. Comparison of L-glutaminase production by marine and terrestrial strains of *Beauveria bassiana*.

A comparative evaluation of the L-glutaminase production by marine and terrestrial strains of *B. bassiana* was conducted using a standard terrestrial strain of *B. bassiana* (NCIM 1216) obtained from National Collection of Industrial Microorganisms (NCIM, Pune, India), under solid state fermentation and submerged fermentation using polystyrene, in distilled water and sea water based media. From the results presented in Table .1 it is evident that sea water based fermentation medium is preferred by marine *B. bassiana* for maximal L-glutaminase production (47 U/ml in SmF and 49.89 U/ml in SSF) than distilled water based medium (17.33 U/ml in SmF and 25.74 U/ml in SSF). Whereas, in the case of terrestrial *B. bassiana*, distilled water based media supported enhanced enzyme production (8.48 U/ml in SmF and 29.25 U/ml in SSF) than sea water (6.43 U/ml in SmF and 21.06 U/ml in SSF). Marine *B. bassiana* produced comparatively higher level of L-glutaminase than terrestrial strain.

3.4. L-Glutaminase production by immobilized spores of marine *Beauveria bassiana*

Initially, the process parameters, such as support concentration, inoculum concentration, $CaCl_2$ concentration, curing time, activation time and retention time required for preparation of stable immobilized fungal spore beads capable of producing extracellular L-glutaminase were optimized.

Table 1

Comparison of L-glutaminase production by terrestrial and marine *Beauveria bassiana* under different production conditions

Strain	Seawater based Medium		Distilledwater based Medium	
	SmF	SSF	SmF	SSF
Marine *B.bassiana*	47	49.89	17.33	25.74
Terrestrial *B.bassiana*	6.43	21.06	8.48	29.25

SmF- Submerged Fermentation
SSF - Solid State Fermentation

3.4.1 Optimisation of process parameters for immobilization of fungal spores

3.4.1.1 Support concentration.

Results presented in Fig 29 indicate that immobilized cells prepared with 3% (w/v) concentration of sodium alginate produced relatively high level of enzyme (8.78 U/ml) and further increase in concentrations of sodium alginate led to decline in enzyme production. These results indicate that 3% is the ideal concentration of sodium alginate for preparation of stable beads with immobilized spores.

3.4.1.2 Inoculum concentration

Influence of spore concentration on formation of stable beads and maximal enzyme production was also evaluated. From the results presented in Fig. 30 it is noted that spore concentrations of $12.0x10^8$ spores/gram of bead supported high level of enzyme production (13.10 U/ml)compared to other concentrations. In fact the level of enzyme production gradually increased along with increase in spore concentration. However, concentrations above $12.0 x10^8$ spores/gram of bead led to rapid decline in enzyme production.

3.4.1.3 Calcium Chloride concentration.

Calcium chloride concentrations used for preparation of stable beads with immobilized spores influenced the glutaminase production by immobilized fungal spores. Maximal enzyme production (28.08 U/ml) was observed with a concentration of

Fig.29
Optimisation of support concentration for the preparation of immobilised beads.

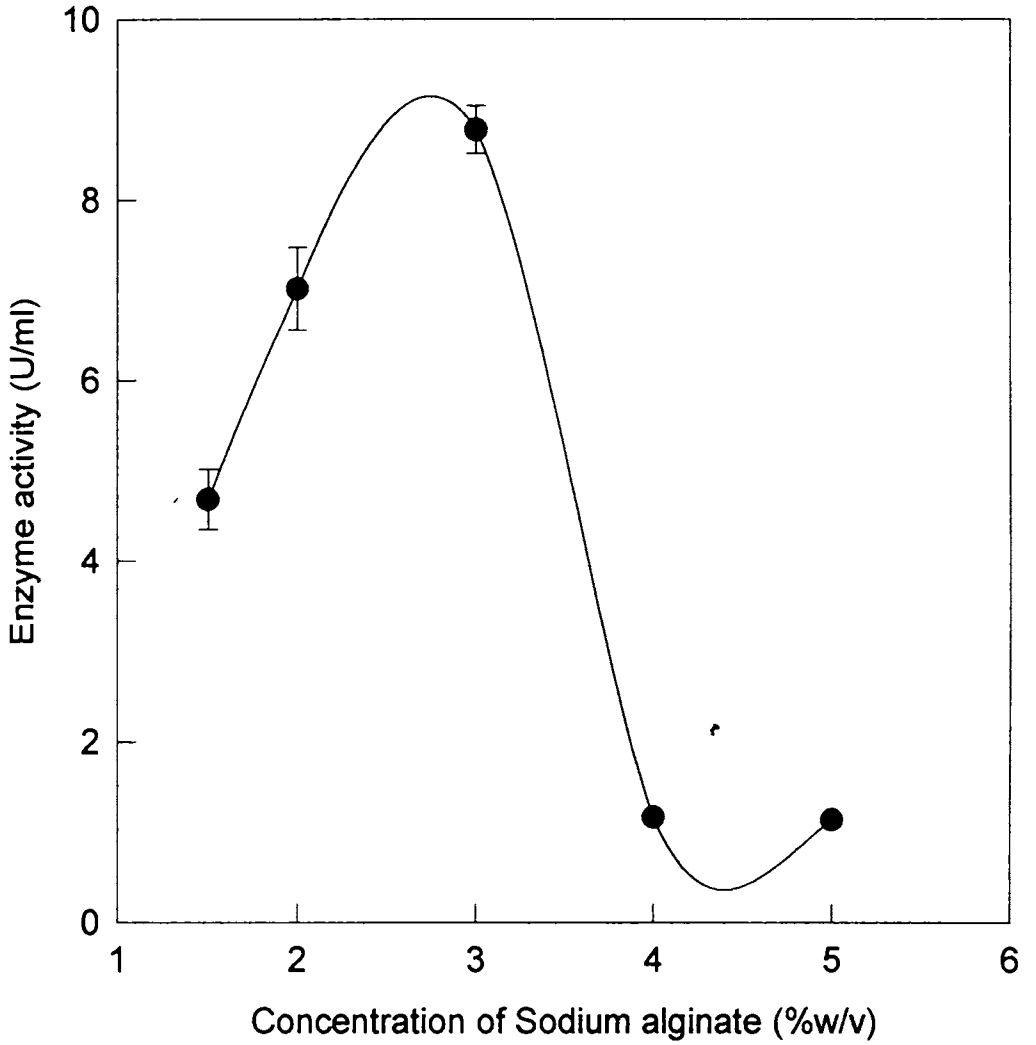
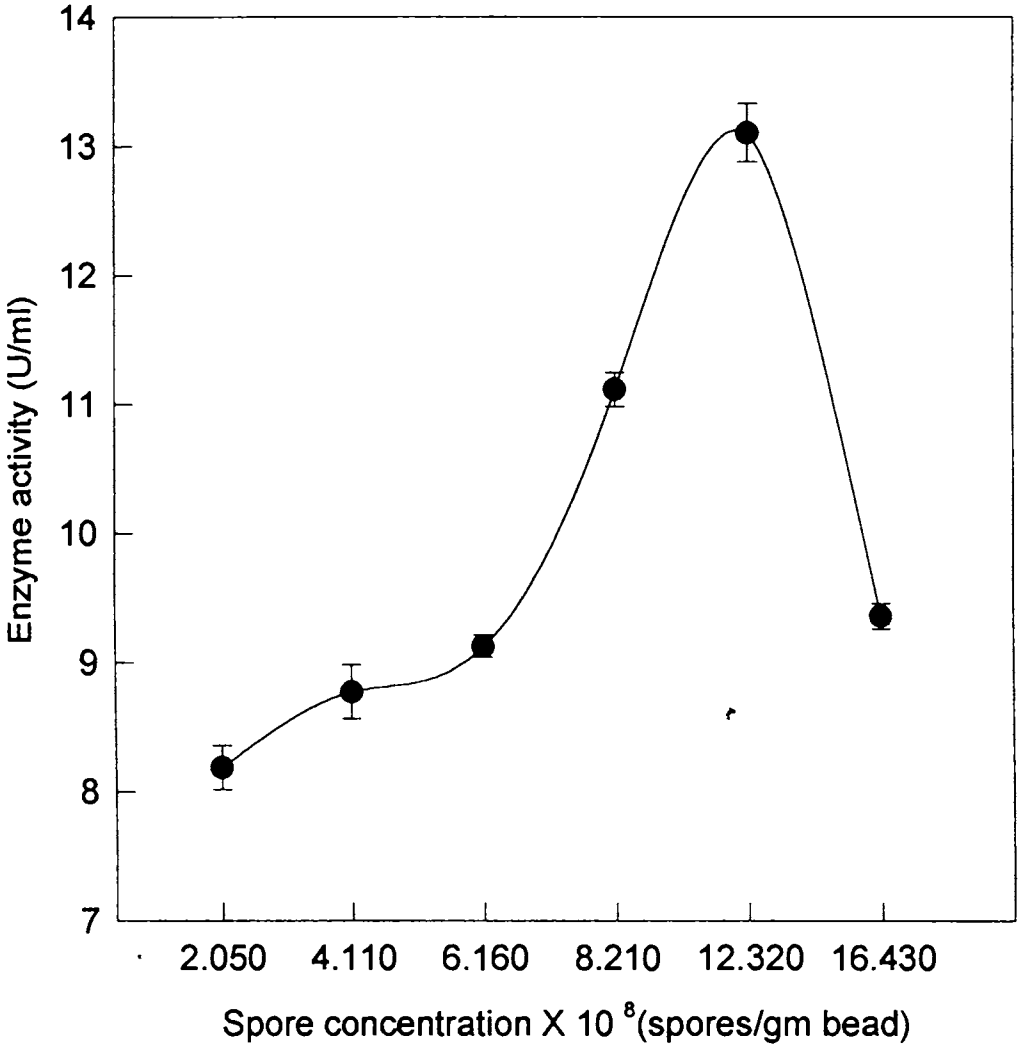


Fig.30
Optimization of spore concentration for enzyme production by immobilized *B.bassiana*



0.10 M $CaCl_2$. Further increase in $CaCl_2$ concentrations led to rapid decline in enzyme production (Fig 31).

3.4.1.4. Curing Time

Beads with immobilized spores cured for a period of 3 hours could support maximal enzyme production (56.16 U/ml). It was also observed that curing periods not less than or more than 3 hours did not support enhanced level of enzyme production (Fig 32) by immobilized spores.

3.4.1.5. Activation Time

After preparation of immobilized spores in alginate beads optimal activation time for maximal enzyme production was determined. It was observed that 15 hours of activation is ideal for maximal enzyme production (58.05 U/ml). Further increase in activation time led to decline in enzyme production. However, activation periods of 5-10 hours did support enzyme production in the range of 35 U/ml (Fig.33).

3.4.1.6 Retention Time.

Optimal retention time for maximal enzyme production by immobilized spores was determined by subjecting the immobilized spores to different time intervals of incubation in enzyme production medium. Data presented in Fig. 34 suggest that 18 hours of retention time could support maximal enzyme production (64.46 U/ml). Although gradual increase in enzyme production was observed along with increase in retention time, and retention time over 18 hours led to decrease in enzyme production.

Fig.31
Optimization of $CaCl_2$ concentration for the preparation of immobilized beads.

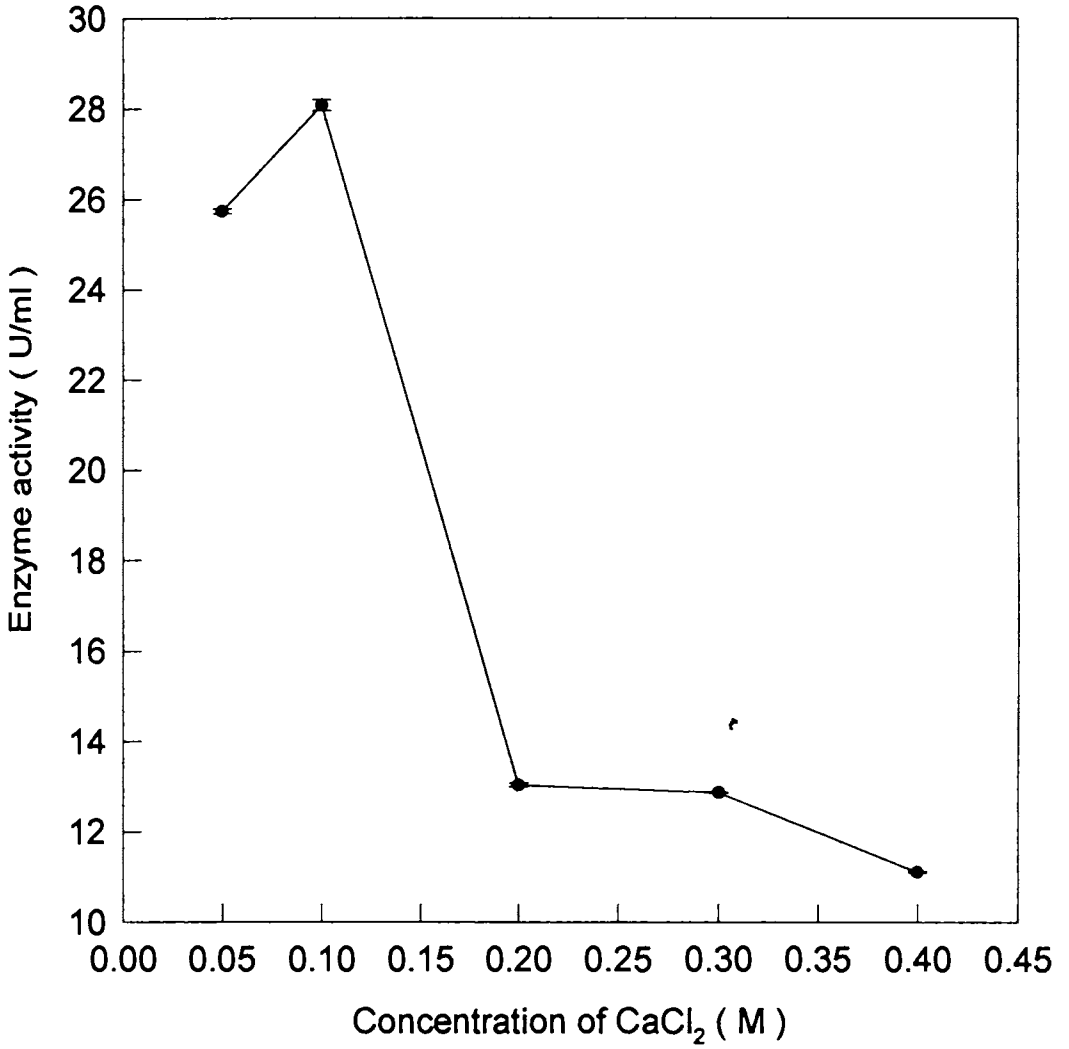


Fig.32
Optimization of curing time for the preparation of immobilized beads

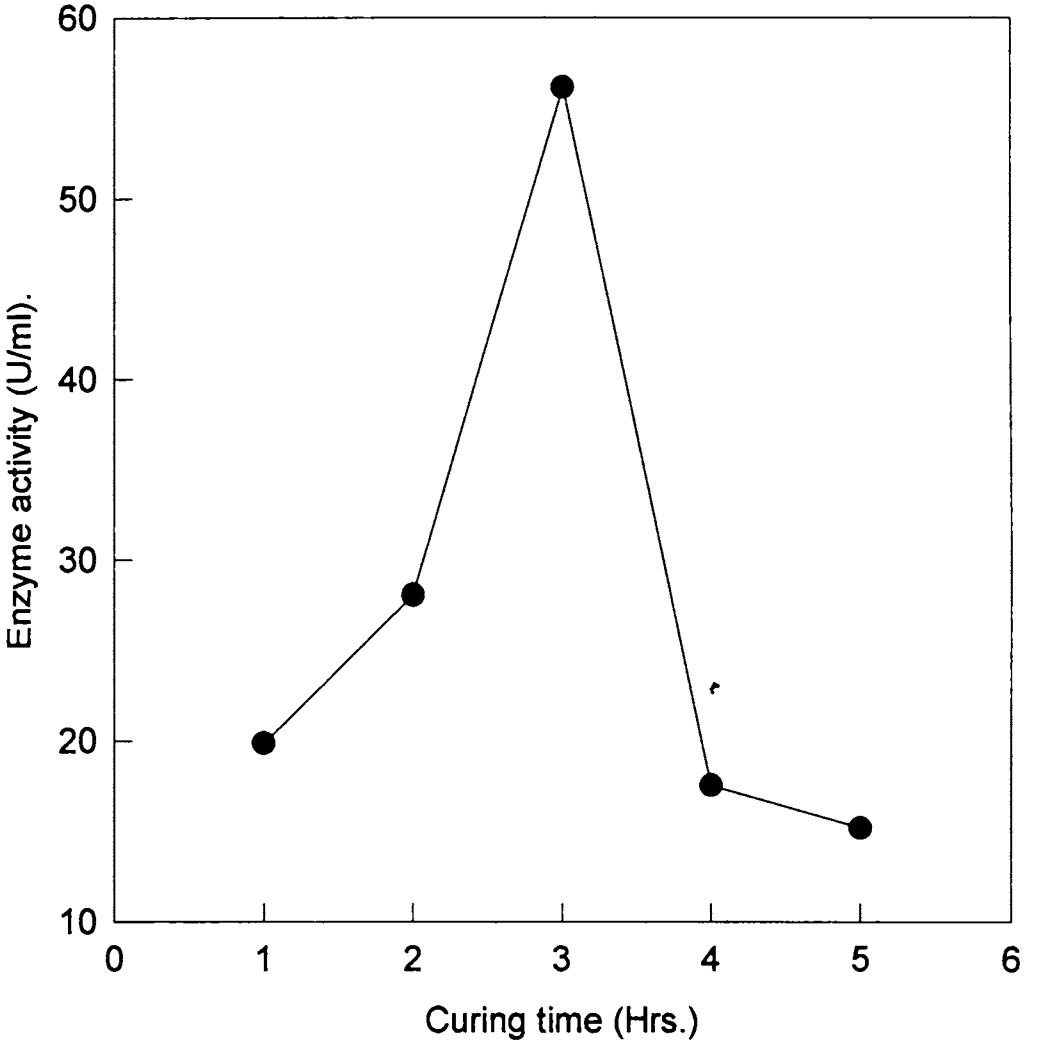


Fig.33
Optimization of activation time for enzyme production by immobilised *B.bassiana*

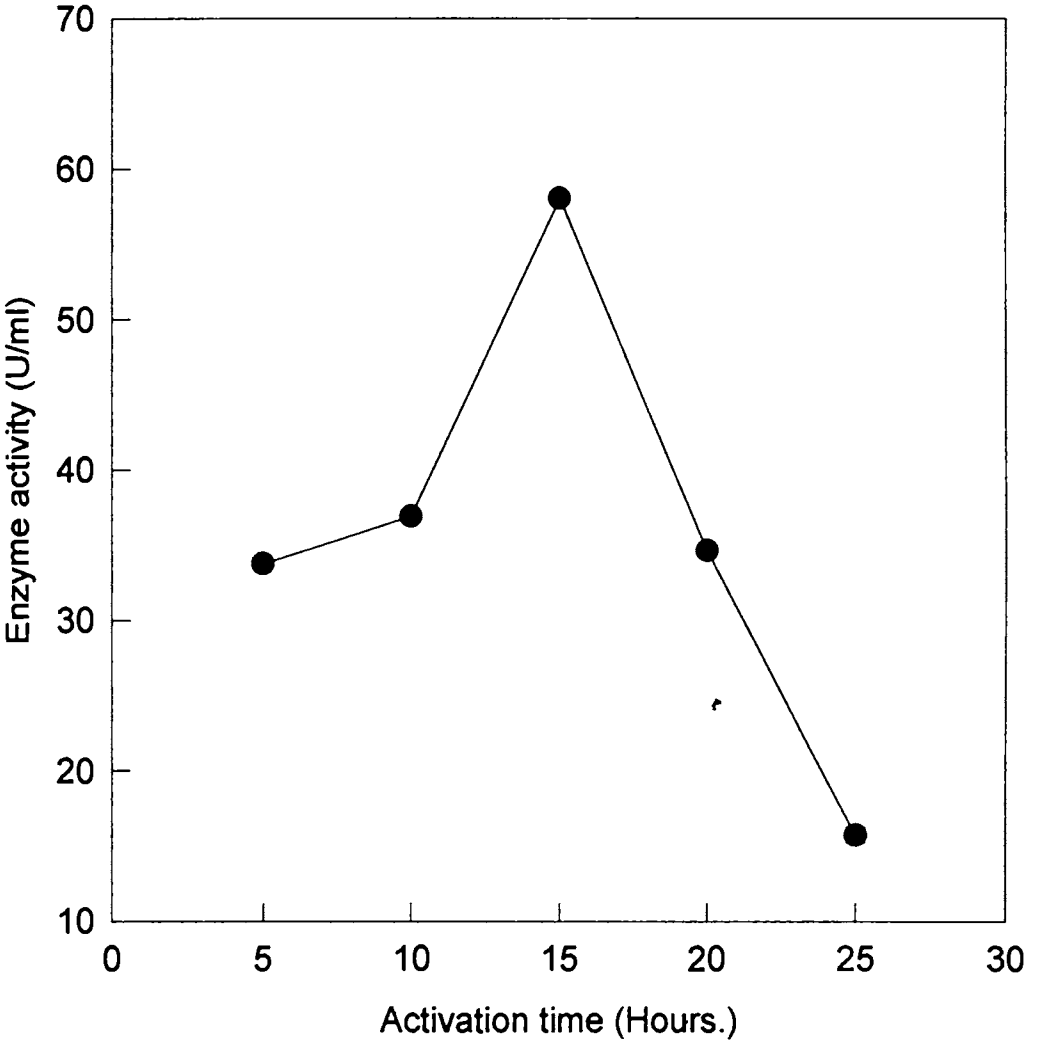
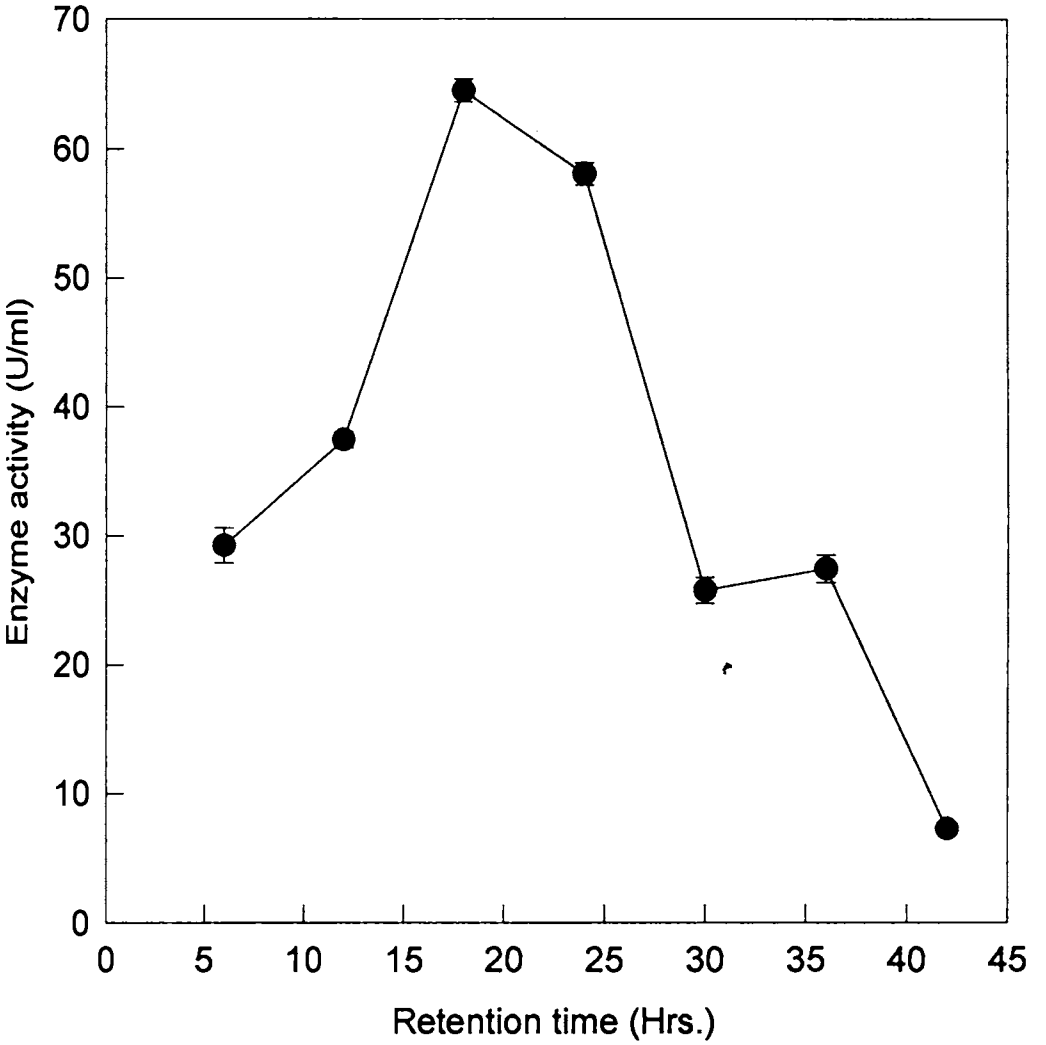


Fig.34
Effect of retention time on enzyme production by immobilised *B.bassiana*



3.4.2. Incubation Temperature

Impact of incubation temperature on enzyme production by immobilized spores of fungi was also studied by incubating the beads with immobilized spores at different temperatures. It was observed from the results presented in Fig. 35 that optimum incubation temperature for maximal enzyme production is 27^0C (64.46 U/ml), although high level of enzyme production could be recorded at 22^0C also. Temperatures above 27^0C led to rapid decrease in enzyme production.

3.4.3. pH of the enzyme production media.

Data documented in Fig. 36 clearly evince the fact that pH of the enzyme production media influence the level of enzyme production by fungal spores under immobilized conditions. Thus, increase in media pH from 7.0 to 9.0 resulted in rapid increase in enzyme production from 17.33 U/ml to 64.46 U/ml. However, further rise in pH to 10.0 led to sharp decline in enzyme production, indicating that specific pH level in the enzyme production medium can only support maximal enzyme production by immobilized spores.

3.4.4 L-glutaminase production by immobilized spores of *Beauveria bassiana* in a packed bed reactor.

In a packed bed reactor the effect of flow rate, substrate concentration, aeration rate and bed height on continuous production of L-glutaminase by marine *B. bassiana* was evaluated.

Fig.35
Effect of incubation temperature on enzyme production by immobilised *B.bassiana*

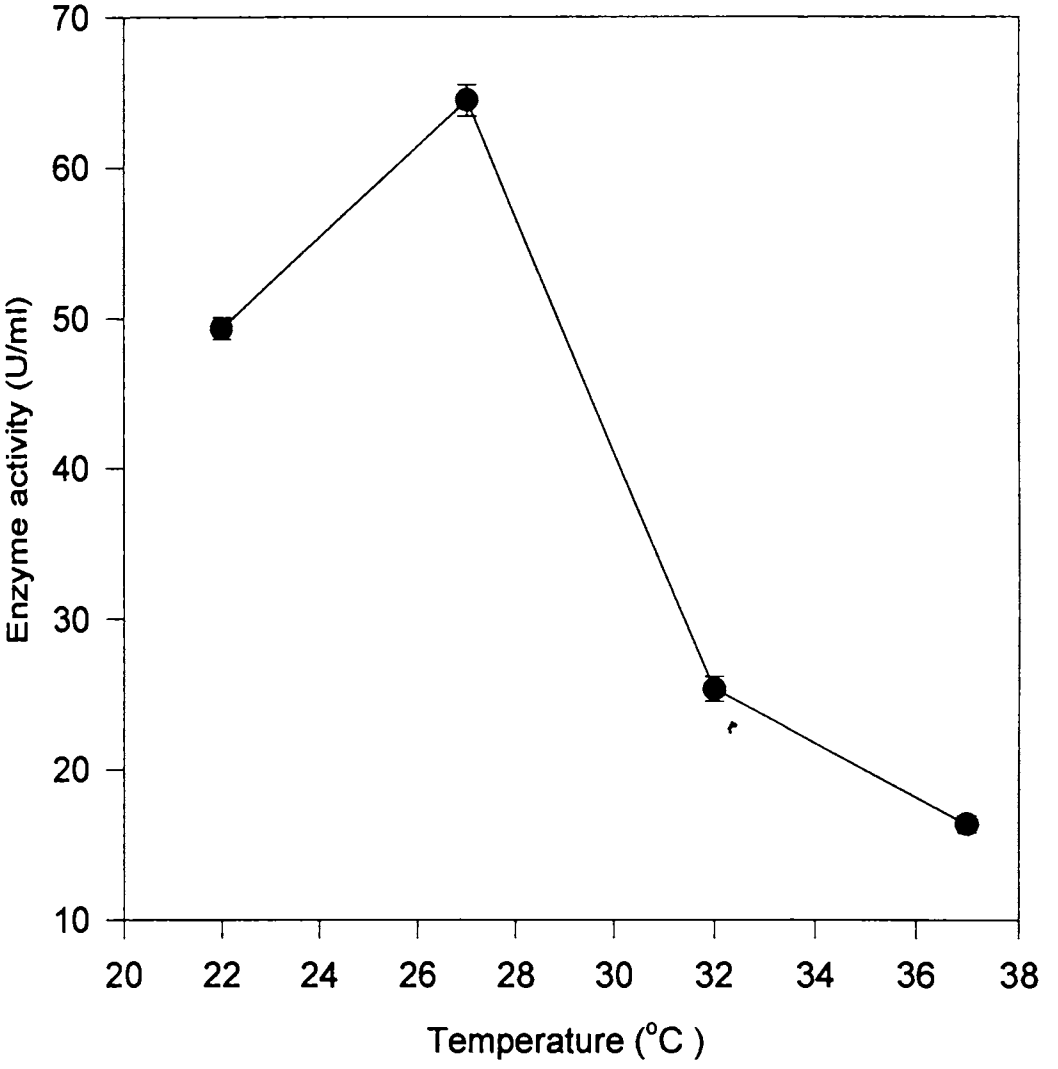
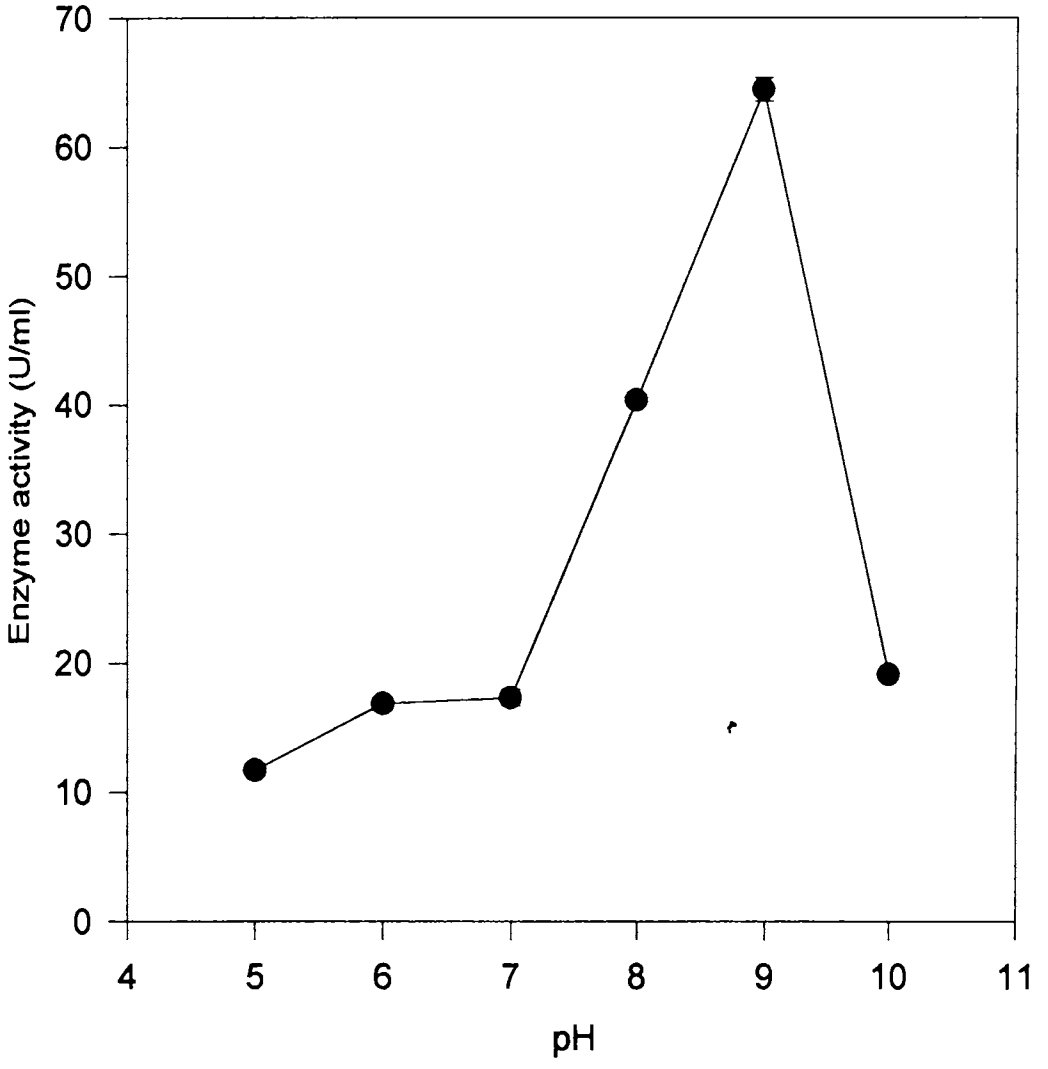


Fig.36
Effect of pH of the media on enzyme production by immobilised *B.bassiana*



3.4.4.1 Flow rate

Effect of flow rate on continuous production of L-glutaminase by immmobilized spores of *B. bassiana* was evaluated at different flow rates of medium in to the packed bed reactor. Results presented in Fig. 37 indicate that slower the flow rate of medium, higher the enzyme yield. Thus maximal enzyme production was obtained at a flow rate of 20 ml/hr (26.68U/ml) followed by 30 ml/hr, 40 ml/hr, 50 ml/hr and 60 ml/hr. The enzyme production decreased rapidly for the higher flow rates of 40-60 ml/hour.

3.4.4.2. Substrate concentration

Effect of substrate concentration in the enzyme production media on L-glutaminase production by immmobilized spores of *B. bassiana* in a packed bed reactor, under continuous mode, was evaluated at different concentrations of glutamine in the medium. From the results presented in Fig. 38 it is inferred that glutamine concentration of 0.25% (w/v) promoted maximal glutaminase production (26.68U/ml), compared to other concentrations tested.

3.4.4.3. Aeration rate

Effect of aeration rate on continuous production of L-glutaminase by immobilized spores of *B. bassiana* in a packed bed reactor, was evaluated by aerating the reactor at different aeration rates. Results presented in Fig. 39 indicated that maximal glutaminase production (15.91U/ml) was effected by immobilized spores in the absence of aeration, and aeration resulted in decrease in enzyme production under immobilized condition of the spores.

Fig .37
Effect of flow rate on enzyme production by immobilized *B.bassiana* in a packed bed reactor

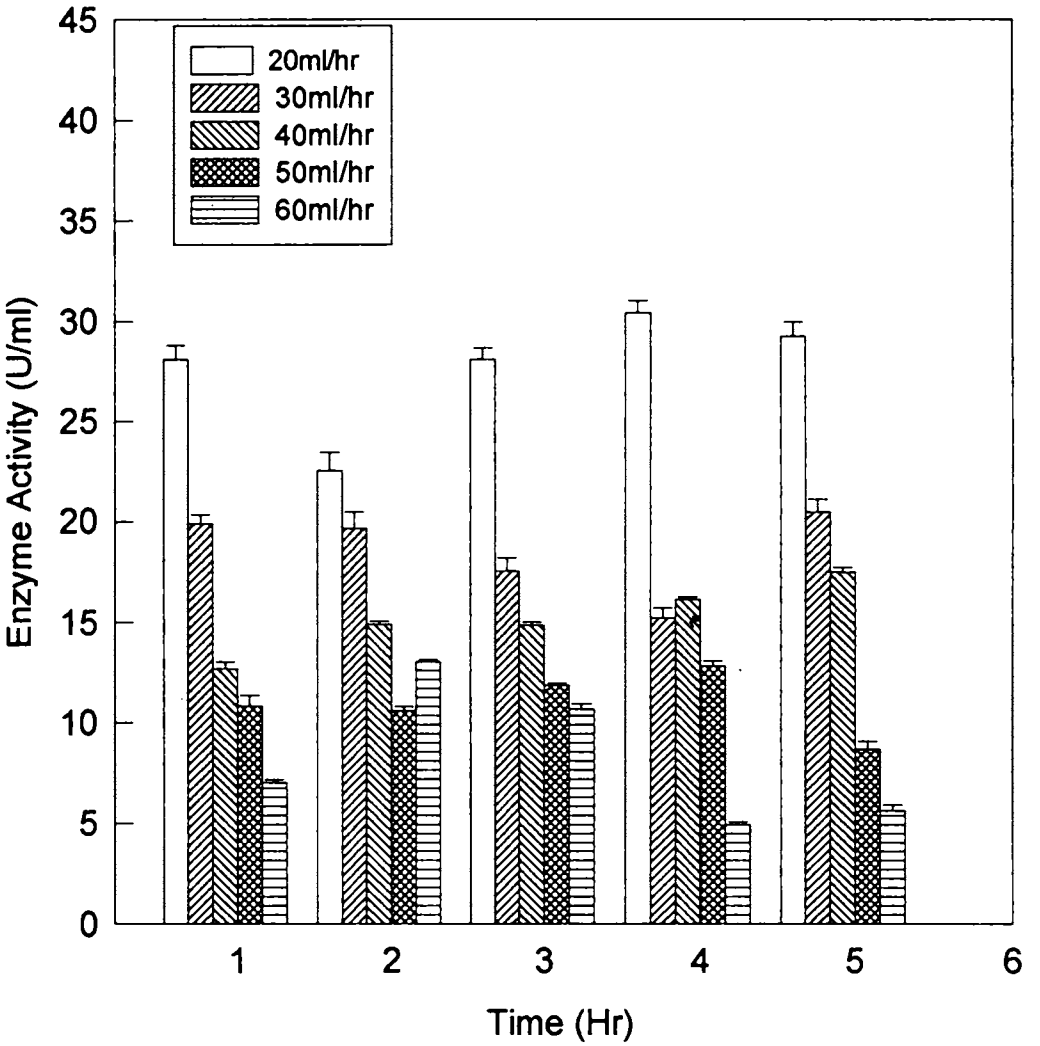


Fig.38
Effect of Substrate concentration on enzyme production by immobilized *B.bassiana* in a packed bed reactor

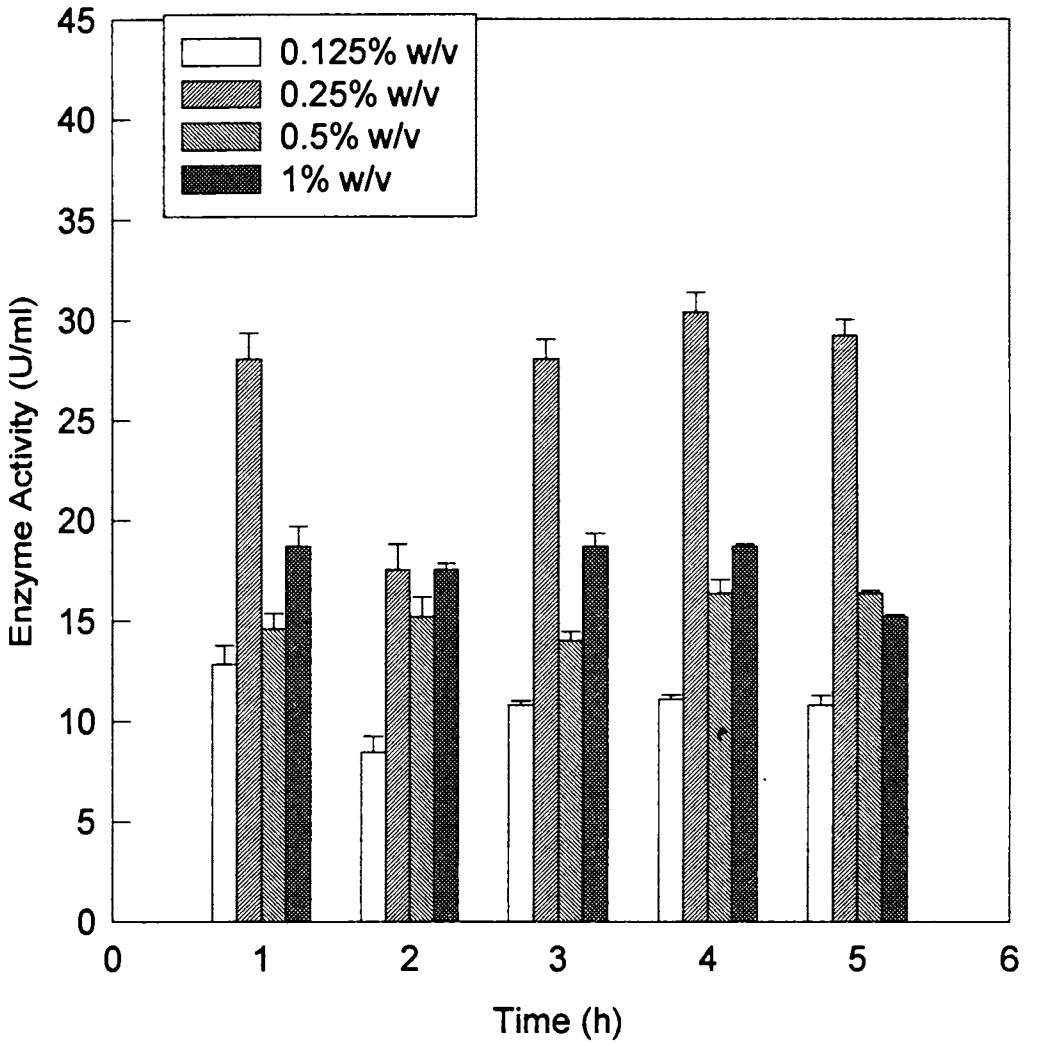
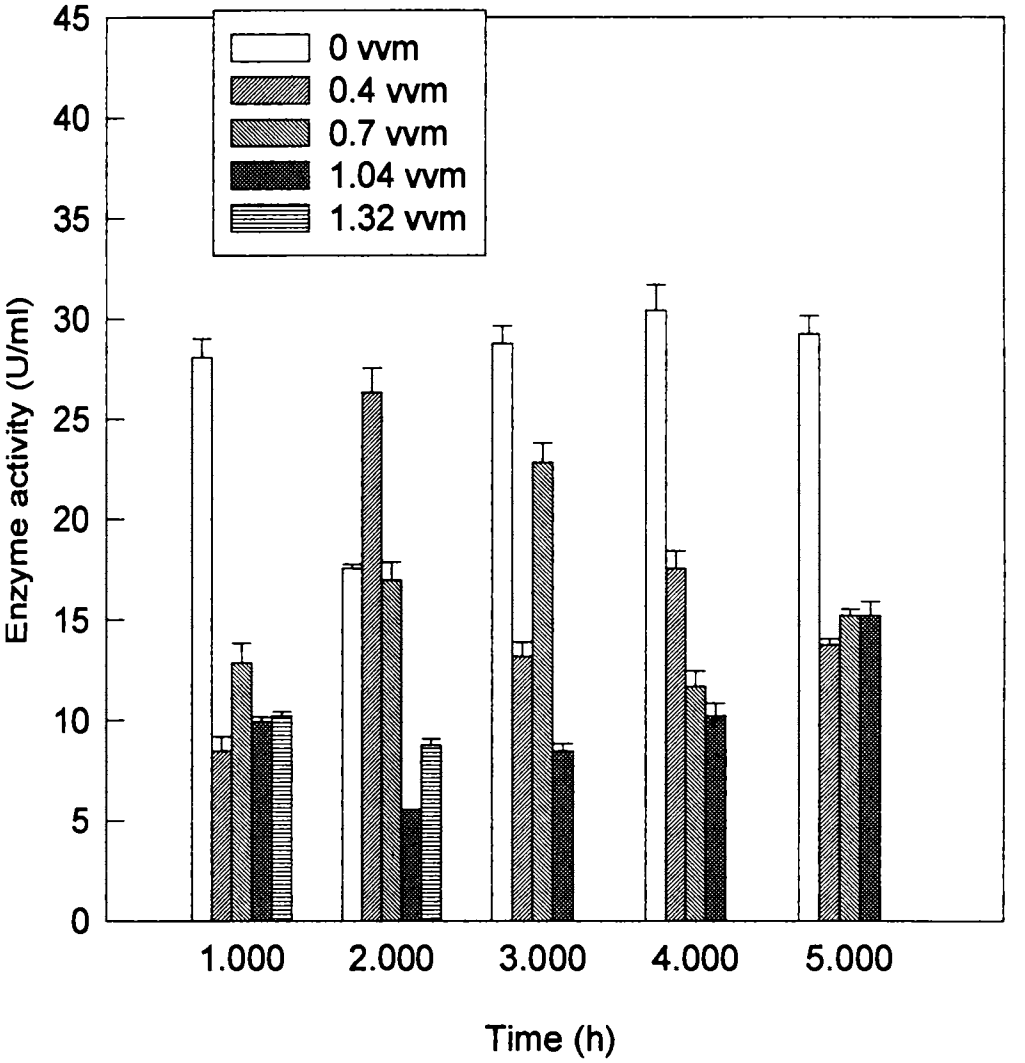


Fig.39
Effect of aeration on continuous enzyme production by immobilized *B.bassiana* in a packed bed reactor



3.4.4.4. Bed height

Effect of bed heights of immobilized spores of *B. bassiana* in a packed bed reactor on continuous production of L-glutaminase was evaluated at three different bed heights. From the results presented in Fig. 40 it is inferred that maximal glutaminase production (26.68 U/ml) could occur with a bed height of 15 cm followed by 10 cm and 5 cm.

3.4.5. Cumulative production of L-glutaminase by immobilized *Beauveria bassiana* in a Packed bed reactor

Cumulative production of L-glutaminase by immobilized spores of *Beauveria bassiana* in a packed bed reactor was also monitored. Total enzyme yield per hour of operation was calculated for different flow rates, substrate concentrations in the enzyme production media, aeration rates and bed heights towards determining the efficiency of the packed bed reactor for continuous production of the enzyme. The results are presented in Table 2. It was observed that for a time interval of 1h, 404.8U/100ml (4.048 U/ml/hour) could be produced at a flow rate of 20ml/hour compared to 397.8U/100ml (3.978 U/ml/hour), 299.2U/ 100ml (2.992 U/ml/hour), 196.42 U/100ml (1.964 U/ml/hour) and 168.5 U/100ml (1.685 U/ml/hour) at the flow rates of 30, 40, 50 and 60 ml/hour, respectively. The enzyme production medium collected after fermentation for one hour thus showed a maximal productivity at lowest flow rate tried (20ml/h). Substrate concentration of 0.25% w/v gave the maximum productivity of 404.8 U/h. Productivity for no other substrate concentrations were comparable to this. Aeration did not make any difference in the productivity of the reactor and an unaerated

Fig.40
Effect of bed heights on continuous enzyme production by immobilized *B.bassiana* in a packed bed reactor

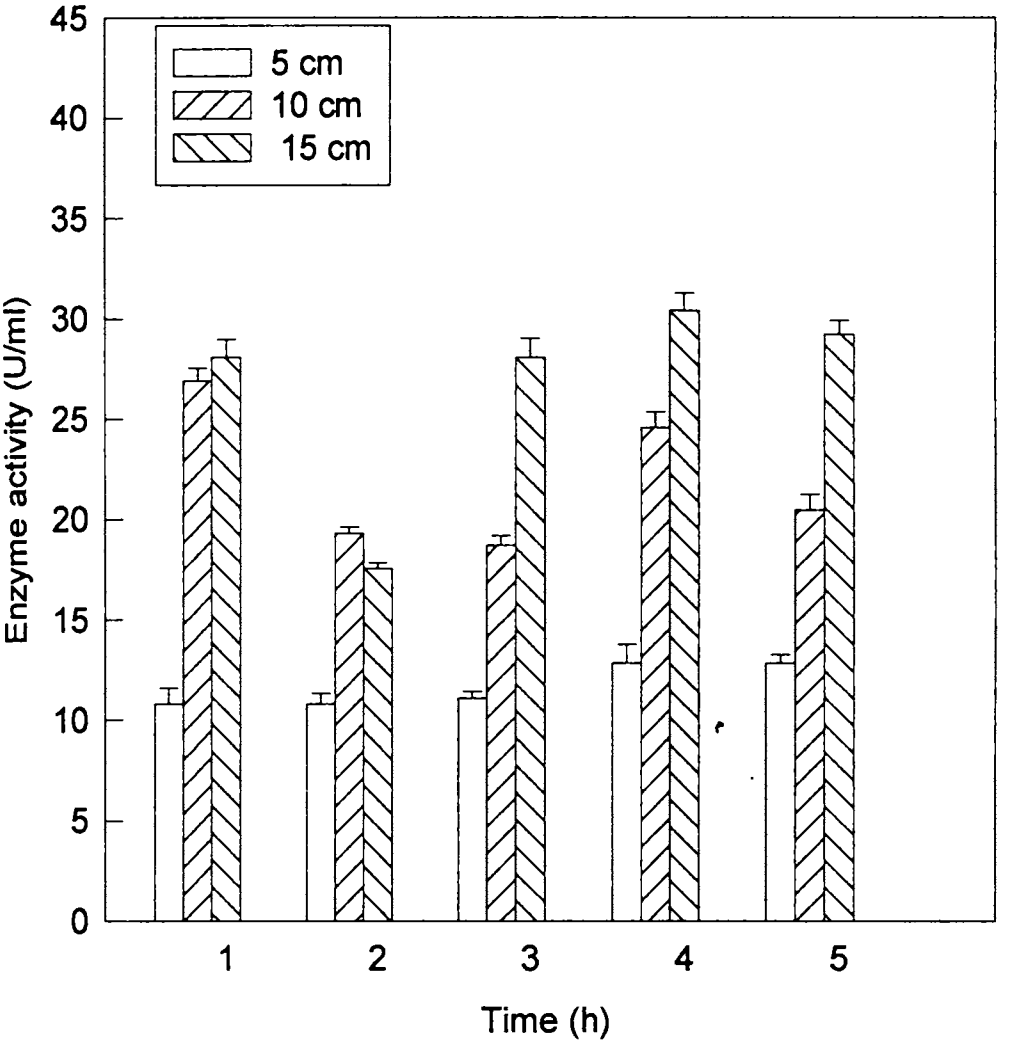


Table 2

Cumulative production of L-glutaminase by immobilized spores of *Beauveria bassiana* under continuous cultivation in a packed bed reactor

Process Variable	Level	Mean Enzyme activity (U/ml)	Cumulative productivity (Enzyme units /hour)	Volumetric productivity U/ml/h
	20	20.24	404.8	4.048
	30	19.89	596.7	3.978
Flow rate (ml/h)	40	14.96	598.4	2.992
	50	9.821	491.05	1.9642
	60	8.33	505.44	1.685
	0.125	9.88	199.6	1.996
Substrate Concentration (%w/v)	0.25	20.24	404.8	4.048
	0.5	14.63	292.6	2.926
	1	14.55	331	3.31
	0	20.24	404.8	4.048
	0.4	15.52	310.4	3.104
	0.7	15.42	308.4	3.084
Rate of Aeration (vvm)	1.04	15.09	301.8	3.018
	1.32	4.92	98.4	0.984
	1.7	0	0	0
	5	15.44	308.8	3.088
Bed Height (mm)	10	20.85	417	4.17
	15	22.25	445	4.45

process could give the maximal productivity of 404.8U/h followed by the least aerated (0.4 vvm) one with 310.4U/h. Increase in bed heights had a clear advantage in cumulative productivity with the highest bed (15cm) giving maximal production (445U/h)

3.5. Comparison of the different fermentation systems for L-glutaminase production by *Beauveria bassiana*

L-glutaminase production by marine *B. bassiana*, under submerged, solid state and immobilized state was carried out and the performance of the fungi in terms enzyme yield was compared for evaluating the efficiency of the system for enzyme production by marine fungi. It was observed that fungal spores immobilized in sodium alginate beads, under batch mode, could enable high enzyme activity (64.46 U/ml) compared to solid state fermentation (49.89 U/ml) and submerged condition (47 U/ml) (Table 3). Whereas, when yield of enzyme in terms of enzyme activity per unit biomass was considered, solid state fermentation supported more yield per unit biomass (121.68 U) compared to immobilized system (78.205 U) and submerged fermentation system (72.31 U) The most interesting observation is that immobilized system required very short time of about 18 hours compared to submerged fermentation system (108 hours) for synthesizing almost similar levels of enzyme. Nevertheless, solid state fermentation system gave a high yield in 96 hrs compared to other two systems.

Table. 3

Comparison of the different fermentation systems for L-glutaminase production by marine *Beauveria bassiana*

Process	Activity (U/ml)	Yield	Optimal Incubation Period
Submerged Fermentation	47	72.31	108 h
Solid State Fermentation	49.89	121.68	96 h
Immobilized System (Batch Mode)	64.46	78.21	18 h

Biomass as total cell protein

SmF - 0.65mg/ml
SSF - 0.41mg/ml
Immobilized spores - 0.82mg/ml (Effective biomass acting on unit volume of production medium)

Discussion

4. DISCUSSION

Submerged fermentation

Submerged fermentation process is routinely used for the production of most of the industrially important microbial enzymes in industry, irrespective of the source, whether it is bacteria or fungi. Hence, in the present study, once it was confirmed that marine *Beauveria bassiana* has the ability to secrete L-glutaminase as extracellular enzyme into the medium, an attempt was made initially to optimize the various process parameters, which influence L-glutaminase production under submerged fermentation conditions. The data presented under the chapter results throws more light on the physiological property of the fungus selected for the study, besides recognizing the optimal bioprocess parameters, for enhanced level of L-glutaminase production.

Results obtained for the optimization study very clearly indicate that bioprocess variables influence the rate of extracellular L-glutaminase synthesis by *B. bassiana*. Thus the initial pH of the medium and incubation temperature were observed to influence L-glutaminase production by *B.bassiana* significantly. The fungal strain showed preference for highly alkaline pH 9.0 (7.78 U/ml) as the optimum pH for maximal enzyme production under SmF. This observation testify the alkalophilic property of the strain, reported earlier (Suresh and Chandrasekaran, 1998). Further, it was noted that, although the fungus was isolated from marine sediment and could be grown at room temperature in the laboratory, it required a temperature of 27^0C for producing enhanced level of L-glutaminase enzyme (14.11U/ml). Nevertheless,

considerable level of enzyme yield could be obtained at other pH and temperatures also. This observation is also in agreement with the report for chitinase production by the same strain (Suresh and Chandrasekaran 1998,1999). These factors are largely characteristic of the organism (Chandrasekaran *et al* 1991). Moreover most microbial extra cellular enzymes are produced in high yields at a growth pH near the maximum for enzyme activity (McTigue *et al* 1994).

In any fermentation medium, nitrogen sources influence significantly the rate of growth and product formation. According to McTigue *et al* (1994) nitrogen can be an important limiting factor in the microbial production of enzymes. In the present study, among the different organic nitrogen sources tested, yeast extract was observed to promote maximal glutaminase production by *B.bassiana* (14.59 U/ml) compared to other nitrogen sources. Among the different inorganic nitrogen sources tested, potassium nitrate was observed to promote maximal glutaminase production by *B. bassiana* (13.06U/ml) compared to other nitrogen sources. Comparatively similar levels of glutaminase yield was observed for both yeast extract and potassium nitrate, irrespective of their organic or inorganic nature.

Further studies on optimization of yeast extract concentration indicated that 2% (w/v) yeast extract concentration is optimal for maximal enzyme production (16.38U/ml). Among the different concentrations of potassium nitrate tested 1(%) (w/v) concentration promoted maximal enzyme production. In both the cases, further increase in concentration resulted in a decrease in enzyme production. Probably the easy availability of additional nitrogen sources along with glutamine in the fermentation medium could have contributed to the rapid growth and enzyme production. In the case

of enzyme production. Thus it was observed that a maximum of 17.32 U/ml was recorded at a concentration of 0.2% methionine. These results very clearly indicate the key decisive role of aminoacids in the fermention medium on L-glutaminase production by marine fungus. There was no L-glutaminase activity in the fermentation broth when amino acids were not added in the fermentation medium. This specific observation suggest that *B. bassiana* requires addition of amino acid to the medium for synthesizing extracellular L-glutaminase. Probably aminoacids added to the fermentation medium play the role of inducer for L-glutaminase synthesis for this marine fungus.

Although the fungus was isolated from marine sediment and anticipated to be strictly marine in nature, out of curiosity an experiment was designed to understand whether the fungus can grow at varied concentrations of NaCl in the medium. It was observed that presence of sodium chloride in the fermentation medium, prepared based on distilled water, influenced rate of glutamine synthesis. Thus the strain showed considerable level of L-glutaminase synthsis even in the absence of NaCl, and also at higher concentrations of NaCl, upto 9 % (w/v) which supported maximal enzyme production. However, with further increase in sodium chloride concentration enzyme production decreased. Results of the present study suggest an euryhaline nature of the strain, in terms of the ability to grow at varying concentrations of NaCl, inspite of its specific requirement for high salt concentrations for maximal glutaminase activity. Probably the strain could be assigned as halophilic with reference to enzyme production.

A time course study on submerged fermentation production of L-glutaminase by *B.bassiana*, after process optimisation, indicated that maximal enzyme production (47.0 U/ml) could be obtained only at fermentation conditions of pH 9.0, 27^0C, 9% sodium chloride concentration, with yeast extract(2%) as additional nitrogen source, sorbitol as additional carbon source, and methionine (0.2%w/v) as unducer, after 108 hours of incubation. It was also noted that the glutaminase production was growth associated for this strain. A similar observation was made for chitinase synthesis by this strain earlier (Suresh and Chandrasekaran, 1998,1999). These observations add evidence to the suggestion that this marine fungus is obviously an alkalophilic and halophilic organism by nature, which have specific requirements for maximal growth and activity for production of chitinase (Suresh and Chandrasekaran, 1998,1999) or glutaminase.

Solid state fermentation

Solid substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitrogen, minerals as well as growth factors. Filamentous fungi are able to penetrate deep into the solid substrate particles for nutrient uptake (Lonsane *et al* 1985), while bacteria and yeast grow by adhering to the surface of the solid substrate particles (Lonsane and Ramesh 1990). Since solid state fermentation processes using nutritionally rich substrates such as wheat bran have inherent problems, use of nutritionally inert materials as supports for solid state fermentation has been recommended so as to overcome these inherent problems (Aidoo *et al* 1982) and a variety of materials were tried as inert supports for SSF processes (Zhu *et al* 1994; Prabhu and Chandrasekaran 1995, 1997).

According to Kobayashi *et al* (1991) a fermentation system using nutritionally inert supports enables to achieve a controlled medium composition and feed rate to separate the product readily from the inert carrier and to perform the SSF process continuously and semi-continuously.

Most marine bacteria can utilize nutrients present in minute concentrations and many of them can only find sufficient food while growing as aufwuchs, a condition during which they remain adsorbed on to solid particles (Chandrasekaran 1996). Marine *Vibrio costicola* was found to get adsorbed on to polystyrene beads and utilize the nutrients supplied in the moistening medium (Prabhu and Chandrasekaran 1995,1996). Similarly *Pseudomonas sp* was observed to synthesise L-glutaminase as an exoenzyme during solid state fermentation using polystyrene as inert support (Keerthi,1990). In the present study also it was observed that marine *B.bassiana* could grow on polystyrene support and produce L-glutaminase as an extracellular enzyme during solid state fermentation.

In order to design a suitable bioprocess for maximal glutaminase production under solid state fermentation using polystyrene as inert support, an effort was made to optimize the important physical, chemical and nutritional parameters that influence the production of L-glutaminase by the marine *B. bassiana* using sea water as the basal fermentation medium. Results obtained in the present study showed positive indications for probable use of SSF using polystrene as inert support, for the production of L-glutaminase with minimal impurities (Prabhu and Chandrasekaran ,1995)

Interestingly it was observed that initial pH of the medium, at 27 ^{0}C, influenced L-glutaminase production by the marine fungal strain, such that two pH optima were

observed that favour high enzyme yield, one at pH 6.0 (23.37U/ml) and another at pH 9.0 (23.95 U/ml). Most microbial extracellular enzymes are produced, at high levels, at a growth pH that is near to the optimal pH required for the maximal enzyme activity (McTigue *et al* 1994). From the results obtained it was inferred that pH does not seriously influence enzyme production by marine *B. bassiana* under SSF, since it could produce invariably considerable level of enzyme over a wide range of pH between 5 to 10.

Incubation at 27 ^{0}C, enhanced enzyme production (32.17U/ml) compared to other temperatures. Nevertheless, considerable level of L-glutaminase production could be obtained at relatively higher levels over a wide range of incubation temperature varying from 22°C (29.42 U/ml) to 47 °C (21.41U/ml). However, it was observed that temperatures above 27°C did not enhance enzyme production compared to lower temperatures. Similar observations were made for the same strain during SSF production of chitinase (Suresh and Chandrasekaran, 1999). These factors are largely characteristic of the organism and vary for each species (Chandrasekaran *et al* 1991). Incorporation of additional carbon sources enhanced the enzyme yield. Among the carbon sources tested, while glucose at 1% (w/v) level enhanced L-glutaminase production to 32.17U/ml, compared to 25.52 U/ml recorded with L-glutamine alone, maltose effected only a marginal increase in enzyme yield. All the other carbon sources tested resulted in a decrease in enzyme yield when compared to control. Comparable results were reported for the production of L-glutaminase by marine *Vibrio costicola* under solid state fermentation using polystyrene as inert support(Prabhu and Chandrasekaran 1997). Optimal glucose concentration was studied by varying the

glucose concentration in the medium and maximum enzyme production (42.12U/ml) was observed at a concentration of 0.5%(w/v). Maltose could promote enhanced L-glutaminase synthesis, by *Vibrio costicola,* in polystyrene system under SSF (Prabhu and Chandrasekaran 1996). Perhaps glucose in the cultivation medium is used by the bacteria as an easy source of carbon for its normal metabolism and L-glutamine must be considered by the bacteria as source of nitrogen in the presence of glucose in the medium. That could have been the reason for enhancement in the enzyme yield compared to control experiment where only L-glutamine alone was added in the medium.

Impact of additional nitrogen sources, on enzyme production was evaluated by incorporating various organic and inorganic nitrogen sources in the medium along with L-glutamine. The results indicated very clearly that addition of other nitrogen sources in the presence of L-glutamine did not enhance enzyme production, and instead these additional nitrogen sources inhibited L-glutaminase synthesis by marine *B. bassiana*, since the level of glutaminase production recorded for the nitrogen sources tested were lesser than what was recorded for the control experiment. Probably under solid state fermentation conditions the fungus can synthesize L-glutaminase only at specific concentration of nitrogen resource, and when the nitrogen concentration exceeds the threshold level it might effect inhibition of growth and enzyme production. Thus perhaps in the presence of glutamine other additional nitrogen sources might have had a negative influence. Further studies could resolve this interesting observation.

Among the different amino acids tested, L- glutamine was observed to enhance L-glutaminase synthesis (32.17 U/ml). Interestingly, there was L-glutaminase

production (32.76U/ml) even in the absence of L-glutamine as well as any additional amino acid in the seawater medium (data not shown). This particular observation suggests that *B. bassiana* could elaborate extracellular L-glutaminase even in the absence of an enzyme inducer, when seawater is used as a medium. Probably, a detailed study on the molecular mechanism involved in the role of seawater components in the biosynthesis of L-glutaminase would draw an insight on the biology of these organisms in natural environment besides designing an economically viable fermentation media.

The effect of glutamine concentration on the production was evaluated in detail, as other amino acids and other nitrogen sources did not show enzyme yield higher than that observed for glutamine. It was observed that a maximal level of 40.95U/ml was recorded at a concentration of 0.25% glutamine) Further, it was also observed that concentrations above 1%, inhibited glutaminase synthesis.

Whether L-glutamine, in the absence of any other carbon source such as glucose, in the sea water based enzyme production medium, can promote glutaminase synthesis was evaluated. It is inferred from the results that L-glutamine promoted production of 25.52 U/ml L-glutaminase at 1 %(w/v) concentration compared to control experiment, (23.4U/ml) in sea water based media without any additives. Further increase in L-glutamine concentration resulted in gradual decrease in the production of L-glutaminase. It was also observed that L-glutamine concentration ranging from 0.25% to 0.75% led to a decline in enzyme yield. This particular observation on the negative impact of lesser concentration of glutamine on glutaminase synthesis also warrants further study to have a clear understanding on the metabolic response of marine fungi to varying concentration of enzyme inducer substance in the medium.

The present study also has thrown open new doors for further research on the role of sea water as fermentation medium, since in the present study it was observed that sea water has promoted synthesis of glutaminase as an extracellular enzyme during solid state fermentation by marine fungus. The aged seawater glutamine medium was selected for further studies not only due to the ease of preparation and the non requirement of expensive chemicals, but also it serves the requirements of a marine fungi with respect to several trace elements and minerals salts, which are naturally present in it. These results indicate the possible role of nutrients present in aged sea water in induction of L-glutaminase compared with distilled water based enzyme production medium, which is almost chemically defined. Of course this observation could not be substantiated with proof since further studies are required to understand the exact role of constituents of sea water in extra cellular enzyme synthesis.

The substrate: media ratio, which determines the moisture content of substrates played a crucial role in the SSF process, as observed with wheat bran and other natural substrates (Prabhu,1996).Since the water absorbency of polystyrene is low (Brydson,1982) increase in the proportion of media in the ratios above 1:1 (w/v; approximately 50-60% moisture content) resulted in the existence of free water and a consequent reduction in the product yield (Prabhu and Chandrasekaran 1997). Zhu *et al* (1994) in a similar study, used 60% moisture content for the SSF production of nuclease P 1 by *Penicillium citrinum* using polyurethane foam as inert support.

Optimal level of initial moisture content in the solid support system required for enzyme production during solid state fermentation was determined by altering the volume of moistening medium added to polystyrene, such that different moisture levels

were achieved. The enzyme production increased along with an increase in moisture content from 15.21U/ml at 60% (v/w) to a maximum 32.17 U/ml at 80%(v/w), after 5 days of fermentation. Whereas, at reduced moisture content (60%) the enzyme production rate was low and moisture content levels above 80%, inhibited enzyme production by marine *B. bassiana.* Any further increase in the initial moisture content resulted in the existence of free water and consequent reduction in enzyme production. This observation is in agreement with that observed for marine *Vibrio costicola* (Prabhu and Chandrasekaran, 1995, 1996).

Inoculum concentration, both spore and vegetative inocula, influenced the rate of L-glutaminase synthesis by marine *B. bassiana.* It was observed that there was a gradual increase in the synthesis of enzyme along with increase in the concentrations of spore inoculum up to $3.7x10^8$ spores/ml, when a maximum of 44.46 U/ml was recorded. However, spore concentrations above this level resulted in a marginal decline in enzyme production. Nevertheless, considerable levels of enzyme production was observed at other spore inoculum concentrations tested during the study. Similarly, an increase in the concentration of vegetative inocula also resulted in an increase in the rate of synthesis of L- glutaminase. Thus, maximal enzyme production (26.32 U/ml) was obtained at 50 ug dry weight of inocula per gram of polystyrene beads. However, further increase in vegetative inocula concentration resulted in a rapid decrease in the synthesis of enzyme. Results obtained in the present study indicate that concentration of inocula is critical for achieving maximal enzyme production and it has to be added at optimal level. Perhaps higher concentrations may lead to competition for nutrients in the medium and consequent reduction in enzyme production level. Lower

concentrations may not be sufficient to effect maximal enzyme production. It was also observed that spore inocula was ideal compared to vegetative inocula in this case for effecting maximal enzyme production, for spores have the advantage of adsorbing onto polystyrene and then germinate rapidly, engulfing the polystyrene beads. In the process they produce enzyme abundantly compared to vegetative inocula, which might not have found the appropriate niche for such an activity. Of course further studies on the nutrient uptake and metabolism by spores and vegetative mycelia would unearth more information on this aspect.

Marine organisms are greatly influenced by the salinity of sea water. The strain used in the present study was isolated from marine sediments and hence it was desired to optimize the strain for its optimal salinity requirement, so that sea water can be used as a fermentation basal medium. Sea water was diluted to various concentrations, with distilled water. Results obtained in the present study indicated that concentration of sea water, used as the basal media component, has significant impact on enzyme synthesis. Thus it was observed that with increase in concentration of sea water there was a gradual increase in the production of L-glutaminase and the maximal production was obtained at full strength sea water (44.46 U/ml). Interestingly, even at 0% seawater concentration (distilled water) *B. bassiana* could produce 25.74 U/ml of enzyme, indicating an euryhaline nature of the strain used in the study.

Since this strain was observed to grow and produce glutaminase at 9% NaCl under SmF, it was desired to evaluate the impact of additional sodium chloride concentration in sea water, contributing to high salt concentration in the fermentation media, and consequent influence on rate of enzyme synthesis. It was noted that addition

of sodium chloride to the sea water based medium did not enhance enzyme production and instead led to a sharp decrease in enzyme activity along with increase in sodium chloride concentration from 0 % to 4.0 %. This suggests that the fungus is not halophilic, but could be a halotolerant one and a natural commensal organism of the marine environment. High NaCl concentration in the enzyme production medium attributed through addition of NaCl over and above that already present in the seawater, affected the enzyme production. Further the results very clearly advocate the use of sea water as an ideal medium for L-glutaminase synthesis under solid state fermentation using polystyrene as inert support.

In order to monitor the rate of L-glutaminase production by *B. bassiana* at optimized conditions, a time course experiment was carried out after optimization of various bioprocess conditions. Results obtained for the said study suggest that L-glutaminase production increased progressively along with increase in incubation time until 96 hours, when maximal enzyme production was recorded (49.89 U/ml). However, the enzyme yield declined during further incubation beyond 96 hours. The reason for the decrease in enzyme yield after 96 h might be attributed to the inactivation of glutaminase by the protease secreted by the fungus. A similar observation was made with *V.costicola* for glutaminase under SSF using polystyrene (Prabhu and Chandrasekaran, 1996,1997). A similar observation was also made during SSF on polyurethane foam for the production of nuclease P1 by *P. citrinum*, where the nuclease yield was reduced after 3 days of fermentation due to enzyme inactivation by protease (Zhu *et al* 1994). Ramesh and Lonsane (1987) also observed reduction in alpha amylase production by *B. megaterium* under SSF on wheat bran after 52 h of incubation and

postulated that this may be due to poisoning, denaturation and/or decomposition of the enzyme as a result of interaction with other components in the medium.

Determination of the total cell protein of the organism during the time course experiment revealed that the fungal strain quickly passed into stationary phase during solid state fermentation after 48 hours. Enzyme production increased along with the increase in total cell protein, during the logarithmic phase, up to 96 h followed by a decline on extended incubation A comparison of biomass and enzyme activity curves indicated that maximal enzyme synthesis occurred during stationary phase. Nevertheless, considerable level of enzyme synthesis was recorded during log phase. This trend evidence that the enzyme production was growth associated.

Comparison of L-glutaminase production by marine and terrestrial strains of *Beauveria bassiana*.

A comparative evaluation of the L-glutaminase production by marine and terrestrial strains of *B. bassiana* was conducted using a standard terrestrial strain of *B. bassiana* obtained from National Collection of Industrial Microorganisms (NCIM, Pune, India), under solid state fermentation and submerged fermentation using polystyrene, in distilled water and sea water based media. It was observed that marine *B. bassiana* preferred sea water based fermentation medium for maximal L-glutaminase production compared to distilled water based medium which supported comparatively lesser glutaminase synthesis, irrespective of the fermentation condition, submerged or solid state fermentation. Whereas, in the case of terrestrial *B. bassiana*, distilled water based media supported enhanced enzyme production compared to sea

water. Marine *B. bassiana* produced comparatively higher level of L-glutaminase than terrestrial strain. This particular observation suggest high potential for marine fungi for industrial enzyme production and their suitability for industrial application. Similar reports are not available for any other enzyme and hence a comparison could not be done.

L-glutaminase production by Ca-alginate immobilized *B. bassiana* spores

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). Emilia Abraham *et al.*(1991) have demonstrated a continuous production of glucoamylase by immobilizing mycelial fragments of *A.niger*. L-glutaminase production by *B. bassiana.* was observed under submerged culture conditions (Keerti *et al.*, 1999), and under solid state fermentation on an inert support-polystyrene (Sabu *et al* ;1999). These observations led to us to investigate the potential of Ca-alginate immobilized *B. bassiana* for continuous production of the enzyme towards developing an ideal bioprocess for industrial production of the enzyme where biomass can be recycled. Hence, initially various process parameters affecting extracellular L-glutaminase production by Ca-alginate immobilized *B. bassiana* spores were optimized under batch process and these optimized parameters were incorporated for studies on continuous production of the enzyme.

Results obtained in the present study indicate the importance of immobilizing support concentration, concentration of $CaCl_2$ solution used for bead preparation, and the bead curing time on L-glutaminase production. At lower support concentrations,

beads were malformed and unstable, with considerable leaching of spores. Enzyme yield increased with increase in the support concentration up to 3% w/v level (8.77 U/ml) and then showed a decline. This might be attributed to the diffusional limitations encountered with higher alginate concentrations. The optimal concentration of $CaCl_2$ for the preparation of beads with maximal enzyme yield (28.08 U/ml) was 0.1M. Nevertheless considerable enzyme yield (25.74 U/ml) was obtained when a 0.05M solution was used for bead preparation. However at that concentration, the beads formed were less stable with leaching of spores and floated in solution. It was also noted that the beads required a curing time of 3 hours for maximum productivity (56.16 U/ml). Increase in curing time beyond 3 hours drastically reduced the enzyme activity indicating that prolonged exposure to $CaCl_2$ may reduce the enzyme yield from beads. Spore concentration influenced enzyme production under immobilized condition. Of the different concentrations of spores tested, a concentration of $12x10^8$ spores/g bead gave maximal enzyme yield of 13.10 U/ml. The enzyme yield increased with increase in spore concentration, characteristic of the immobilized cell fermentations. However there was a decrease in enzyme yield with the higher spore concentration of $16x10^8$ spores/g bead, which might be due to substrate limitation at that spore concentration.

Activation time of the saline preserved beads also influenced the rate of enzyme production by immobilized spores. Beads stored in saline at 4 ^{0}C, required an optimal activation of 15 hours, at 27^{0}C, in 1% glutamine solution, to bring them into the active production phase. 15hours of activation yielded an enzyme activity of 58.05 U/ml. Decrease in enzyme yield of beads activated for more than 15 hours might be accounted with the fact that the beads already entered the production phase, in the

activation medium and hence the enzyme activity could not be detected in the enzyme production medium.

It was observed that the immobilized *B. bassiana* produced maximal enzyme (64.46 U/ml) at a pH of 9 and at room temperature (27 $\pm$ 0.2^{0}C). It may be noted that this fungus recorded the maximal enzyme production at pH 9 under solid state fermentation using polystyrene as inert support (Sabu *et al.*, 1999), as well under submerged fermentation. This may be due to the alkalophilic nature of the fungus and specific preference for alkaline pH 9.0(Suresh and Chandrasekaran,1998). However considerable enzyme production was recorded over a wide range of pH and temperature. It was also observed that the optimal incubation time for maximal enzyme yield (64.46 U/ml) by immobilized *B. bassiana* is 18h, beyond which there was a probable inactivation of the enzyme and the yield reduced significantly. In general results obtained in the present study suggest that calcium alginate immobilization of fungal spores is effective for rapid production of L-glutaminase by marine *B.bassiana* similar to that observed for *Bacillus polymyxa* for alpha amylase(Mohandass 1993)

Continuous production of L-glutaminase by Ca-alginate immobilized spores of *B. bassiana* in a 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 washout at the relatively high flow rates that are used. Packed bed reactors being studied 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 (Margaritis and Bajpai,1983).

Hence, in the present study continuous production of L-glutaminase by immobilized spores of marine *B. bassiana* was performed incorporating the optimal level of parameters obtained in the batch production studies. Parameters which directly influenced continuous studies were studied at their different levels. Different flow rates tested included 20, 30, 40, 50, and 60ml/hour. It was found that the lowest flow rate of 20 ml/hour gave maximum yield of L-glutaminase. Production of enzyme remained almost steady throughout the time of observation and showed a linear decline with increase in flow rate. Cumulative production of L-glutaminase by immobilized spores of *Beauveria bassiana* in a packed bed reactor was also monitored. Total enzyme yield per hour of operation was calculated for different flow rates, substrate concentrations in the enzyme production media, aeration rates and bed heights towards determining the efficiency of the packed bed reactor for continuous production of the enzyme. It was observed that 404.8U/100ml (4.048 U/ml/hour) could be produced at a flow rate of 20ml/hour compared to other flow rates. This can be attributed to the fact that at lower flow rates the residence time is more and the beads are in contact with the medium for a longer duration. Often in continuous production, the medium is in contact with the immobilized cells for shorter duration; and unless recycled most of the substrate remains unutilized So the study was performed to find out the optimal substrate concentration in the enzyme production medium, at a flow rate of 20 ml/hour when

used for continuous production of L-glutaminase in a packed bed reactor. A substrate concentration of 0.25% w/v was recorded as ideal concentration for continuous production of L-glutaminase. Aeration did not make any difference in the productivity of the reactor and an unaerated process could give the maximal productivity. This might be due to the fact that the air bubbles fill in the voids of beads, thereby reducing the contact between the beads and medium.

Increase in bed heights had a clear advantage in cumulative productivity with the highest bed (15cm) giving maximal production (445U/h). Maximal enzyme production was obtained for the highest bed height (15 cm), for the given flow rate indicating that with increase in bed height, the residence time also increases for the given flow rate, increasing the contact time between beads and medium and thereby effecting a higher productivity.

Under the optimized conditions for continuous production, the reactor gave a volumetric productivity of 4.048 U/ml/hour, when compared to the productivity for Ca-alginate immobilized *B. bassiana* spores (3.58 U/ml/hour) under batch production and (0.52 U/ml/hour) for *B. bassiana* under SSF on inert support.

This indicates that continuous production of the enzyme by Ca- alginate immobilized spores is well suited for the *B.bassiana* and results in a higher yield of enzyme within a shorter time compared to the other fermentation systems. Similar observations were made for amylase production using *Bacillus polymyxa* (Mohandass and Chandrasekaran,1994), municipal effluent treatment using *Bacillus* sp (Anbusaravanan *et al*,1994) and for rubber effluent treatment (Jayachandran *et al* 1994).

Comparison of the different fermentation systems for L-glutaminase Production by *Beauveria bassiana*

Performance of marine *B. bassiana*, in terms L-glutaminase production, under submerged, solid state and immobilized conditon was evaluated with reference to the enzyme yield. Of the three fermentation systems tried for enzyme production, fungal spores immobilized in sodium alginate beads, under batch mode, could enable high enzyme activity (64.46 U/ml) compared to solid state fermentation (49.89 U/ml) and submerged condition (47 U/ml). Whereas, when considered in terms of enzyme activity per unit biomass, solid state fermentation was observed to support more enzyme yield (121.68 U/unit biomass) compared to immobilized system (78.21 U/unit biomass) and submerged fermentation system (72.31 U/unit biomass)

The most interesting observation is that immobilized system required very short incubation time, about 18 hours, compared to submerged fermentation system (which required 108 hours) for synthesizing almost similar levels of enzyme. Nevertheless, solid state fermentation system gave a high yield in 96 hours compared to other two systems. Immobilized spores could yield more enzymes in very short period under continuous mode of operation than any other system.

Further, when the various process conditions were compared, interesting observations were made for the three fermentation systems. The marine *B.bassiana*,

irrespective of the fermentation system whether submerged or solid state or immobilized showed preference for same pH (9.0), and incubation temperature (27^0C), whereas several variations were also noted for other variables. Thus while glutamine was preferred as substrate for glutaminase production under solid state fermentation, methionine was observed as preferred substrate under submerged fermentation. While sorbitol supported glutaminase production under submerged fermentation, it was glucose that supported glutaminase production under solid state fermentation. While yeast extract and potassium nitrate supported glutaminase production as additional nitrogen sources under submerged fermentation, there was no need for additional nitrogen sources for glutaminase production under solid state fermentation. In fact it is very difficult to draw conclusions on this particular preferences showed by this marine fungus in response to variation in the physical state of fermentation, and only a detailed study at the molecular level alone can resolve this mysterious behaviour of marine *B.bassiana.*

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

Beauveria bassiana, isolated from the marine sediment and available in the culture collection of the Department was used in the present study for evaluation of its potential for L-glutaminase production, as an extra cellular enzyme. Enzyme production was carried out under three fermentation conditions namely submerged, solid state and immobilized.

Initially the various process parameters that influence the rate of enzyme production under SmF were optimized using distilled water as basal medium. pH of the medium, incubation temperature, additional nitrogen source, additional carbon source, aminoacid requirement, and NaCl concentration in the medium were optimized. pH 9.0, 27^0C, sorbitol (1% w/v), yeast extract (2%w/v), potassium nitrate(1% w/v), methionine(0.2% w/v), 9.0% NaCl concentration were observed as optimal conditions for maximal enzyme production. After optimization atime course study was conducted and found that incubation for 108 hours of incubation was required for maximal enzyme production under submerged fermentation.

Solid state fermentation production of L-glutaminase was carried out using polystyrene as inert solid support, using aged sea water as basal medium. Initially the various process parameters that influence the rate of enzyme production under SSF were optimized. pH of the medium, incubation temperature, additional carbon source, additional nitrogen source, aminoacid requirement, L-glutamine concentration, initial

moisture content, inocula concentration, sea water concentration and additional NaCl concentration in the medium were optimized. Two pH optima one at 6.0 and another at 9.0, 27^{0}C, D-glucose(0.5%), Glutamine concentration(0.25% w/v), 80% moisture content, spore inocula (3.7 x 10^8 spores /ml) and 100% sea water concentration were observed as optimal conditions for maximal enzyme production. Absence of additional nitrogen source and NaCl in the medium were observed as ideal condition for maximal enzyme production. After optimization a time course study was conducted and found that incubation for 96 hours of incubation was required for maximal enzyme production under submerged fermentation.

B.bassiana could produce L-glutaminase both in distilled water medium sas well as in sea water based medium. Sea water could act as a ideal fermentation medium without any additives.

A comparison was made between marine and terrestrial strains of *B.bassiana* using a standard strain *B.bassiana* NCIM 1216. It was observed that marine strain produced higher levels of L-glutaminase compared to terrestrial strain. Further marine strain required sea water as ideal fermentation medium for maximal enzyme production under both submerged and solid state fermentation conditions, Whereas terrestrial strain required distilled water based medium as ideal fermentation medium for maximal enzyme production under both submerged and solid state fermentation conditions,

Spores of *B.bassiana* were immobilized in calcium alginate beads. Optimal conditions for preparation of stable immobilized spore beads were standardized. 3% sodium alginate as support concentration, 12 x10^8 spores /gram bead, 0.1 M $CaCl_2$, 3 hours of curing time, 15 hours activation time, 18 hours retention , pH 9.0, 27^{0}C were

identified as optimal conditions for preparation of stable beads. After optimization L-glutaminase production under continuous mode in a packed bed reactor was carried out at different flow rates (20ml/hour - 60ml/hour), substrate concentration, aeration and bed heights. 20ml/hour flow rate, 0.25% glutamine concentration and 15cm bed height were observed as ideal conditions for maximal glutaminase production. No aeration of the packed bed reactor was required.

Of the three fermentation systems, immobilized system supported maximal level of enzyme by the selected fungal strain compared to submerged and solid state fermentation.

CONCLUSIONS

Based on the results obtained in the present study the following conclusions are drawn. *Beauveria bassiana* isolated form marine sediment has immense potential as an industrial organism for production of L-glutaminase as an extracellular enzyme employing either submerged fermentnation or solid state fermentation. Spores of this fungus are capable of synthesising L-glutaminase in abundant quantity at high rate, under immobilized condition entrapped in calcium alginate beads. High rate of enzyme synthesis could be achieved by immobilized spores under continuous mode of operation in a packed bed reactor. Further it was observed during the course of the present study that aged sea water could be used as an ideal fermentation medium for L-glutaminse synthesis using polystyrene as inert solid support system.

The results obtained for the media optimization studies and use of sea water alone as media for L-glutaminase synthesis indicate the possible role of nutrients present in aged

sea water in induction of L-glutaminase compared with distilled water based enzyme production medium, which is almost chemically defined. Further studies are warranted to substantiate the exact role of constituents of sea water in extra cellular enzyme synthesis, and to understand the differential nutritional requirement shown by this strain under submerged and solid state fermentation.

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