# CHITINASE PRODUCTION BY MARINE FUNGI

A THESIS

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COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
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## Doctor of Philosophy

IN BIOTECHNOLOGY
IN THE FACULTY OF SCIENCE

by

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**1996**

*Affectionately dedicated to*
*my beloved mother*

# DEPARTMENT OF BIOTECHNOLOGY

**COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY**

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10th October 1996

## CERTIFICATE

This is to certify that the work reported in this thesis entitled *Chitinase production by marine fungi* was carried out by Mr. Suresh P.V under my supervision for the requirement of the degree of **DOCTOR OF PHILOSOPHY** in Biotechnology in the Faculty of Science, Cochin University of Science and Technology and that no part there of has been presented before for the award of any degree, diploma, association or recognisation in any university.


Dr. M. Chandrasekaran

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## DECLARATION

I hereby declare that the work presented in this thesis entitled "Chitinase production by marine fungi" is based on the original work done by me under the supervision of DR. M. Chandrasekharan, (UGC Career Awardee), Reader, Department of Biotechnology, Cochin University of Science and Technology and that no part of this has been included in any other thesis submitted previously for the award of any degree in any university.

Suresh P.V.

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# PREFACE

I have been inspired to work on marine microbial enzyme, especially in chitinolytic enzyme from marine fungi, by Dr. M. Chadrasekharan, my Supervisor who worked on marine microbial enzyme production with various fermentation techniques. In fact, Dr. M. Chandrasekaran and co-workers at Cochin University of Science and Technology are the pioneers working in the area of Marine Microbial Biotechnology in India.

Traditionally mycologists have devoted most of their efforts to study terrestrial fungi. Consequently filamentous fungi is well recognised in industrial processes and has gained more importance with the current awareness and rapid expansion of Biotechnology. In fact terrestrial fungi are used almost exclusively for this purpose.

In this context it is worthwhile to consider the marine fungi from the vast marine environments, which remain untapped so far. Further, in the light of resurgence of interest in solid substrate fermentation system all over the world and the growing interest in the recycling of agro-industrial waste in developing countries, an attempt was made to explore the potential of marine fungi for the production of chitinolytic enzymes and to recognize the ability to hydrolyse native chitin through submerged as well as solid substrate fermentation culture conditions, using `wheat bran and shellfish processing waste such as `prawn waste' as solid substrates.

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# CHAPTER - ONE

# INTRODUCTION

The enzyme is nature's own way of effecting biochemical synthesis, breakdowns, transformations and separations. It's role is only one step down the chain of command from the gene itself and like the gene it is a highly diverse structure. The current world market for industrial enzymes is approximately US $ 600 million (sales value). More than half of the market is in the USA (Arbige and Pitcher, 1989). Among the source of enzymes, plants, animals and microbes, the latter serves as a promising resource with diverse range of enzymes for varied purposes. Today microbial enzymes have become a commodity, produced by the industries in a large scale through the process of fermentation using potential microorganisms.

Microbial enzymes have several advantages over the enzymes derived from plants or animals by virtue of their great variety of catalytic activities, cheaper in cost, regular abundant supplies at even quantity and relatively more stability. Further production is usually convenient and safe compared to that from plant and animal sources (Cheethem, 1985)

In spite of their utilization in industries for many centuries only recently a detailed knowledge relating to their nature, properties and functions have become evident. In

fact, the commercial success of alkaline protease and amyloglucosidases in the early twentieth century formed a basement for subsequent research and development in the area (Wiseman, 1978). Although the use of microbial enzymes may not have expanded at a quite the rate expected a decade ago, there is nevertheless intense activity and considerable interest in the whole area of enzyme technology evidenced by reports on intensive screening pursued all over the world for enzymes with novel properties and functions from various microorganisms inhabiting different environments (Fogarty and Kelly, 1990).

Despite the fact that the terrestrial environment has contributed to the overwhelming growth of enzyme industry, through providing potential microorganisms, bacteria and fungi, as source for several industrial enzymes in all these years, there are reports on marine microorganisms which are potent producer of DNase, lipase, alginase, proteases, chitinases and glutaminase (Austin, 1992, Renu and Chandrasekaran, 1992, Moriguchi *et al.*, 1994, Prabhu and Chandrasekaran, 1995, 1996).

Although the oceans cover more than two third of the World's surface, our knowledge of marine organisms, in particular fungi, is still very superficial (Molitoris and Sehumann, 1986). Further, as on date marine microorganism

remain as untapped sources of many metabolites with novel properties (Faulkuer, 1986; Chandrasekaran, 1996). Thus there is an urgent need to undertake investigations exploring the probabilities of deriving new products of economic importance from potential marine microorganisms.

Chitinase, the subject of the present study, is found in a variety of living organisms such as snails, bean seeds, insects and microbes (Reynold, 1954; Clarke and Tracey, 1956; Jeuniaux, 1966). Among the various sources, microbial chitinases are drawing the interest of the Industry and agriculture currently. Microbial chitinases are being qualified for use as biocides for the control of fungal pathogens and entomopathogens in agriculture. The production of microbial chitinases has received attention as one step in a bioconversion to treat shellfish waste chitin (Revash and Carroad, 1981). As in the bio conversion of cellulose to Single Cell Protein, the production of the chitinase system is thought to be one of the primary economic variable estimated to account for 12% of the total production cost (Cosio *et al.*, 1982).

Since the enzyme industry is highly competitive, reliable estimates of the extent to which different fermentation techniques are used are not easy to establish. Although specific procedures adopted by different manufactures

will vary to a degree there remains only two principal methods of cultivation i.e, solid substrate and submerged fermentation.

Today's industry, however, is dominated by deep tank or submerged culture fermentation with its lower cost of production, process control capabilities and reduced contamination problems. Although solid state fermentation, which is known as traditional method, is used less frequently 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).

Solid substrate fermentation (SSF) refers to growth of microorganisms on solid-nutrients with moisture existing within the solid matrix in an absorbed form but without the presence of a free liquid phase (Cannel and Moo-Yong, 1980). Many microorganisms are capable of growing on solid substrate. The filamentous fungi represent the most common class of microorganisms used in SSF because of their ability to penetrate and colonise the substrate by apical growth and tolerate the low amount of water available (Lambert, 1983; Smith and Aido, 1988).

One of the most successful exploitation of SSF technique is for the commercial production of different exoenzymes such as pectinase, amylase, amyloglucosidase and cellulose, since it offers many advantages over submerged fermentation (Lonsane and Karanth, 1990).

The success of SSF largely depends on the type of the substrate and the nature of the microorganisms used. SSF processes will utilize a wide range of solid organic materials, such as wood, straw etc. Such compounds are invariably polymeric molecules and insoluble in water. However, they are cheap, readily available and provide concentrated sources of nutrients (Smit and Aido, 1988). The selection of a raw material for bio-processing is dictated by the price, availability, composition and the oxidation state of the carbon sources (Hacking, 1986). The most widely exploited substrates for SSF are mainly materials of plant origin and include food crops (grains, roots, tubers, and legumes) plant residues, and lignocellulosic materials (wood, straw, hay and grasses) (Smith and Aido, 1988). By far the most popular substrate used in solid substrate fermentation for exo-enzyme production by filamentous fungi is wheat bran (Lambert, 1983).

Majority of the microorganisms used in SSF processes are native of terrestrial environments and the use of marine

microorganisms in SSF is not known except for the few reports on the use of marine bacteria (Renu and Chandrasekaran, 1992; Prabhu and Chandrasekaran, 1995, 1996).

Chitin, widely distributed in nature, particularly in marine vertebrates, insects, fungi and algae is at present the subject of a number of project, directed towards the economical exploitation of various chitinous resources (Muzzarelli, 1977).

Due to chitin's important biological role as a structural component its synthesis and degradation have been the subject of extensive research. Most of the studies have been focused on fungal morphogenesis, bio-insecticides and bio-fungicides and the commercial utilization of chitinous waste for SCP production (Zikakis, 1989; Shaikh and Deshpande, 1993).

The chemical similarity between cellulose and chitin has suggested a process scheme for the bioconversion of chitin to yeast SCP production which draws upon the technology developed for cellulose bioconversion (Revash and Carroad, 1981).

India, one of the leading shrimp and Crab landing countries, generates large quantities of chitinous solid waste

from numerous shell fish processing industries. The chitinous solid waste fraction of the average Indian landing of shellfish range from 60,000 to 80,000 tonnes (Madhavan *et al.*, 1986). The solid waste from shellfish processing is composed mainly of chitin, $CaCO_3$ and protein. Ashford *et al.* (1977) demonstrated that chitin represents 14-27% and 13-15% of the dry weight of shrimp and crab processing respectively. Madhavan and Nair (1978) reported that dry prawn waste and dry squilla contained 23% and 15% chitin respectively.

Current method of handling shellfish solid waste include fishmeal production (Nirmala, 1991) and bioconversion of waste chitin to yeast single cell protein (SCP) using chitinolytic enzyme (Revash and Carroad, 1981). Whereas in India, most of the shellfish solid waste are subjected to ocean dumping and land filling, except for the small quantity (about < 10%) which is used for chitin production.

In this context, considering the potentials of marine microorganisms for deriving industrially important enzymes and the tremendous scope for bioprocessing of chitinous shrimp wastes towards economic utilisation employing solid state fermentation, in the present study, an attempt was made to isolate a potential chitinase producing fungus from marine environment and to develop an ideal bioprocess for the production of chitinase using prawn wastes as solid substrate.

# REVIEW OF LITERATURE

## Chitin

Chitin is the most abundant renewable natural resource after cellulose (Deshpande, 1986; Gooday, 1991; Nicol, 1991). It is a white, hard, inelastic, nitrogenous polyasaccharide, widely distributed in nature, particularly in marine invertebrates, insects, fungi and algae (Muzzarelli, 1977). It is the second most abundant organic compound next to cellulose on earth. It was first isolated by (Braconnot,1811) from mushrooms and was named "fungine" The name chitin, derived from Greek "chiton," mean `a coat of mail' due to its prominence in the mantles of the insects.

Chitin occurs widely in nature as a structural polymer in the integument of insect and crustacea and in the cell wall of many fungi (Jeuniaux, 1971).

Chitin is a polymer of unbranched chains of $\beta$ (1->4) linked 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine, GlcNAc). It may thus be regarded as a derivative of cellulose, in which the C-2 hydroxyl group have been replaced by acetamido residues (Foster and Weber, 1960) In the case of chitosan the acetylated amino group of chitin is deacetylated, polymer of $\beta$ (1 -> 4) linked-D-glucosamine (McGahren *et al.*, 1984).

Chitin occurs in three polymeric crystallographic forms viz: $\alpha$ chitin, $\beta$ chitin and $\gamma$ chitin, which differ in the arrangement of molecular chains within the crystal cells (Richards, 1951, Muzzarelli, 1977).

Based on the purity of chitin, it is differentiated into three states "native", decalcified chitin and chitin. Pure chitin, rarely found in nature, normally occurs in combination with protein and certain inorganic salts, especially of calcium salts.

Chitin and chitosan are the only abundant basic polysaccharides. The chitin is highly hydrophobic and is insoluble in water, dilute acids, dilute and concentrated alkalies, alcohols, and other organic solvents (The Merk Index, 1983). It is soluble in concentrated HCl, $H_2SO_4$, 78-97% $H_3PO_4$ and anhydrous formic acid. Chitosan is insoluble in water, but soluble in dilute acids. The properties of chitin and chitosan vary considerably depending on the source and production process.

The major sources of chitin are shell fish, krill, clams, oyster, squid, fungi and insects. The annual availability (Global) of chitinous materials is presented in Table I. Chitin and Chitosan have been produced on a pilot plant scale from shrimp and *Aspergillus niger* in Norway and

Italy (Nicolaysen, 1980; Muzzarelli, 1983). Chitin represents 14-27% and 13-15% of the dry weight of shrimp and crab processing respectively (Ashford *et al.*,1977). Dry prawn waste and dry squilla contain 23% and 15% chitin respectively (Madhavan and Nair, 1978).

Table 1 Annual availability (Global) of chitinous materials (Allen *et al.*, 1978)

| Resources | Quantity harvested, $10^3$ tonnes | Chitinous waste, $10^3$ tonnes | Chitin potential, $10^3$ tonnes |
|---|---|---|---|
| Shell fish | 1700 | 468 | 39 |
| Krill (potential landing) | 18200 | 3640 | 56 |
| Clam/oysters | 1390 | 521 | 52 |
| Squid | 660 | 99 | 1 |
| Fungi | 790 | 790 | 32 |
| Total | 22,740 | 5,118 | 150 |

At present, the commercial sources of chitin is the shrimp wastes from shell fish processing plant, crab wastes and squilla caught during trawling of shrimps (Madhavan and Nair, 1974, 1975; Madhavan *et al.*, 1986) The chitinous solid waste fraction of the average Indian landing of shellfish range from 60,000 to 80,000 tonnes (Madhavan *et al.*, 1986) It is estimated that the world wide annual recovery of chitin

from the processing of marine invertebrates, for example, is 37,300 tonnes (Nicol, 1991).

## Chitinases

The complete enzymatic hydrolysis of chitin to free N-acetyl-D-glucosamine is performed by a chitinolytic system, the action of which is known to be synergistic and consecutive (Jeuniaux, 1966). The chitinolytic enzymes are generally induced as a multi enzyme complex and are traditionally classified into two main types (1) endochitinase (poly-β-1->4 -(2-acetamido-2-deoxy)-D-glucoside glyconohydrolase, EC: 3.2.1.14) (2) chitobiase (β-D-N- acetyl-glucosaminidase, chitobiase acetamido deoxy glucohydrolase, EC: 3.2.1.30 formerly EC: 3.2.1.29). Endo chitinase randomly hydrolyses the polymer of N-acetyl-D-glucosamine, including tetramers and to a lesser extent trimers, and eventually yields diacetyl chitobiose as the major product. Chitobiase hydrolyses chitobiose and chitotriose (Deshpande, 1986; Shaikh and Deshpande, 1993). Same chitobiase cleaves N-acetyl glucosamine units from the non-reducing end of chitin chain (Gooday, 1990).

The existence of a third class of enzyme, exo chitinase has also been suggested (Robbins *et al.*, 1988) The exo chitinase catalyses the progressive release of diacetyl chitobiase (or GlcNAc ?) units from the non-reducing end of

the chain. The classification of exo and endo enzyme depends mainly on the nature of the substrate (Lindsay and Good, 1985).

Not much is known about the mechanisms involved in the break down of chitin (as observed in cellulose degradation) mainly because many chitinases take part in a synergistic relationship (Ohtakara, 1964) and only a few have been purified to homogeneity (Charpentier and Perheran, 1983). Still the exoglycanase known in cellulolytic system has not been implied in the case of chitinolytic system (Deshpande, 1986). Nevertheless some evidences are considered for the presence of prehydrolytic factor in the chitinolytic system (Ch $_1$) in the cellulolytic (C $_1$) degradative mechanisms (Monreal and Reese, 1969, Deshpande, 1986).

**Enzymatic mechanisms involved in the degradation of chitin**

A number of enzymes are known to be responsible for the breakdown of chitin by an extracellular enzyme system and the end products assimilated and is utilized for intracellular metabolic processes by the cell. Based on a number of reports, Hood and Meyar (1977b) suggested the following pathway for the enzymatic breakdown of chitin (Chart I)

Chitin —(Prehydrolytic factor)→ Chitin (Susceptible)

Chitin —(Chitinase)→ Chitodextrins

Chitodextrins —(*Chitinase)→ Chitobiose
(short chain soluble chitin residue)

Chitobiose —(*Chitobiase)→ N-acetyl glucosamine

N-acetyl glucosamine —(* Deacetylase)→ glucosamine

glucosamine —(*Deaminase)→ glucose

* cellular uptake occurs.

Chart I Possible pattern for the enzymatic degradation of Chitin (Hood and Mayer, 1977b).

Ohtakara (1964) suggested a possible pattern for the enzymatic degradation of chitin and glycolchitin with the partially purified chitinase from *Aspergillus niger* (chart II).

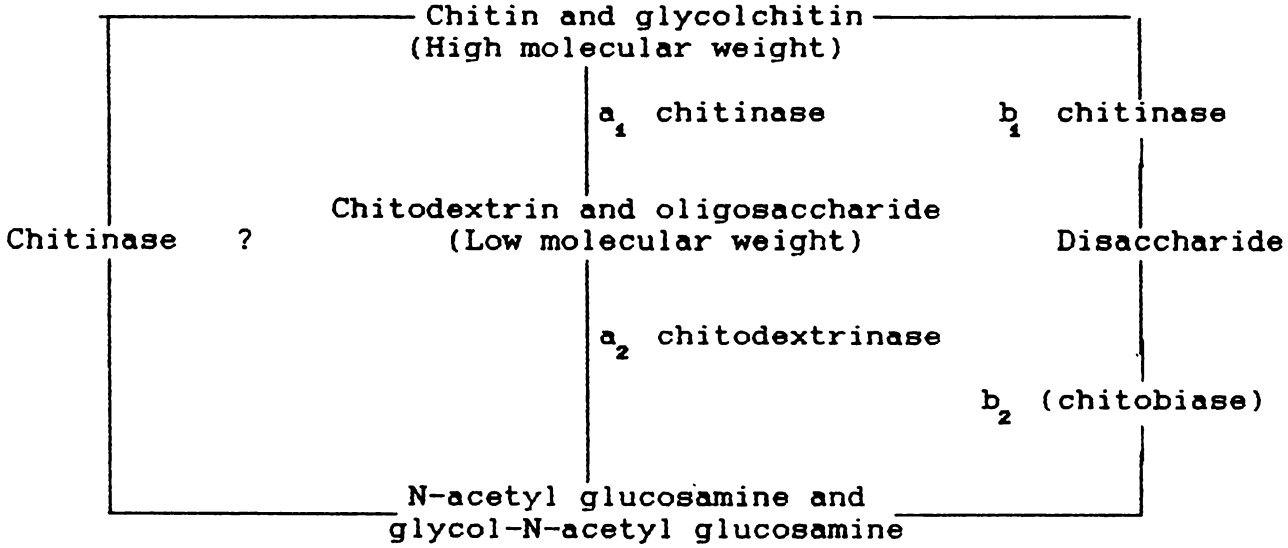


Chart II Possible pattern for the enzymatic degradation of chitin and glycolchitin (Ohtakara, 1964).

Crystalline chitin was efficiently hydrolyzed only by a mixture of endo and exo glycanases (Monreal and Reese, 1969) It was found that exo and endo chitinases could hydrolyse phosphoric acid swollen chitin. The endochitinase hydrolyse the derivatives such as CM chitin (Hultin, 1955), glycolchitin, (Ohtakara, 1961), phosphoric acid swollen chitin, (Deshpande, 1986), or chitosan acetate prepared by the partial deacetylation of chitin (Tracy, 1955), and act randomly splitting the chitin into smaller chain, eventually producing chitobiose.

The hydrolysis of native chitin could be the result of the synergistic action of the endo and exo chitinases, But the question "which enzyme initiates the attack" ? remains unanswered. Reese (1975) suggested that the first step in the hydrolysis of cellulose as well as chitin involve a prehydrolytic factor ie, $C_1$ for cellulose hydrolysis (Rees *et al.*, 1950; Sadana and Patil, 1985) and $Ch_1$ for chitin hydrolysis (Monreal and Rees, 1969; Fermar and Grant 1985). Of course in the case of chitin hydrolysis, though it is parallel to cellulose hydrolysis, a lot of work will have to be carried out before any postulation about the mechanism of degradation of chitin can be made (Deshpande, 1986).

The structure and function of lysozyme's active site are well known. According to Hara *et al.*(1989) a chitinase

from *Streptomyces erythraceus* and hen egg-white lysozyme have a similar binding mode to N, $N^4$- diacetyl chitobiose. Using various partially O methylated disaccharides to elucidate the substrate binding mode of the chitinase, they found that the hydroxyl groups in the $C_6$ position of the reducing end sugar and in the $C_3$ and $C_4$ positions of the non-reducing end sugar are not important for the enzymatic action. Whereas the acetamide group in the $C_2$ position and the hydroxyl groups in the $C_3$ position of the reducing end sugar and in the $C_6$ position of the non-reducing end sugar are oriented towards the enzyme molecule (Hara *et al.*, 1989).

**Marine filamentous fungi**

The early history of marine mycology start with the report of Saccardo (1883), and Ellis and Everhart (1885) who reported species of *Ophiobolus* on plant remains in marine environment.

The degradative process of marine fungi involving the production of intra and extracellular enzymes have received considerable attention. Mayers and Reynolds (1959a, b, 1960, 1963), Mayers and Scot (1968), and Mayers *et al* (1960) were among the first to study the cellulolytic activity of marine lignicolous fungi in detail, Meyers (1968) and Jones and Irvine (1972) discussed the degradative role of filamentous marine fungi in the marine environment. Pisano *et*

*al.*(1964) screened 14 marine fungi for the gelatinase activities and found such activity in the culture filtrates of 13 isolates. Rodriguese *et al.* (1970) studied the dehydrogenase pattern in marine filamentous fungi, while Vembu and Sguros (1972) examined citric acid cycle and glycoxylate by pass in glucose grown filamentous marine fungi.

Schumann (1974) demonstrated, in 20 marine fungi, the production of cellulase by applying the viscometric and agar plate method. The clearing of cellulose containing agar by 14 marine fungi was also used by Henningsson (1976) as a measure of cellulase and xylanase production. Eaton (1977) demonstrated the ability to degrade wood cell wall components of several marine fungi belonging to the genera *Cirrenallia, Halosphaeria, Humicola, Niaculcitlna and Zalerion.* They compared them with fresh water and terrestrial fungi and found production of cellulase, xylanase and mannase in all species tested.

Detailed information on the extra-cellular enzyme production by marine fungi has been provided by Molitaris and Schaumann (1986), and Schauman *et al.* (1986). Nair *et al.* (1977) studied the distribution and activity of L-asparaginase producing fungi in the marine environment of Port Novo, east coast of India.

Recently Grant and Rhodes (1992) studied the production of cell-bound and extra-cellular laminarinase by *Dendryphiella salina* and five other marine fungi. They reported that the ratio of cell bound to extra-cellular enzyme activity was little altered by changes in growth condition, and suggested the role of marine fungi, in the break down of sea weed, due to its ability to degrade laminarine.

**Distribution of chitinase system**

Chitinases are known to be distributed widely in bacteria, fungi, plants, invertebrates and vertebrates, (Flach *et al.*, 1992). However a review of literature on chitinase from plants, invertebrates and vertebrates is out of scope of the present study. Hence attention is paid only on micro-organisms while reviewing the literature on chitinase.

Chitinase producing bacteria and fungi reported in the literature is presented in Table 2 and Table 3 respectively.

Table 2 Chitinase producing bacteria.

| Organisms | Reference |
|---|---|
| *Bacillus* sp. | Seino *et al.*, 1991 |
| *B. licheniformis* | Takayanagi *et al.*, 1991<br>Shimabara *et al.*, 1991<br>Trachuk *et al.*, 1996 |
| *B. thuringiensis* | Morozov *et al.*, 1994 |
| *Alteromonas* sp. | Tsujibo *et al.*, 1992 |
| *Aeromonas* sp. | Ueda and Arai, 1992 |
| *A. hydrophila* | Yabuki *et al* 1986.,<br>Mitsutomi *et al.*, 1990 |
| *A.caviae* | Inber and Chet, 1991 |
| *Vibrio* sp. | Takabashi *et al.*, 1993 |
| *V. alginolyticus* | Murao *et al.*, 1992 |
| *V. harveyi* | Michael and David, 1994 |
| *V. vulnificus* | Wartman *et al.*, 1986 |
| *V. cholerae* | Fujshima *et al.*, 1995 |
| *Klebsiella* sp. | Jeuniaux, 1959 |
| *Pseudomonas* sp. | Gassher and Bromel, 1984 |
| *Clostridium* sp. | Clarke and Tracey, 1956 |
| *Beneckea* sp. | Takahashi *et al.*, 1982 |
| *Arthrobactor* sp. | Latzko *et al.*, 1992 |
| *Serratia marcescens* | Monreal and Reese, 1969;<br>Reid, and Ogrydziak, 1981;<br>Cabib, 1988 |
| *Streptomyces* sp. | Skujius *et al.*, 1970 |

| | |
|---|---|
| *S. griceus* | Reynold, 1954; Berger and Reynold, 1958; Smucker and Kim, 1984 |
| *S. violaceus* | Wigert, 1962 |
| *S. orientalis* | Tominaga and Tsujisaka, 1976 |
| *S. kurssanovii* | Ilyna *et al.*, 1993; Stayachenko and Varlamov, 1993 |
| *S. viridificans* | Gupta *et al.*, 1995 |
| *S. antibioticus* | Jeuniaux, 1958, 1959, 1966, |
| *S. thermoviolaceus* | Horoshi *et al.*, 1993 |
| *S. olivoceviridis* | Romaguera *et al.*, 1992 |
| *S. lydicus* | Lee, 1993 |

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**Production and properties of microbial chitinase**

**Culture condition and production of fungal chitinases**

The first step in the study of microbial chitinases is the identification of conditions which promote production of enzyme. Such culture condition were reviewed by Monreal and Reese (1969). The factors which are known to affect the production of chitinases in microbial cultures include (1) sources of chitin, (2) type of chitin, (3) particle size of chitin (4) initial concentration of chitin, (5) incubation period, (6) temperature, (7) pH and (8) certain organic and inorganic chemicals.

**Table 4** chitinase producing fungi

| Organisms | References |
|---|---|
| *Aspergillus* sp. | Monreal and Reese, 1969; Polachek and Rosenberger, 1978 |
| *A. niger* | Ohtakara, 1963; Thomsan *et al.*, 1979 |
| *A. nidulans* | Reyes *et al.*, 1988 |
| *A. carneus* | Sherief *et al.*, 1991 |
| *Chyrtrimyces hyalinus* | Reisert, 1972 |
| *Verticillium albo-atrum* | Vessey and Pegg, 1973; Pegg, 1988 |
| *Sclerotina sclerotiorum* | Rai and Dhawann, 1978 |
| *Lycoperdon* sp. | Tracey, 1955 |
| *Fussarium* sp. | Nuero, 1995 |
| *Beauveria bassiana* | Leopold and Samsinkova, 1970; Smith and Grula 1983 Hung and Danmei, 1986; Hung *et al*; 1988, Hung, 1991 |
| *Pencillium* sp. | Monreal and Reese, 1969; Monaghan *et al.*, 1972; Rodriguez *et al.*, 1995 |
| *Neurospora* sp. | Elango *et al.*, 1982; Zarain-Herberg and Arrayo-Begarich, 1983 |
| *N. crassa* | Angle, 1988 |

| | |
|---|---|
| *Mucor mucedo* | Monreal and Rees, 1969; Bemmann *et al.*, 1982; Humphreys and Gooday, 1984; Suimi *et al.*, 1988 |
| *M. rauxii* | Rast *et al.*, 1991; |
| *Candidobolus* sp. | Monreal and Rees, 1969; Ishikawa *et al.*, 1981 |
| *Agaricus* sp. | Fermor and Grant, 1985 |
| *Paecilomyces varioti* | Gautam *et al.*, 1996 |
| *Aphanocladium ulbum* | Kunz *et al.*,; 1992; Srivastava, 1986 |
| *Aeromonium abclavatum* | Gunaratna and Balasubramanian, 1994 |
| *Rhizopus oligoporus* | Yanai *et al.*, 1992 |
| *Stachybotry elegans* | Russel *et al.*,1994 |
| *Pycnoporus cinnabarinus* | Ohtakara, 1988 |
| *Myrothecium verrucariae* | Vyas and Deshpande, 1989 |
| *Metahizinus anisophiac* | St. Leger *et al.*, 1991 |
| *Piromyces comunis* | Sakurado *et al.*,1995 |
| *Talaromyces emersonii* | Hendy *et al.*, 1990; Mc Cormarck *et al.*, 1991 |
| *Trichoderma* sp. | Monreal and Reese, 1969; Tiunova, 1983; Hou and Tong, 1985 |
| *T. harzianum* | Ridout *et al.*, 1988 Ulhoa and Peberdy, 1993; Harman *et al.*, 1994 |
| *T. viride* | Tiunova *et al.*, 1983 |

The initial pH of maximal enzyme production varied with the species of micro-organisms. Generally fungi showed optimum chitinase production at acidic pH at pH 4.5 (Monreal and Reese, 1969). Maximal levels of chitinase production was observed at pH 5.0 with *Talaromyces emersoni* (Mcarmack *et al.*, 1991), *Aspergills niger* (Sherif *et al.*, 1991). *Strachytrotrys elegans* a mycoparasite of *Rhizoctonia solani* (Russel *et al.*, 1994). Maximum chitinase yield was obtained after 4-5 days for *Beauveria bassiana* (Leopold and Saminkova, 1970). Thermophilic fungus *Talaromyces emersonii* CBS 814.70 produced maximum levels of chitinase activity after 14 days of growth (Hendy *et al.*, 1990). *Fussarium solani* recorded peak of extra-cellular activity of chitinase on the 9th day, while the mycelial activity was recorded on the 6th day after growth in potato dextrose broth (Mathivanan and Balasubramanian, 1990.)

Presence of chitinase without inducers in the enzymatic preparations from the culture fluid of 25 day old autolyzed culture 17 *Fusarium* sp. was reported. *Aphanocladium album* produced ~ 4 nkat /ml of chitinase after 14-21 days of growth in a glucose medium containing 1% chitin (Srivastava *et al.*, 1986). *Strachybotrys elegans* Mycoparasite of *Rizoctonia solani* under liquid culture produced maximal levels of chitinase after 3 days of growth in a medium containing chitin (Russel *et al.*, 1994). *Talaromyces emersonii* showed maximum chitinase and N-acetyl beta-glucosaminidase activities after 2

days of growth (McCarmack *et al.*, 1991). *Myrothecium vurrucaria* produced maximal chitinase after 7 days of fermentation in a medium containing chitin and 1% oxygoll (Vyas and Deshpande, 1989).

Reyes *et al.* (1977) studied autolysis of *Neurospora crassa* under different culture conditions and the release of chitinase. Chitinase activity was never found in fermentor cultures where as in shake culture this enzyme was found after 22 days of autolysis, while in stationary culture it was excreted after 8 days of autolysis.

Cell wall degrading enzymes $\beta$ 1—>3-glucanase and chitinase production by *Trichoderma* sp. under solid-state fermentation using wheat bran as a solid substrate was observed at 25$^{o}$C after 6 days (Suimi *et al.*, 1988).

Thermophilic fungus *Talaromyces emersonii* CBS 814.70 produced thermostable extracellular chitinolytic system in a medium containing chitin (Hendy *et al.*, 1990). A hyperparasitic fungus of wheat *Aphanocladium album* was reported to produce maximal chitinase of ~ 4 nkal/ml in a glucose medium containing 1% chitin. Chitinase and chitobiase were produced by *Trichoderma harzianum* when grown in a chitin containing medium. However, enzyme production was repressed with easily metabolized carbon sources, such as glucose or

N-acetyl-glucosamine (Ulhoa *et al.*, 1993). *Piromyces communis*, an anaerobic rumen fungi isolated from a fistulated goat, grew well and produced chitinase in a basal medium with and without colloidal chitin, and chitin powder and showed no N-acetyl-$\beta$-glucosaminidase activity (Sakurado *et al.*, 1995).

The activity of chitinase was found essentially in the supernatant from culture solution and very little in the periplasm of *Beauveria bassiana* Eu-120 and during the growth of the mycelium it is expected into the culture fluid. (Hung, 1991).

*Tularomyces emersonii* CBS 814.70 produced chitinase, under submerged fermentation, in a medium containing 1-2% chitin (w/w) at 45$^{o}$C at 200 rpm and with an aeration rate of 8 litre/minute (McCarmack *et al.*, 1991). *Aspergillus carneus* cultivated on a medium containing colloidal chitin, under submerged condition, recorded a maximum yield of 47.3 U/ml of chitinase and 0.38 U/ml of chitosanase at 10 g/litre chitin (Sherief *et al.*, 1991). *Myrothecium rucari* produced highlevels of chitinase in a medium containing chitin as sole carbon source, while addition of 0.03 urea increased the enzyme yield by 4 fold in days compared to control However, addition of oxygoll (0.1%) to the cultivation medium yielded maximal activity (Vyas and Deshpande, 1989)

*Strachybotrys elegans*, a mycoparasite of *Rhizoctonia solani* produced chitinase and $\beta$ 1—>3 glucanase under liquid culture on chitin or laminarin as the sole carbon source (Russel *et al.*, 1994). The production of both chitinase and glucanase was significantly higher in a medium containing sodium nitrate when compared to that with ammonium nitrate, ammonium tartarate and asparagine. They found out that the production of chitinase and glucanase by *S. elegans* was significantly influenced by the carbon sources incorporated in to the medium (Russel *et al.*, 1994).

*Beauveria bassiana* showed maximal chitinase activity when grown in a medium containing ammonium sulphate, peptone or lactic acid and demineralized crab sells as inducer (Hung and Danmei, 1986). It was also reported that *Beauveria bassiana* produced chitinase without the addition of chitin to the medium, indicating the constitutive nature of the enzyme (Leopold and Saminkova, 1970).

**Properties of microbial chitinase**

Properties of chitinase, produced by various microorgaisms, reported in the literature is presented in Table 4. From the Table it is seen that the optimum temperature varied between 25$^{o}$C - 90$^{o}$C depending upon the source of enzyme. However majority of the microorganisms produce chitinase that show optimal temperature above 40$^{o}$C. Similarly the optimal pH of the enxyme varied from 4.0 to 9.0.

Table 4 Properties of microbial chitinases

| Organisam | optimum $T^{o}c$ | optimum $P^{H}$ | PI | M.wt KDa | Reference (s) |
|---|---|---|---|---|---|
| *Bacillus thuringensis* | 60 | 8.0 | — | —— | Chigaleichik, 1976 |
| *B. licheniformis* | 50 | 8.0 | 3.9 | 70 | Tsujibo *et al.*,1992a |
| *B. licheniformis* | 70-80 | - | -- | Ch1-189<br>Ch2-76<br>Ch2-66<br>Ch3-59 | Takyanagi *et al* 1991 |
| *B. licheniformis* | 70<br>90<br>70.<br>70<br>70 | 4.5-5.5 and 9-9.5 | -- | 66<br>62<br>53<br>49<br>42 | Trachuk *et al.*, 1996 |
| *Pseudomonas* aeruginosa | 60 | 6.0 | —— | — | Nagahata and Shimahara, 1979 |
| *vibrio* sp. | - | 6.8 and 9.0 | 3.7 | 63 | Mowlah *et al.*, 1979 |
| *Vibro* sp. | — | 6.8 and 10.5 | 3.7 | 63 | Ohtakara *et al.*, 1978 |
| *Vibrio* sp. | 40 | 5.0 | — | 10 | Takahashi, *et al.*, 1993 |
| *V. angullarium* | 60 | 6.0 | — | — | |
| *V. alginolyticus* | 45 | 4 and 9 | 4.3 | 66 on SDS PAGE | Murao *et al* 1992 |
| *V. alginolyticus* | 56 | 5.5 | — | — | Aribisala and Goody, 1978 |
| *Vibro gerris* | 40 | 7.0 | — | — | |

| | | | | | |
|---|---|---|---|---|---|
| *Alteromonas* | 50 | 8.0 | 3.9 | 70 | Tsujibo *et al.*, 1992 |
| *Aeromonas* sp. | | | | | |
| (Chi-I) | 50 | 4 | 7 | 112 SDS | Ueda and Arai, 1992 |
| (Chi II) | 60 | 4. | 8.1 | 115<br>114 GF | |
| *A. hydrophila* | — | —— | 4.6 | 110 | Yabuki, 1986 |
| *Serratia marcescens* | 50 | 6.4 | — | — | Monreal and Rees, 1969 |
| *Beneckea neptuna* | 40 | 7.0 | — | — | Hood and Meyer, 1977a |
| *Streptomyces kursovii* | — | —— | — | chi1 42<br>chi2 40<br>chi3 26<br>chi4 20 | Stoyachenko and Varlamove, 1993 |
| *S. olivaceo-viridis* | — | — | — | chi1 20.5<br>chi2 30<br>chi3 47<br>chi4 92 | Romaguera *et al.*, 1992 |
| *S. thermo-violacus* | 70-80 | 8-10 | — | —— | Hiroshi *et al*, 1993 |
| *S. cineroruber* | 50 | 4.5 | 8.6 | 19 | Okazaki and Togawa, 1991 |
| *S. antibioticus* | — | 6.2 | — | 29 | Skujins *et al*, 1990 |
| *S. orientalis* | | | | | |
| (chi i) | — | 5.5-6.5 | 8.8 | 33 | Tominaga and |
| (chi 2) | | | 8.65 | 25 | Tsujsako, 1976 |
| *Chitrimyces hyalina* | 25 | 5.5 | — | — | Reisert, 1972 |
| *Mucor mucedo* | — | 5.65<br>5.5 | — | — | Humphreys *et al.*, 1984 |

| | | | | | |
|---|---|---|---|---|---|
| *Aeremonium abclavatiom* | 50 | 3-4 | — | 45 on SDS PAGE | Gunaratha and Balasubramaninan, 1994 |
| *Pencillium oxalicum* | 35 | 5.0 | 4.5 | 54.9 SDS PAGE 21.5 on GF | Rodrigues *et al.*, 1995 |
| *Pycanoporus cinabarinus* | — | 4.5 | 3.6 | 38 on GF | Ohtakara, 1988 |
| *Neurospora crassa* | — | 6.7 | — | 20.6 on GF | Angel, 1988 |
| *Verticillium albo-atrum* | 51 | 3.7 | — | (1) 64 (2) 58 | Pegg, 1988 |
| *Metarhizium* | — | 5.3 | 6.4 | 33 | St. Leger *et al.*, 1991 |
| *Piromyces comunis* | 40 | 5.5 | —— | —— | Sakurado *et al.*, 1995 |
| *Talaromyces emersoni* | 75 | 5.0 4.8 | 3.6 | — | Hendy *et al.*,1990 |
| *Trichoderma harzianum* | 50 | 5.5 | — | 118 on GF 64 on SDS PAGE | Ulhoa and Preberdy, 1991 |
| *T. harzianaum* | — | — | 3.9 | 40 | Harman *et al.*, 1994 |

GF Gel filtration

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

## Application of chitinolytic enzymes

### Chitinase in fungal protoplast technology

Isolation of fungal protoplast has gained considerable importance over the decade, in view of its various biotechnological applications. Since chitin is the structural component in the cell wall of most fungi, chitinolytic enzymes play significant role in protoplast isolation (Peberdy, 1985; Koga *et al.*, 1988; Vyas and Deshpande, 1989). Hamlyn *et al.* (1981) evaluated various commercial mycolytic preparations for protoplast isolation and found that high chitinase levels permit effective mycelia degradation. Yanagi and Takebe (1984) who used various enzyme preparations, single or in combination to isolate protoplast from *Coprinus macrorhizus* and other bacidiomycetes, reported the notable contribution of chitinase activity to efficient protoplast release. Recently Gautam *et al.* (1996) reported the isolation of protoplast of a thermophilic fungus *Malbranchea sufara* using two thermostable enzymes produced by the thermophilic fungus *Paecilomyces varioti*. They found out that the frequency of protoplast regeneration observed (35%) was considerably higher than that obtained using commercial lytic enzymes.

### Chitinase in single cell protein production

The production of microbial chitinases has received attention as one step in a bioconversion process to treat

shellfish waste chitin (Revah-Moiseev and Carroad, 1981). The enzymatic conversion of waste chitin, produced by the shell fish processing industry, to yeast single cell protein (SCP), has investigated (Revah-Moisev and Carroad, 1981; Vyas and Despande, 1991). The SCP can be used as an animal and aquacluture feed supplement. *Serraciea marcescens* chitinase system was used to hydrolyse the chitin and *Piechia kudriavezevii* to yield the SCP (with 45% protein and 8 to 11% nucleic acid) Revah-Moiseev and Carroad (1981). *Myrothecium verrucaria* chitinase complex and *Saccharomyces cerevisiae* was used for SCP production from the chitin waste (Vyas and Deshpande, 1991). The total protein and nucleic acid content of their SCP were 61 % and 3.1% respectively.

**Chitinase as bio-control agent**

Chitin is an essential structural component of the fungal and insect pathogens of vascular plants. As it is absent from the vascular plants themselves, chitin could be used as a target molecule for fungicidal and insecticidal agent. In this regard, the role of chitinase has been studied extensively (Tanaka *et al.*, 1970; Barrow-Broaddus and Kerr, 1981; Sapiro *et al.*, 1989).

In plants microbial infections, other injuries or treatment with ethylene or salicylic acid, induce chitinase (Mauch and Stachelin, 1989; Koga *et al.*, 1992). The plant and

bacterial chitinases differ markedly in anti-fungal activity. This difference may be attributed to the difference in their substrate specificities / or modes of action (Schlumbaum *et al.*, 1986; Roberts and Seliternnikoff, 1988).

Several microbial chitinases have been used as anti-fungal agents with β-glucanase, propan-2-ol and polyethelene lauryl ether, has been sprayed on a rice field to control rice blight caused by Pyricularia *oryzae* (Tanaka *et al.*, 1970). A chitinase producing *Arthrobactor* sp. inhibited the growth of *Fussarium moniliforme* var. *subglutinans*, the causative agent of pine pitchy canker (Barrows-Broddus and Kerr, 1981). Ordentlich *et al.* (1988) and Shapiro *et al.* (1989) reported that *S. marcescens* is an effective biocontrol agent of *Sclerotium rolfsii* infecting beans and of *Rhizoctonia solani* infecting cotton, under green house conditions.

Smirnoff (1975) reported the contribution of chitinase in the control of spurce budwarm infection of balsam fir trees.

*Trichoderma harzianum* is a potential biocontrol agent against a wide variety of plant pathogens encountered in commercial agriculture. The mycoparasitism of pathogenic fungi involve volatile/ non-volatile antibiotic and hydrolytic

enzymes such as glucanases, proteinases and chitinases (Ridout et al., 1988). Entamopathogenic fungi (eg. *Beauveria bassiana, Metahizium anisopliae* and *Verticillium lecanii* are parasities of various pests, including potato beetle, sugar cane frag hopper and aphids. Entry of the parasite through the insect cuticle is a combination of mechanical pressure and enzymatic digestion. chitinase and protease play a role in the cuticle degradation (St. Leger *et al.*, 1986). Fungal formulation containing mycelial fragments and conidia are perceived as safe alternative of chemical pesticides (Thakur et al., 1991)

**Preparation of chito-oligossacharides**

There is a growing appreciation for biologically active chito-oligosaccharides. For example chitohexose and chitoheptose show anti-tumour activity and are efficient elicitors of chitinase activity in melon plants. (Shaikh and Deshpande, 1993). Due to hydrolytic N-deacetylation, conventional partial acid hydrolysis of chitin gives only lower yield of the desired pentamers, hexamers and heptamers. Chitinase from *Bacillus* sp. has been used for chitosan oligosaccharide production which and gave high yield, (> 60%) of oligomers (diamers to pentamers) (Izume and Ohtakara, 1987). A novel chitinase from *vibrio alginolyticus* yield chitotriose and chitopentose from colloidal chitin Muraro *et al.* (1992).

Nanjo *et al.* (1989) described the transglycosylation reaction of *Nocardia orientalis* chitinase when the tetramers or pentamers were incubated with the enzyme, accumulation of the hexamer or heptamer, respectively was observed. A chitinase from *Trichoderma reesi* also showed efficient transglycoslylation reaction with the tetramer, yielding the hexamer and dimer as the major products (Usci et al., 1990). They also observed chain elongation from dimer to hexamer and heptamer, using lysozyme catalysis in the presence of 30% $(NH_4)_2SO_4$ in a buffer medium. Takayanagi *et al.* (1991) purified three chitinase from *Bacillus licheniformis*, catalyzed a trasglycosylation reaction that convert $(GlcNAc)_4$ into $(GlcNAc)_6$.

**Cytochemical localization of chitin/chitosan using chitinase, chitosanase-gold complex.**

Chitin and chitosan are the most ubiquitous polymers of fungal cell wall and although biochemical analysis can provide precise information about their structure, cytochemical localization studies can reveal the functional specialization of these polymers.

The use of lectins, with specific binding affinities for monosaccharides, in conjunction with colloidal gold has permitted the localization of sugar residues in thin section of plants and fungi (Benhamou, 1988; Chamberland, 1985) studied the localization of chitin ultrastructurally in tomato

roots cells infected by *Fuarium oxysporium* using chitinase-gold complex. Wheat germ agglutin-gold complex and chitinase-gold complex have been used as probes for the detection of GlcNAc residues in the secondary cell wall of plants and in pathogenic fungi (Benhamou, 1988, 1989; Benhamou and Asselin, 1989). Grenier *et al.* (1991) reported the tagging of barley chitosanase with colloidal gold particles for the localization of chitosan in spore and hyphal cell wall of fungi. Using this technique they demonstrated the presence of chitosan in the cell wall of *Ophiostomo ulmi* and *Aspergillus niger*.

**Shrimp waste**

Shrimp waste constitutes mainly shrimp heads and tail hulls, besides broken or imperfectly peeled or discoloured shrimps. Depending on the species and method of processing, waste might exceed 60% of the landed weight (Nirmala, 1991)

Solid shrimp waste contains mainly valuable components including shrimp meat, protein and chitin. Shrimp waste have been mainly used for the preparation of shrimp meal, a useful component in live stock and aquaculture feed (Nirmala, 1991)

Pollution due to shrimp waste has become an increasing problem and hence, reducing processing waste or finding alternative uses for these waste is gaining popularity

as a means to improve profitability in the seafood industry (Nirmala, 1991)

**Chitinase production using shrimp waste**

Paul and Raymond (1978) produced an extracellular chitinase enzyme system by a submerged culture of *Serratia marcescens* on chitin waste. Cabello *et al.* (1988) cultivated *Streptomytes* sp.in media containing powdered decalified shrimp shell to produce chitinase and chitosanase. They found out that the yield and activities of the enzyme were equal to that obtained with commercial chitin in the medium.

Sabry (1992) reported the chitinase production by *Alcaligenes denitificons, Bacillus amyloliquefaciens, B. megasterium* and *B. subtilis* using shrimp shell waste under submerged fermentation. He studied chitinase production from 40 strains of bacteria isolated from the seafood packing industry using shrimp shell waste under submerged fermentation. Cosia *et al.* (1982) reported production of chitinase by *Serratia marcescens* QMD 1466 with shrimp-shell chitin waste as substrate, and subsequent hydrolysis of the waste for SCP production by the yeast *Pichia kudriavzvii.*

**Significance of the present study**

Potential source of microorganisms hyperproducing chitinase is the need of the hour for economic utilization of

the abundant chitinous wastes generated from the shellfish processing industry towards bioprocessing of chitin for production of SCP. Chitinase is also used as a biocide. Marine microbes could contribute to industrial production of chitinase, if potential strains are screened and suitable bioprocess is developed. However, not much information is available in this subject, but for the reports on the distribution of chitinolytic bacteria in the marine environmens of India.

Hence in the present study, an attempt was made to screen potential microorganisms hyperproducing chitinases from marine sources and to develope an ideal bioprocess for the production of chitinases.

The specific objectives include,

1) to isolate chitinolytic fungi from marine environments.
2) to select potential strains that produce chitinase, with the ability to hydrolyze native chitin, under both SmF and SSF conditions.
3) to standardize the growth conditions of the selected strain.
4) to study the chitinase synthesis under SmF
5) to study the chitinase synthesis under SSF and
6) to purify and characterize the isolated enzyme.

# CHAPTER - TWO

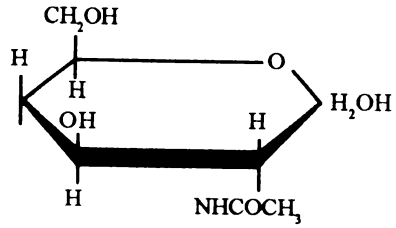
# MATERIALS AND METHODS

## 2.1 Substrate

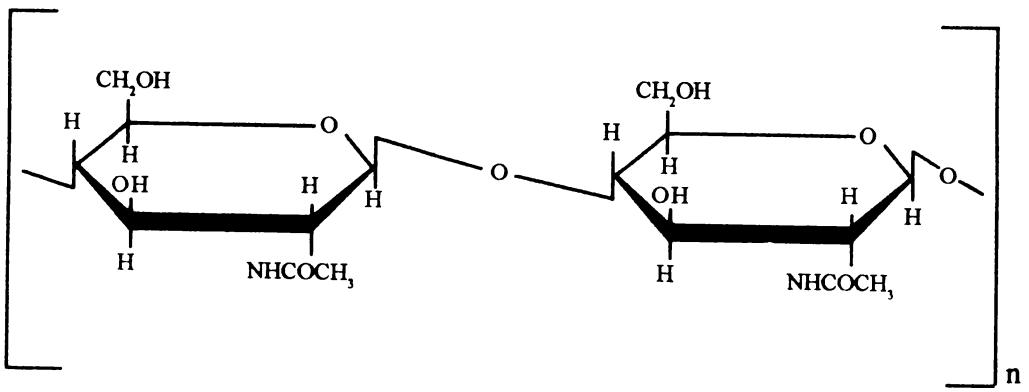
Colloidal chitin was used as the substrate for the enzyme production by chitinolytic fungi (Hsu and Loockwood, 1975)

Chitin is a nitrogenous polysaccharide a polymer of unbranched chain of β (1—>4) linked 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine, GlcNAc) (Figure 1) The emperical formula for chitin is $(C_8H_{13}O_5NHCOCH_3)_n$,(`n' is the repeating units of GlcNAc)

**Figure 1** The structure of chitin




N-Acetyl-D-glucosamine (GlcNAc)




The chitin (chitobiose) repeating unit

Purified chitin, obtained from prawn shell, used in the present study was obtained as a gift from Central Institute of Fisheries Technology, Kochi, India.

## 2.2 Preparation of colloidal chitin

The colloidal chitin was prepared according to Willoughby (1968) with some modifications as described below.

Five gram of the purified chitin was taken in a beaker and added with 140 ml of 50% concentrated $H_2SO_4$, with continuous stirring in an ice bath for 60 minutes, until the suspension became very clear. The insoluble fraction was filtered off by passing through glass wool, and the chitin solution was poured into 2 litre distilled water in order to precipitate the chitin. The suspension was kept overnight at $4^{o}C$ to settle the colloidal chitin. The supernatant was decanted out and the remaining mixture was centrifuged at 5000 rpm at room temperature. The sedimented residue was resuspended in distilled water. The suspension was allowed to settle again and decanted repeatedly with distilled water until the pH of the liquid became neutral. The chitin was collected by centrifugation as mentioned above and stored as a paste at $4^{o}C$, after heating for 15 minutes in boiling water bath.

## 2.3 Isolation of chitinoclastic fungi from marine environments

Chitinoclastic fungi from marine environments were screened following the procedure described by Jones (1971).

### 2.3.1 Samples

Both water and sediment samples of marine and estuarine environments of Kochi (between $09^{o}$ 58 N-$10^{o}$ 10 N and $76^{o}$ 15 E-$76^{o}$ 25 E) were collected and screened for the chitinoclastic fungi.

### 2.3.2 Collection of samples

Water samples were always collected from a depth of 0.5 metre below the surface level to avoid aero-aquatic interface microbial population using sterile glass containers. Sediment samples were collected as described by Borut and Jonson (1962) with an Ekman dredge. Inner walls of the grab were surface sterilized with alcohol. The sediment in the grab was sliced open with a sterile spatula and the middle portion of the sediment was immediately transferred to sterile bottles. Both water and sediment samples, were transported to the laboratory in an ice box and subjected to microbiological analysis immediately.

### 2.3.3 Media

Colloidal chitin agar medium originally proposed by Lingappa and Lockwood (1962) was prepared according to the procedure described by Booth (1971) and used for the isolation of chitinoclastic marine fungi.

The composition of chitin agar medium

| | |
|---|---|
| Colloidal chitin | 1 g |
| Agar | 2 g |
| 50% aged sea water | 100 ml |
| pH | 7.5 |

The medium was autoclaved, cooled, added with a mixture of filter sterilized antibiotics (0.5 mg Penicillin G. and 0.5 mg streptomycin sulphate per plates) and used.

### 2.3.4 Plating procedure

Conventional pour plate technique was used for the plating of samples. Water samples were directly (without dilution) used for inoculating the isolation medium. Whereas for the sediment samples, a suspension of 1:100 dilution was prepared using sterile 50% aged sea water and one ml of the suspension was inoculated into the isolation medium.

All the inoculated plates were incubated at 28$^{o}$C in an incubator, under dark condition for 3 weeks.

### 2.3.4 Isolation and purification

Colonies which showed clear lytic zones around them were treated as chitinolytic. Such colonies were picked and subcultured to Bennet's agar (HiMedia, India) slants prepared with 50% aged sea water. The isolates were purified repeatedly using conventional streakplate method on Bennet's agar plates prepared with 50% aged sea water.

### 2.3.6 Maintenance of purified culture

All the purified isolates were maintained on Bennet's agar slants prepared with 50% aged sea water and stored at $4^{o}C$ in the laboratory.They were subcultured once in three month into fresh medium. The selected strains, used in the later studies, were stored as stock culture under mineral oil at room temperature (Agnes, 1971).

### 2.4 Classification and identification

All the isolates were classified based on the scheme proposed by Ainsworth (1971) and adopted in The Fungi volume IV A and IV B (Ainsworth *et al.*, 1973), and all the isolates were identified upto generic levels based on the key suggested by Kendrick and Charmichel (1973). The selected strain, which was used in the later studies, was identified upto the species level.

## 2.5 Screening of potential strain for chitinase production

All the strains, isolated from marine environments, were subjected to intensive screening towards selection of potential strains that can grow well utilizing chitin as substrate and produce chitinase under fermentation conditions (Molitoris and Schaumann, 1986).

The strategy adopted for selection included two stages.

Stage I :- Ability to grow and demonstate chitinolytic activity on colloidal chitin agar plate (qualitative approach).

Stage II :- Ability to produce chitinase under solid-substrate fermentation conditions (quantitative approach).

### 2.5.1 Stage I

The solid medium formulated by Hankin and Anagnostakis (1975) was used for testing the ability to grow and produce chitinolytic activity on agar medium.

Composition of Hankin and Anagnostakis solid colloidal chitin medium

| | |
|---|---|
| Mineral salt solution* | 500 ml |
| Yeast extract | 0.2 g |
| colloidal chitin | 24 g |
| Agar | 15 g |
| 50% aged sea water | 500 ml |
| pH | 6.0 |

*Composition of mineral salt solution

| | |
|---|---|
| Ammonium sulphate | 2 g |
| Pottassium hydrogen phosphate | 6 g |
| Disodium hydrogen phosphate | 6 g |
| Ferric sulphate | 0.2 g |
| Calcium chloride | 0.001 g |
| Boric acid | 10 μg |
| Manganese sulphate | 10 μg |
| Zinc sulphate | 70 μg |
| Copper sulphate | 50 μg |
| Molebdenum oxide | 10 μg |

Growth on agar medium was determined quantitatively by the measurement of increase in colony size (expressed in terms of diameter of colony), where as enzyme production was determined quantitatively by measuring the size of the clear zones of digested chitin around the colony (measured in terms of radius of the clear zone and expressed in milimeter).

### 2.5.2 Stage II

Those strains which showed significant chitinase production in the chitin agar medium during the first stage were further screened by testing their ability to produce chitinase under solid substrate fermentation (SSF) by adopting the method suggested by Ramesh (1989) with some modification, when required, as detailed below.

### 2.5.2.1 Preparation of solid substrate

The commercial wheat bran (WB), as 5 g aliquot, containing particles of size > 425 $\mu$, taken in petriplates (10 x 1.5 cm) was mixed with colloidal chitin at 1% levels (w/w), moistened with 5 ml of aged sea water (pH 8), autoclaved at 121$^{o}$C for 60 minutes and allowed to cool down to room temperature (28 $\pm$ 2$^{o}$C ).

### 2.5.2.2 Inoculum preparation

Fully sporuluted (two weeks old) slant culture, in test tubes (50 ml capacity), newly raised on Bennet's agar prepared with 50% aged sea water, was added with a 20 ml of sterile physiological saline (0.85% NaCl) containing 0.1% Tween 80 by means of a sterile pipette. The spores were scraped using an inoculation needle under strict aseptic conditions and the spore suspension obtained was adjusted to a concentration of 16x10$^{6}$ spores/ml using sterile physiological saline. The prepared spore suspension was used as inoculum.

### 2.5.2.3 Inoculation and incubation

The wheat bran medium (WB medium) prepared in petridishes was inoculated with 2 ml of spore suspension per plate (inoculum level, randomly selected). The contents of the medium were mixed thoroughly, with a sterile glass rod in order to spread the spores uniformly all over the wheat bran,

and then incubated in an incubator with 65-70% relative humidity at 28°C for desired incubation period.

### 2.5.2.4 Extraction and recovery of enzyme

Chitinase from the fermented wheat bran (mouldy bran) was recovered by employing the simple contact method of extraction using distilled water as extractant. The fermented wheat bran, in each plate, was transferred to a 250 ml conical flask, added with 25 ml (5 volume based on the initial weight of wheat bran) of distilled water, kept on a rotary shaker, and agitated at 150 rpm for 30 minutes at room temperature (28 ± 2°C). The slurry was then squeezed through a cheese cloth. The process was repeated twice, extracts pooled and centrifuged at 4°C in a refrigerated high speed centrifuge for 20 minutes at 10,000 rpm. The clear supernatant was collected and used as crude enzyme preparation for assay of chitinase.

### 2.5.2.5 Enzyme assay

The chitinase was assayed using colloidal chitin as substrate (Ohtakara, 1988), by estimating the reducing sugars produced during the hydrolysis of substrate with N-acetyl-D-glucosamine as a reference compound according to the method of Jones and Grainger (1983) a modification of Miller's (1959) dinitrosalycylic acid (DNS) method.

The reaction mixture, which contained 0.5 ml of 0.5% colloidal chitin, 0.5 ml of McIlvanine's citrate buffer (0.1 M) pH 6.0 and 1 ml of appropriately diluted enzyme solution was incubated at 37$^{o}$C for 2 h with occasional shaking. The reaction was arrested by keeping the tube in boiling water bath for 5 minutes. The undigested materials were removed by centrifugation at 5000 rpm for 5 minutes. 1 ml of the supernatant taken in a test-tube was added with 0.5 ml of DNS reagent, and the tubes were heated for 10 minutes in a boiling water bath. After cooling, the reaction mixture was added with 4 ml of distilled water, and the absorbance was measured at 500 nm against appropriate substrate and enzyme blank.

One unit of chitinase activity was defined as the amount of enzyme that catalyzed the release of 100 mg of reducing sugars as N-acetyl-D-glucosamine/ ml reaction mixture under the assay conditions (Pegg, 1985). The enzyme yield per gram initial weight of solid substrate was calculated and expressed as units per gram initial dry substrate (U/gIDS).

### 2.5.3 Selection of potential strain

All the strains subjected to stage II screening programme were ranked in terms of their enzyme yield and the top ranked strain was selected for further studies.

## 2.6 Growth studies

The selected strain was characterized for its growth conditions. Optimal conditions required for maximal growth were determined by subjecting the organism to various incubation temperatures, different levels of pH, NaCl concentration, colloidal chitin concentration and incubation period. The effect of various carbon and nitrogen sources and salinity on growth was also studied.

### 2.6.1 Media

Two media were used for cultivation of the fungus in liquid culture.

Medium I

A sea water complex medium, GPYS medium suggested by Molitoris and Schaumann (1986) was used as broth without agar.

Composition of GPYS broth

| | |
|---|---|
| Glucose | 1 g |
| Peptone | 0.5 g |
| Yeast extract | 0.1 g |
| Natural sea water | 1 Litre |
| pH | 7.6 |

Medium II

The mineral salt solution proposed by Hankin and Anagnostakis (1975) was used as a Mineral Salt Medium (MSM)

with little modification.

The composition of MSM

| | |
|---|---|
| $(NH_4)_2SO_4$ | 2 g |
| $KH_2PO_4$ | :6 g |
| $Na_2HPO_4$ | 6 g |
| $FeSO_4.7H_2O$ | 0.2 g |
| $CaCl_2$ | 0.001 g |
| $H_3BO_4$ | 10 µg |
| $MgSO_4.7H_2O$ | 10 µg |
| $ZnSO_4.\ 7H_2O$ | 70 µg |
| $CuSO_4.\ 5H_2O$ | 50 µg |
| $MoO_4$ | 10 µg |
| Distilled water | 1000 ml |
| pH | 7.6 |

### 2.6.2 Preparation of inoculum

Two hundred and fifty millilitre of GPYS broth, taken in a one litre Erlenmyer conical flask, was inoculated with 10 ml of spore suspension prepared in distilled water containing 0.1% Tween °80, from two week old agar slope culture, subcultured and raised on Bennet's agar slants (prepared with 50% aged sea water). The inoculated broth was incubated at room temperature (28 $\pm$ 2°C) on a rotary shaker at 150 rpm. After two days of incubation the mycelia was collected aseptically by centrifugation at 10,000 rpm for 10 minutes and washed repeatedly with sterile physiological

saline (0.85% NaCl). The sedimented mycelial pellets were broken down by vigorous agitation, with sterile glass beads (0.3 cm diameter) using a vortex mixture, and suspended in 100 ml of the same saline. The concentration of the prepared suspension was approximately 25.0 $\mu$g dry weight equivalent of mycelia per ml.

### 2.6.3 Inoculation and incubation

The prepared inoculum was added at 2% level to the cultivation medium (unless otherwise specified). The inoculated flasks were incubated on a rotary shaker at 150 rpm at 28 $\pm$ 1$^{o}$C (unless otherwise specified) for 60 h (arbitrarily selected) unless other wise mentioned.

### 2.6.4 Measurement of growth

After incubation for the specified period, growth of the organism was measured, as described below, either in terms of dry weight of the biomass or in terms of protein content of the biomass (unless otherwise specified).

#### 2.6.4.1 Estimation of dry weight of the biomass

After incubation for specified incubation period the mycelia was separated from the culture broth by centrifugation at 10,000 rpm for 10 minutes and washed repeatedly, with sterile distilled water to remove residual medium constituents and products of growth by consecutive centrifugation and

decantation. The washed mycelial biomass was taken in a pre-weighed petri-dish, dried to a constant weight in a hot air oven at 100-105°C for overnight, cooled in a desicator and the dry weight was calculated.

2.6.4.2. **Estimation of total protein content of biomass**

Growth was estimated in terms of increase in total protein content of biomass as suggested by Herbest *et al.* (1971) using Folin ciocalteu reagent (Lowery *et al.*, 1951) as detailed below.

After incubation for the desired period the mycelia were harvested by centrifugation from the culture broth, at 10,000 rpm for 10 minutes, washed repeatedly, by consecutive centrifugation, with sterile distilled water to remove the residual medium constituents and the products of growth. The washed mycelial biomass was homogenized with a tissue homogenizer and suspended in sterile distilled water.

From the mycelial suspension 2 ml was taken in a test-tube and added with 2 ml of 1 N NaOH. The tube was placed in a boiling water bath for 5 minutes. After cooling to room temperature, the undissolved residue was removed by centrifugation at 5000 rpm for 10 minutes. one ml of the supernatant was mixed with 2.5 ml of alkaline reagent, allowed to stand for 10 minutes and rapidly added with 0.5 ml of

Folin-Ciocalteru reagent. After standing for 30 minutes, the blue colour developed was measured by taking the absorbance at 750 nm in a UV-visible spectrophotometer (Shimadzu-160A, Japan) against the reagent blank. Bovin serum ablumin was used as standard for computation of protein content and expressed as g/litre.

### 2.6.5 Incubation temperature

Optimal growth temperature was determined by incubating the inoculated GPYS broth at various temperatures (22 - 47$^{o}$C) Growth was estimated as described under section 2.6.4.1.

### 2.6.6 Initial pH of the medium

Optimal pH for maximal growth was determined by subjecting the strains to various pH levels 2 - 12 adjusted in the GPYS broth using either 1N NaOH or 1N HCl. Growth was estimated as described under section 2.6.4.1.

### 2.6.7 Sodium chloride

Optimal sodium chloride concentration required for maximal growth of the strain was determined by incorporating sodium chloride concentration at various levels (0-16% w/v) in GPYS broth. Growth was estimated as described under section 2.6.4.1.

### 2.6.8 Colloidal chitin concentration

Optimal requirement of colloidal chitin concentration as substrate for maximal growth was studied by subjecting the strain to various levels of colloidal chitin (0-10% w/v) incorporated in the mineral salt medium. Growth was estimated as described under section 2.6.4.2.

### 2.6.9 Carbon source

Effect of carbon source, on the growth of fungus was studied by cultivating the fungus in the mineral medium supplemented with various carbon sources (at 1% levels) viz., organic (arabinose, ribose, xylose, glucose, mannose, galactose, sucrose, maltose lactose, glycerol) and inorganic (trisodiumcitrate). Growth was estimated as described under section 2.6.4.1.

### 2.6.10a Nitrogen source

Effect of various nitrogen sources on the growth of the fungus was studied by supplementing various nitrogen sources (at 1% levels) in the mineral salt medium. They included organic (yeast extract, beef extract, caseine, peptone, liver extract, and malt extract), inorganic ($NH_4Cl$, $NaNO_3$ and $(NH_4)_2SO_4$) and amino acids (glycine, asparagine, glutamine, lycine, arginine, proline, and tryptone). Growth was estimated as described under section 2.6.4.1.

### 2.6.10b Glucose

Effect of glucose on growth was studied by subjecting the strain to various levels (0 - 15 %)

incorporated in the mineral salt medium.

### 2.6.11 Effect of salinity on growth

The effect of salinity on growth of the fungus was studied in both liquid and solid media as detailed below.

#### 2.6.11.1 On liquid medium

The effect of salinity of sea water on growth in liquid medium was studied by subjecting the organism to GPYS broth prepared with various concentration of aged sea water, and distilled water plus 5% (w/v) NaCl and distilled water. Growth was estimated as described under section 2.6.4.1.

#### 2.6.11.2 On solid medium

The effect of salinity on growth of the fungus in solid medium was studied using Bennet's agar plates prepared at various concentrations of aged sea water, distilled plus 5% (w/v) NaCl and distilled water. The inoculated plates were incubated at 27$^{o}$C in a BOD incubator in inverted position. The growth of the organism was determined, by linear growth measurement, as increase in colony diameter at various intervals of incubation.

### 2.6.12 Incubation time

Optimal incubation period for maximal growth was determined by incubating the inoculated GDYS broth at the optimised growth conditions for a total period of 96 hours and determining the growth at regular intervals by means of dry

weight of the biomass as per the procedure described earlier under section 2.6.4.1. From the results obtained growth curve was constructed.

## 2.7 Chitinase production by marine fungus under submerged fermentation (SmF)

Chitinase production by marine fungus under submerged fermentation condition was carried out under shake flask culture and static culture conditions. The culture conditions required for maximal chitinase production was optimized.

### 2.7.1. Medium

Since no media was reported in literature for the chitinase production by marine fungus under SmF conditions, The mineral salt solution proposed by Hankin and Anagnostakis (1975) supplemented with colloidal chitin and yeast extract was used, with little modification.

Composition of mineral salt colloidal chitin (MSCC), medium.

| | | |
|---|---|---|
| $NH_4SO_4$ | | 2 g |
| $Na_2HPO_4$ | | 6 g |
| $KH_2PO_4$ | | 6 g |
| $MgSO_4$ | $7H_2O$ | 1 g |
| $CuSO_4$ | $5H_2O$ | 10 $\mu$g |
| $FeSO_4$ | $7H_2O$ | 1 mg |

| | |
|---|---|
| $ZnSO_4$ $7H_2O$ | 70 µg |
| $MnCl_2$ $4H_2O$ | 7 mg |
| Yeast extract | 6 g |
| Colloidal chitin | 10 g |
| Distilled water | 1000 ml |
| pH | 7.6 |

The prepared medium was dispensed as 25 ml aliquots in 250 ml Erlenmeyer conical flasks, autoclaved at 121°C for 15 minutes and used.

### 2.7.2 Preparation of inoculum

Young vegetative mycelial inoculum (48 h old) was prepared as described under section 2.6.2 and used.

### 2.7.3 Inoculation and incubation

The prepared medium was inoculated with the prepared inoculum (5% v/v) and incubated at room temperature (28 $\pm$ 1°C) on a rotary shaker at 150 rpm for 5 days (unless otherwise specified).

### 2.7.4 Harvesting of enzyme

After incubation the fermented broth was centrifuged, at 10,000 rpm for 20 minutes, at 4°C, in a refrigerated centrifuge (Kubota Model 6700, Japan) and the cell free supernatant was collected and used for chitinase assay and biochemical assays.

### 2.7.5 Enzyme assay and measurement of enzyme production

The enzyme activity was estimated using colloidal chitin as substrate as per the procedure described under section 2.5.2.5 The enzyme activity was expressed in terms of unit /mililitre (U/ml).

### 2.7.6 Biochemical assays

The cell free supernatant, obtained after centrifugation was assayed for soluble protein, reducing sugars and pH.

#### 2.7.6.1 Soluble protein

The total extracellular soluble protein in the cell free extract (crude enzyme preparation) was measured according to the method of Lowery *et al.* (1951) using Folin-Ciocaltaeu's reagent.

#### 2.7.6.2 Reducing sugars

The reducing sugars in the cell free extract was estimated using dinitrosalicylic acid (DNS) reagent according to the procedure described by Jones and Grainger (1983), a modified method of Miller (1959), using glucose as standard as described below.

One mililitre of the sample, mixed with 0.5 ml DNS reagent was taken in a test-tube and heated in a boiling water

bath for 10 minutes. After cooling to room temperature, the contents were diluted with 4 ml of distilled water and the absorbance was measured at 500 nm using a uv - visible spectrophotometer (Shimadzu-160A, Japan) against respective blank.

#### 2.7.6.3 Determination of pH

The pH of the fermented broth, at the end of the incubation period, was monitored using a digital pH meter (Systronic, India).

### 2.7.7 Effect additional carbon sources on chitinase production by *B. bassiana*

Effect of additional carbon sources, other than colloidal chitin, on enzyme production was tested with both organic (glucose, maltose, mannose, mannitol, dextrin, sorbitol, sucrose, soluble starch) and inorganic (trisodiumcitrate) carbon sources incorporated in at 1% levels (w/v) in the MSCC medium. After 5 days of incubation the culture broth was analyzed for chitinase activity, soluble protein, reducing sugar and pH.

### 2.7.8 Influence of various chitinous substrates on chitinase production by *B. bassiana*

Influence of different chitinous substrates on chitinase production, under submerged condition was studied using purified crab shell chitin (Sigma, USA), purified prawn

shell chitin (CIFT, India), colloidal chitin prepared from prawn shell chitin (section 2.2), powdered prawn waste (section 2. 10.1), chitosan power (CIFT, India ) and glucosamine as substrates, at 1% (w/v) level in the enzyme production medium.

### 2.7.9 Impact of different concentrations of prawn waste on chitinase production by *B. bassiana*

Prawn waste was found to support enhanced level of chitinase production compared to others (section 2.7.8). Hence its optimal concentration required for maximal chitinase production was determined by testing the enzyme production at various concentrations ranging from 1-10% (w/v) as described above (section 2.7.8)..

### 2.7.10 Optimization of physico-chemical parameters for chitinase production by *B. bassiana* under SmF

Optimum physico-chemical condition required for maximal chitinase production was determined by subjecting the organism to various levels of incubation temperature, pH, substrate concentration, and NaCl concentration, additional nitrogen sources, concentration of yeast extract, inoculum concentration and incubation period.

#### 2.7.10.1 Incubation temperature

Optimal incubation temperature for maximal enzyme production was determined by incubating the inoculated MSCC

medium at various incubation temperature ranging from 22 - 42°C.

### 2.7.10.2 Initial pH of the medium

Optimal pH for enzyme production was determined by subjecting the organism to various pH levels ranging from 4 - 12 adjusted in the (MSCC) medium, using 1 N NaOH/1 N HCl.

### 2.7.10.3 Substrate (colloidal chitin) concentration

Optimal concentration of substrate required for maximal production of enzyme was tested in MSCC medium supplemented with different concentration of colloidal chitin ranging between 0-10% (w/v).

### 2.7.10.4 Sodium chloride concentration

Effect of sodium chloride on enzyme production was determined by subjecting the fungus to various levels of NaCl concentration 0-10% (w/v) adjusted in the MSCC medium.

### 2.7.10.5 Additional nitrogen sources

Effect of additional nitrogen sources, other than colloidal chitin, on the rate of enzyme production was studied by incorporating various nitrogen sources, mentioned below, at 1% (w/v) level in MSCC medium. Both organic and inorganic nitrogen sources were tried, independently and in combination.

| | | |
|---|---|---|
| a) | Organic | yeast extract, peptone, tryptone, beef extract |
| b) | Organic plus Organic | yeast extract- peptone, yeast extract-peptone - beef extract |
| c) | Inorganic | $NH_4Cl$, $(NH_4)_2 SO_4$, $NaNO_3$ |
| d) | Organic plus Inorganic | yeast extract - peptone - $(NH_4)_2SO_4$, yeast extract-$(NH_4)_2SO_4$. |

### 2.7.10.6 Yeast extract

Since yeast extract was found to promote maximal production of chitinase (Section 2.7.10.5) optimal concentration of yeast extract, as additional nitrogen source, was determined by subjecting the organism to different concentrations of yeast extract (0-15% w/v) incorporated into the MSCC medium.

### 2.7.10.7 Inoculum Concentration

The optimal inoculum concentration for maximal enzyme production was determined by using various concentrations of inoculum from 63 $\mu$g to 399 $\mu$g dry weight equivalent of mycelia per 100 ml of fermentation medium.

### 2.7.10.8 Incubation time

Optimal incubation time required for maximal enzyme production determined by incubating the inoculated the medium upto a maximum of 8 days and analysing for enzyme yield at regular intervals.

### 2.7.11 Effect of $Mg^{++}$ ion

The effect of $Mg^{++}$ ions on enzyme production was studied by subjecting the organism to different concentration of $Mg^{++}$ ions incorporated as $MgSO_4$ $5H_2O$ (0.05 - 1% w/v), into the MSCC medium.

### 2.7.12 Effect of phosphate

The effect of phosphate on chitinase yield was studied by the addition of phosphate as $K_2HPO_4$ or $KH_2PO_4$ individually and in combination as $K_2HPO_4$ plus $KH_2PO_4$ at various concentration (% w/v) into the MSCC medium as described below.

| | |
|---|---|
| $K_2HPO_4$ (w/v) | 0.5 |
| $KH_2PO_4$ (w/v) | 0.5 |
| $K_2HPO_4$ $KH_2PO_4$ (w/v w/v) | 0.5:0.5, 1:1, 1:2, 2:0.5, 3:2, and 2:3 |

### 2.7.13 Chitinase production by B. bassisna under SmF - static culture

Chitinase production under static culture conditions of submerged fermentation was carried out employing the same set of physico-chemical parameters at the level optimized for shake flask culture described in the previous sections.

The enzyme production medium, dispensed as aliquots of 25 ml, in 250 ml Erlenmeyer conical flask and sterilized at 121°C for 15 minutes was inoculated with the vegetative

inoculum (at 5% v/v level), prepared as described under section 2.6.2. The inoculated flasks were incubated at room temperature (28 $\pm$ 2°C) without shaking upto 10 days. Samples were analyzed at regular intervals for chitinase yield. The fermented broth was centrifuged at 10,000 rpm at 4°C for 20 minutes in a refrigerated centrifuge (Kuboto, Japan). The supernatant was collected and analyzed for chitinase yield, soluble protein, reducing sugars and pH as per the procedure described previously.

The sedimented mycelial pellet was washed repeatedly with sterile distilled water and used for the measurement of growth as described under section, 2.6.4.2.

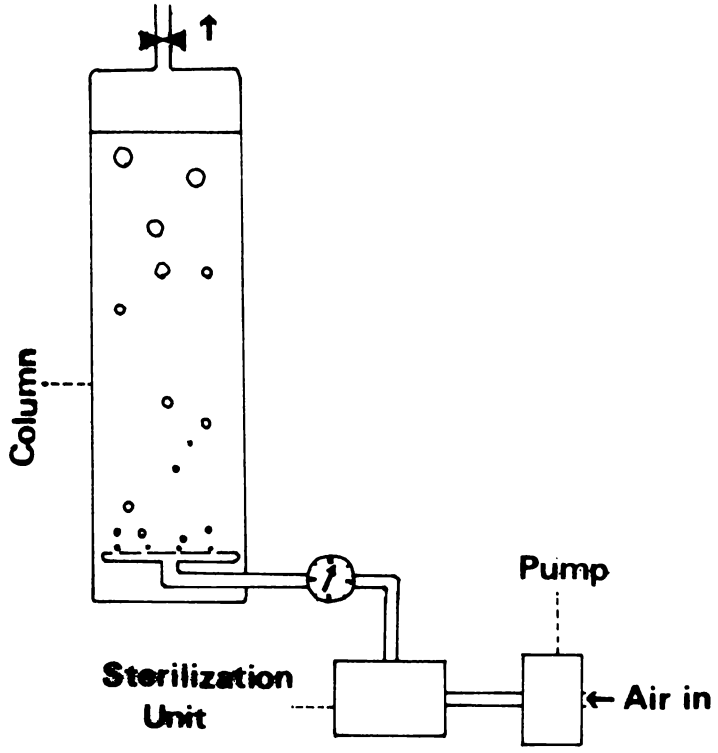
### 2.7.14 Effect of N-acetyl-D-glucosamine on enzyme production

The effect of GlcNAc on chitinase production was determined by supplementing the MSCC medium with GlcNAc at various concentration (0 - 4% w/v) in addition to the colloidal chitin (after optimization studies).

## 2.8 Chitinase production by *B. bassiana* in a bubble column bioreactor

Chitinase production by the fungus in a bubble column bioreactor was assessed by conducting the studies in a cylindrical glass column (4.5 cm diameter and 40 cm long (Figure 2).

Figure 2 Diagram of a bubble column bioreactor




The column bioreactor with the accessories was sterilized either in hot air oven or under steam sterilization and allowed to cool down to room temperature. The enzyme production MSCC medium (section 2.7.1 after optimization) was used for this experiment.

200 ml of the sterilized medium was asceptically transferred into the column, mounted vertically on a laboratory stand. The mouth of the column was closed with a sterilized cotton plug. The outlet of the column was connected to an air pump. The medium was inoculated with the vegetative inoculum (at 5% (v/v level), prepared as described under section 2.6.2. Sterilized air was passed through the column, using an air pump. The fermentation in the column

bioreactor, was conducted at room temperature (28 $\pm$ 2°C). Samples were drawn, aseptically, from the top of the column using sterile pipette at regular intervals. The samples were centrifuged at 10,000 rpm at 4°C for 15 minutes in a refrigerated centrifuge and the supernatant collected was analyzed for chitinase activity, soluble protein, and reducing sugars. The mycelial precipitate was collected, washed repeatedly with sterile distilled water and used for the measurement of growth as per the procedure described under section 2.6.4.2.

## 2.9 Fermentative production of chitinase by solid substrate fermentation (SSF) using wheat bran as solid substrate

### 2.9.1 Substrate

Wheat bran, which is generally used for extracellular enzyme production by fungi under solid-substrate fermentation, was used in the present study. The composition of wheat bran is shown in Table 5.

### 2.9.2 Preparation of solid substrate medium

The wheat bran (WB) medium was prepared as per the procedure described under section 2.5.2.1.

### 2.9.3 Inoculum preparation

Spore inoculum was preferred for inoculating the WB medium, since it is a widely accepted method of inoculating

fungi. The inoculum was prepared as per the procedure described previously under section 2.5.2.2.

Table 5 Composition of wheat bran (Renu, 1991)

| Constituents | Percentage |
|---|---|
| Moisture | 8.80 |
| Total nitrogen | 2.33 |
| Fat | 4.10 |
| Fibres | 10.80 |
| Pentosan | 25.10 |
| Ash | 6.38 |
| Total sugar | 5.40 |
| $P_2O_5$ | 3.15 |
| Carbohydrate content | |
| Starch | 14.10 |
| Sugar | 7.60 |
| Cellulose | 35.20 |
| Hemicellulose | 43.10 |

### 2.9.4 Inoculation and incubation

The sterilized WB medium was inoculated and incubated as per the procedure described earlier under section 2.5.2.3.

### 2.9.5 Extraction and recovery of enzyme

Enzyme from fermented wheat bran (mouldy bran) was extracted by simple contact method using distilled water as the extractant, as described earlier under section 2.5.2.4.

### 2.9.6 Chitinase assay

Chitinase activity was measured as per the procedure described earlier under section 2.5.2.5.

### 2.9.7 Biochemical assays

#### 2.9.7.1 Determination of soluble protein and reducing sugars

Soluble protein and reducing sugars in the enzyme extract was measured as per the procedure described undersections 2.7.6.1 and 2.7.6.2 respectively.

#### 2.9.7.2 Determination of pH of the extract

pH of the enzyme extract was monitored using a digital pH meter (Systronics, India).

#### 2.9.7.3 Determination of pH of the solid substrate

pH of the solid substrate was measured as detailed below.

One gram of the solid substrate was added to 50 ml of distilled water. The mixture was homogenized with a tissue homogenizer for 5 minutes and allowed to settle for 30

minutes. Later the pH of the supernatent was measured using a digital pH meter (Systronics, India).

### 2.9.8 Optimization of process parameters for enzyme production by fungus under SSF using wheat bran medium

#### 2.9.8.1 Initial moisture of the substrate

Optimal requirement of initial moisture content of the WB medium, before sterilization, for the maximal enzyme production was studied by adjusting the moisture content of the substrate to various levels. This was achieved by altering the ratios of the sea water to wheat bran (v:w), ranging from 0:5 to 12:5 by varying the volume of sea water used for moistening the WB medium

#### 2.9.8.2 Incubation temperature

Requirement of c imum incubation temperature for maximal production of enzyme was assessed by incubating the inoculated WB medium at different temperatures ranging from 22 to 42$^{o}$C.

#### 2.9.8.3 Initial pH of the solid substrate

Optimum pH of the solid substrate, after autoclaving, for maximal enzyme production was determined at various pH levels adjusted in the substrate using sea water with different pH, adjusted with 1 N NaOH/1 N HCl.

### 2.9.8.4 Effect of NaOH

Adjusting the pH of the solid substrate to a highly alkaline condition with sea water, that was adjusted to a high pH, was found to be a difficult process with wheat bran. It was also noted that chitinase production was more at alkaline conditions. Hence, an attempt was made with NaOH as solvent for adjusting the pH of the wheat bran medium. NaOH with varying normality (0.1, 0.5, 1 and 2 N) was used to moisten the wheat bran. The WB medium, adjusted to various alkaline pH with NaOH was autoclaved and used.

### 2.9.8.5 Colloidal chitin concentration

Optimal concentration of colloidal chitin for enhanced enzyme production was determined by incorporating colloidal chitin at various levels (0-15% w/w) into the WB medium The colloidal chitin added to the wheat bran was mixed thoroughly and humidified either with sea water/NaOH.

### 2.9.8.6 Additional NaCl concentration

Requirement of additional NaCl for enhanced enzyme production by fungus was determined by incorporating NaCl at various levels (0 - 25 % w/w) in the WB medium and humidified either with sea water/NaOH solution.

### 2.9.8.7 Effect of phosphate

Requirement of phosphate for enhanced enzyme production by fungus was studied by incorporating phosphate to the WB medium, as $K_2HPO_4$ and $KH_2PO_4$, at various levels ( % w/w) as described below.

| | | |
|---|---|---|
| $K_2HPO_4$ | | 1, 2.5 and 5 |
| $KH_2PO_4$ | | 1, 2.5 and 5 |
| $K_2HPO_4$ | $KH_2PO_4$ | 1:1, 1:2.5, 1:5, 2.5:1, 2.5:5, 5:1 and 5:2.5 |

### 2.9.8.8 Type of inoculum

Ideal type of inoculum, spore or mycelial suspension, required for effecting enhanced enzyme production by fungus was determined by using both the types. Both spore and vegetative inocula were prepared as described under sections 2.5.2.2 and 2.6.2 respectively. The prepared inocula were adjusted to various concentrations using physiological saline. Spore inocula tested include concentrations of 32 x $10^6$ 16 x $10^6$ 8 x $10^6$ and 4 x $10^6$ number of spores per 5 g of wheat bran. The vegetative inocula used for inoculating WB medium were approximately 1000, 500, 250, and 20 mg dry weight equivalent of mycelia per 100 g of wheat bran.

### 2.9.8.9 Effect of autoclaving cum cooking

The effect of pretreatment of wheat bran by cooking on enzyme production was studied by conducting the SSF with WB medium autoclaved for various periods (20, 40, and 60 minutes).

### 2.9.8.10 Particle size of wheat bran and inoculum type

Optimum particle size of the solid substrate that promote maximal enzyme production was determined by using wheat bran of different particle size. Commercially available wheat bran was graded into various fractions of their particle size using standard sieves (Filter well, India). The fractions having particle size of < 425 micron (fine particle), between 425-625 micron (medium particle), between 600-1000 micron (coarse particle) and > 425 micron (mixed particle) were used in the present study. The WB medium prepared with different particle size was inoculated separately, with spore and vegetative inocula at their optimized level (section 2.9.8.8, 32 x $10^6$ spores /5 g WB and approximately 1000 mg dry weights equivalent of vegetative inocula per 100 g WB respectively).

### 2.9.8.11 Incubation time

Requirement of optimum incubation time for maximal enzyme production was estimated by incubating the WB medium, inoculated with both type of spore and vegetative inocula, at their optimized levels, for a total period of 96 hour and determining the enzyme yield at regular intervals.

### 2.9.8.12 Additional nitrogen sources

Since yeast extract and $NH_4Cl$ were found to promote maximal enzyme production by the fungus under SmF (section

2.7.7.5), only the yeast extract and $NH_4Cl$ were tested as additional nitrogen sources for maximal enzyme production under SSF. They were added at various levels (0-4% w/w) into WB medium and tested for their influence on enzyme yield.

### 2.9.8.13 Effect of salinity

Effect of salinity of sea water, used for moistening the WB medium, on enzyme production was determined by preparing the WB medium with sea water adjusted to various dilutions (50, 75, and 100% diluted with distilled water). A comparison was made using tap water and distilled water also.

### 2.9.8.14 Effect of culture vessel volume to medium volume

The effect of fermentation vessel volume to wheat bran medium volume on enzyme production was studied by taking WB medium in petriplates or conical flasks as described below.

a) Petriplate (100 x 15 mm)
   - i) 5 g WB medium/ petriplate
   - ii) 10 g WB medium/ petriplate

b) Conical flask
   - iii) 5 g WB medium/ 100 ml conical flask
   - iv) 5 g WB medium/ 150 ml conical flask
   - v) 5 g WB medium/ 250 ml conical flask
   - vi) 5 g WB medium/ 500 ml conical flask

The prepared WB medium, taken in the different vessels were inoculated with the spore inoculum (32 x $10^6$ spores/5 g wheat bran medium (section 2.5.2.2). While the inoculated petriplates were incubated as such (not inverted), the inoculated flasks were incubated in a slanting position on their sides.

## 2.10 Fermentative production of chitinase by solid substrate fermentation using prawn waste as solid substrate

### 2.10.1 Substrate

The sundried prawn waste used in the present study was obtained from CIFT, Kochi, India as a gift. The composition of prawn waste is shown in Table 6. It was stored at room temperature until used.

Table 6 Composition of prawn waste (Madhavan and Nair, 1975)

| Constituents | Percentage |
|---|---|
| Moisture | 76.62 |
| Ash (dry basis) | 31.13 |
| Protein | 39.76 |
| Chitin | 23.08 |
| Fat | 5.054 |

### 2.10.2 Pre-treatment of substrate

The chitinous prawn waste was further dried in a hot air oven at 50°C for 30 minutes and milled to get small particles of varying size, in an electric blender. Particles with different size were used in the study.

### 2.10.3 Preparation of solid substrate for enzyme production

The prawn waste of particle size > 425 micron was used. 5 g of prawn waste taken in a petriplate (10 x 1.5 cm) was thoroughly mixed with 3 ml of sea water (pH 8.5) (unless otherwise specified). Later the plates containing the prawn waste (PW) medium were autoclaved at 121°C for 60 minutes, and allowed to cool down to room temperature 28 $\pm$ 2°C.

### 2.10.4 Inoculum preparation

The spore inoculum was prepared as described under section 2.5.2.2.

### 2.10.5 Inoculation and incubation

Inoculation and incubation for desired incubation period were carried out as described under section 2.5.2.3.

### 2.10.6 Recovery of enzyme

Chitinase produced by fungus during SSF on PW medium was extracted by simple contact method using distilled water as extractant as described under section 2.5.2.4.

### 2.10.7 Enzyme assay

The enzyme activity was measured as described under section 2.5.2.5.

### 2.10.8 Estimation of soluble protein, reducing sugars and pH

The soluble protein, reducing sugars and pH of the enzyme extract was estimated as per the procedure described under section 2.9.7.

### 2.10.9 Optimization of process parameters for enhanced enzyme production by the fungus under SSF using prawn waste

2.10.9.1 Optimal requirement of moisture content of the substrate, initial pH of the substrate, NaCl concentration and phosphate concentration for maximal enzyme production was determined as per the procedures described earlier under sections 2.9.8.1, 2.9.8.3, 2.9.8.6 and 2.9.8.7 respectively.

#### 2.10.9.2 Particle size of prawn waste and inoculum type

Optimal particle size of prawn waste that support maximal chitinase production by fungus during SSF was studied by using different particle size of prawn waste. The particle size tested were (1) < 425 micron (fine particle), (2) 425-600 micron (medium particle), (3) 600-1000 micron (course particle) and (4) > 1.425 micron (mixed particle). Both the spore and vegetative inocula were prepared and adjusted to a

concentration of 32 x $10^6$ number of spores/5 g prawn waste and approximately 50 mg cell dry weight equivalent per 5 g prawn waste respectively (section 2.5.2.2 and 2.6.2 respectively). Inoculation, incubation, enzyme recovery and analyses of enzyme yield, soluble protein, reducing sugars and pH were done as described above.

## 2.11 Enzyme studies

### 2.11.1 Source of enzyme

Chitinase produced by *Beauveria bassiana* under solid substrate fermentation, using wheat bran medium, was purified and characterized. The crude enzyme extract recovered after SSF was also analyzed for enzyme profile.

### 2.11.2 Enzyme profile of the crude enzyme preparation

The crude enzyme recovered after SSF was analysed for the presence of chitinase, (EC 3.2.1.14), chitobiase (EC :3.2.1.30), amylase (EC 3.2.1.1), CM cellulase (EC: 3.2.1.4), laminarinase (EC 3.2.1.6), protease (EC: 3.4.21.14), muramidase (EC 3.2.1.17) and chitosanase (EC 3.2.1.99)

#### 2.11.2.1 Chitinase

The chitinase activity was measured as described under section 2.5.2.5.

### 2.11.2.2 Chitobiase/ β - 1,4-N-acetyl-D-glucosaminidase

Chitobiase activity was measured using an artificial substrate, *p*-nitrophenyl-N-acetyl-β-D-glucosamide (*p*-NAG) according to Hendy *et al.* (1990) as detailed below.

The reaction mixture containing 0.5 ml of 0.75 mM *p*-NAG in 0.1 M sodium acetate buffer (pH 5.0) with 20 µl of enzyme solution was incubated at 32°C for 15 minutes. The reaction was terminated by the addition of one ml of 0.1 M glycine /NaOH buffer pH 12.5 and the amount of *p*-NAG released was detected by measuring the absorbence at 430 nm using *p*-nitrophenol as a reference. One unit of N-acetyl glucosaminidase activity was defined as that amount of enzyme which liberated 1 micro mol. of *p*-nitrophenol under assay conditions per minute.

### 2.11.2.3 Amylase (dextrinizing activity)

The dextrinizing activity of the enzyme extract was measured following the method of Medda and Chandra (1980) as detailed below.

The reaction mixture containing 0.5 ml of 1% soluble starch solution 0.4 ml of 0.1 M phosphate buffer (pH 7.0) and 0.1 ml of enzyme solution was incubated at 32°C for 10 minutes. The reaction was terminated by boiling the mixture for 5 minutes. After cooling down to room temperature, 0.1 ml

of iodine solution (0.3% I + 3.0% KI) was added and diluted with 15 ml of distilled water. The absorbance of the blue colour developed was measured at 620 nm within 30 minutes against appropriate reaction blanks. One unit of dextrinizing activity was defined as the amount of enzyme that hydrolyzed 1 mg of soluble starch under the assay condition.

### 2.11.2.4 CM cellulase

CM cellulase activity was estimated by the method of Ohtakara (1988) with a slight modification for the estimation of reducing sugar produced by the hydrolysis of CM cellulase using DNS reagent (Jones and Grainger, 1983) as detailed below.

Reaction mixture containing one ml of 1% CM cellulose, 2 ml of 0.1 M phosphate buffer (pH 6.5) and one ml of enzyme solution was incubated for 20 minutes at 32°C. The reaction was terminated by boiling the mixture for 5 minutes. The amount of reducing sugar released in one ml of the reaction mixture was estimated as per the procedure described under section 2.7.6.2. One ml of CM cellulase activity was defined as the amount of enzyme which produced one micro mol. of reducing sugar as glucose per minute under the assay condition.

### 2.11.2.5 Laminarinase

Laminarinase activity was estimated as suggested by Polacheck and Rosenberger (1978) based on the estimation of reducing sugars released by the hydrolysis of soluble laminarin, with DNS reagent (Jones and Grainger, 1983).

Reaction mixture containing 0.5 ml of laminarin, and 0.5% in McIlvane's citrate buffer (pH 5.8) was incubated with 0.5 ml of enzyme solution at 32$^{o}$C for 2 h. The reaction was terminated by boiling the reaction mixture for 5 minutes. The reducing sugars produced in one ml reaction mixture was estimated as described under section 2.7.6.2 using DNS reagent. One unit of laminarinase activity was defined as the amount of enzyme which produced one micro mol. of reducing sugar as glucose under the assay condition.

### 2.11.2.6 Protease

Protease activity was assayed following the casein digestion method of Kunitz (1940). One ml of 0.6% casein in 0.05 M Tris-HCl buffer (pH 7.0) was incubated with one ml of the enzyme solution at 32$^{o}$C for 30 minutes. The reaction was arrested with 2.5 ml of 6.44 M TCA solution. The precipitated protein was removed by centrifugation (10,000 rpm, 10 minutes at room temperature) and the absorbance of the filtrate was measured at 280 nm against tyrosine as a reference compound. A unit of proteolytic activity was defined as the amount of

enzyme that liberated 1 μg of tyrosine per ml of the reaction mixture per minute under the assay conditions.

### 2.11.2.7 Muramidase

Muramidase activity was estimated using the freeze dried *Micrococcus lysodeikticus* cells as substrate (David, 1988). 2.8 ml suspension of *Micrococcus lysodeikticus*, freshly prepared in sodium phosphate buffer (0.1 M) pH 7.0 (0.3 g /ml), was mixed thoroughly with 0.2 ml of enzyme solution and incubated at 32°C for 1 h. The absorbence was measured at 450 nm. One unit of activity was defined as the amount of enzyme required for a decrease of 0.001 *A* at 450 per minute per ml of reaction mixture.

### 2.11.2.8 Chitosanase

Chitosanase activity was measured as suggested by Uchida and Ohtakara (1988) using colloidal chitin as substrate, with a slight modification on the estimation of reducing sugars produced by the hydrolysis of the substrate using DNS reagent (Jones and Grainger, 1983). Glucosamine was used as a reference compound.

Soluble chitosan, 1% (w/v): one gm of chitosan ( > 80% deacetylated) suspended in 40 ml of distilled water was dissolved with 9 ml of 1 M acetic acid under stirring for 2 h.

Later the pH was adjusted to pH 6 with 1 M sodium acetate and made upto 100 ml with 0.1 M acetate buffer (pH 6).

A mixture of 1 ml of 1% soluble chitosan and 1 ml of enzyme solution was incubated for 10 minutes at 32°C. The reaction was terminated by boiling for 5 minutes. The amount of reducing sugar produced per ml of reaction mixture was estimated using DNS reagent as mentioned earlier (section 2.7.6.2). One unit of chitosanase activity was defined as the amount of enzyme which produce 1 micro mol. of reducing sugar as glucosamine under the assay conditions.

### 2.11.3 Purification of enzyme

The crude enzyme extract obtained after extraction and centrifugation (section 2.9.5 ) was purified following the conventional purification method detailed below.

#### 2.11.3.1 Ammonium sulphate fractionation

Non-enzyme protein in the crude enzyme preparation was first removed by fractionation with solid $(NH_4)_2SO_4$ precipitation at 50% (w/v) saturation The chitinase present in the supernatent was precipitated by slowly adding solid $(NH_4)_2SO_4$ to 50% saturation with mechanical stirring at 4°C and kept overnight at 4°C. The precipitated enzyme was separated by centrifugation at 10,000 rpm at 4°C for 30 minutes in a refrigerated centrifuge, dissolved in a minimum

volume of 0.1 M citrate phosphate buffer pH 6.0 and dialyzed overnight against several changes of the same buffer at 4°C. The dialyzed solution was then centrifuged at 4°C for 10 minutes to remove the undissolved gummy material left over in the dialyzed solution. The protein and chitinase activity were determined as described earlier, under sections 2.7.6.1 and 2.7.5 respectively.

### 2.11.3.2 Gelfiltration on Sephadex $G_{100}$ and Sephadex $G_{200}$

#### 2.11.3.2.1 Packing and equilibration of columns

Sephadex $G_{100}$ and Sephadex $G_{200}$ (Sigma, USA) was soaked in McIlvane's citrate buffer (0.05 M) pH 6.0. Fine particles were decanted and the gel was suspended in the same buffer with 0.1 M NaCl. A 2 x 47 cm glass column with a sintered disc base mounted vertically on a table laboratory stand, using a spirit level, was tilted and one third of the column was filled with buffer. To this well mixed gel suspension was poured down the inside wall of the column and allowed to settle for 15 minutes without flow. Then the excess buffer was allowed to drain through the growing gel bed. The gel slurry was continuously added until a bed of desired height was attained. A buffer reservoir was connected to the inlet of the column and 2 to 3 column of buffer was allowed to pass through the column for stabilization of the bed and equilibration of the column with the elution buffer.

Uniformity of the column packing and void volume of the column were checked by Blue Dextran 2,000.

#### 2.11.3.2.2 Sample application and elution on sephadex $G_{100}$ column

The partially purified enzyme, obtained after $(NH_4)_2SO_4$ precipitation was loaded (2 ml) on to the sephadex column and eluted with 0.05 M citrate buffer pH 6.0 containing 0.1 M NaCl, with a flow rate at 30 ml/h. Fractions (2 ml) were collected using a fraction collector (Redi Frac, Pharmacia).

#### 2.11.3.2.3 Analysis of fractions

Collected fractions were analyzed for protein by measuring the absorbence at 280 nm using a uv-visible. spectrophotometer (Shimadzu 160A, Japan). The enzyme activity was determined as described under section 2.7.5. The active fractions were pooled, dialized against 0.05 M citrate buffer (pH 6) and concentrated by lyophylization (Yamata, Neocool, Japan). The specific activity of the pooled fraction were calculated.

#### 2.11.3.2.4 Sample application and elution on sephadex $G_{200}$ column

The pooled and concentrated enzyme fractions after eluting through Sephadex $G_{100}$ was loaded (2 ml) on to sephadex

$G_{200}$ column. The column was eluted with 0.05 M McIlvine's citrate buffer (pH 6.0) containing 0.1 M NaCl with a flow rate of 28 ml/h. Two ml fraction were collected using a fraction collector. The fractions were analysed and concentrated as described above under section 2.11.3.2.3.

### 2.11.3.3 Determination of homogeneity of purified enzyme

Homogeneity of the purified enzyme was determined by polyacrylamide gel electophoresis (PAGE) as described by Davis (1964). The electrophoresis was conducted using a slab gel apparatus (modified Studiar model) with a noteched glass plate system. Gels of 1.5 mm thickness were prepared by using perspex spacers of uniform thickness.

#### 2.11.3.3.1 Preparation of reagent

All the reagents (stock solutions and buffers) were prepared according to the procedure described by Hames (1990).

Acrylamide solution

Dissolved 30 g of acrylamide and 0.8 g of N,N$^{1}$ methylene bisacrylamide in distilled water and made upto 100 ml. Filtered with Whatmann No 1 filter paper and stored at 4$^{o}$C in dark bottle.

Ammonium persulphate (APS) 1.5%

Dissolved 150 mg of APS in 100 ml distilled water. Solution was prepared just before use.

TEMED

N,N, $N^1$,$N^1$- tetramethyl ethylene diammonium was used as supplied. Stored at $4^oC$.

Staking gel buffer stock (0.5 M Tris-HCl, pH 6.8)

Dissolved 6 g of Tris in 40 ml of distilled water and adjusted to pH 6.8 with 1 M HCl, made up to 100 ml, filtered and stored at $4^oC$.

Resolving gel buffer stock (3 M Tris-HCl, pH 8.8):

Dissolved 36.3 g of Tris and 48 ml of 1 M HCl in distilled water, made upto 100 ml, filtered and stored at $4^oC$.

Electrode buffer (0.25 mm Tris, 1.92 glycine pH 8.3)

Dissolved 30.3 g of Tris and 140 g of glycine in distilled water, made upto 1 litre using distilled water, and stored at $4^oC$. Diluted to 1:9 (v/v) ratio with distilled water at the time of use.

Sampling buffer

Stacking gel buffer - 12.5 ml, glycerol - 10% level and 5% Bromophenol blue - 1 ml were mixed and made up to 25 ml using distilled water.

Staining reagent

Dissolved 0.5 g of Coomassie Brilliant blue in 500 ml of water methanol glycial acetic acid (5:5:2) v/v ratio mixture and stored at room temperature.

2.11.3.3.2 Preparation of sample and electrophoresis

The sample for electrophoresis was prepared by mixing 1 ml of purified enzyme (1 mg/ ml) in citrate buffer (0.05 M) with 1 ml of sample buffer.

Resolving gels of 7.5 and 10% strength and 1.5 mm thickness were prepared and electrophoresis were performed at $4^{o}C$ at a constant voltage of 75 volt until the sample entered the resolving gel and then the voltage was increased to 100-125 V.

2.11.3.3.3 Staining and destaining

After electrophoresis the gels were stained for protein using staining reagent. The gels were placed in a gel staining tray and stained for about 6 h at room temperature. After the staining was completed, excess stain was removed by placing the gel in a destaining solution (30% methanol and 10% acetic acid solution). The destained gel, was preserved in 7% acetic acid solution.

### 2.11.3.4 Characterization of purified enzyme

The purified enzyme was characterized for its activity and stability with respect to various pH, temperature, metal ions and determined the molecular weight both at native and denatured conditions of protein.

#### 2.11.3.4.1 Effect of pH on the activity

Effect of pH on chitinase activity was assayed at different levels of pH using various buffers viz 0.1 M concentration of citrate-phosphate buffer (3-7), sodium phosphate buffer (7-8) and glycine - NaOH buffer (8.6-10.6). Enzyme assay was performed as described under section 2.7.5.

#### 2.11.3.4.2 Effect of pH on the stability

Effect of pH on the stability of chitinase was determined by maintaining the enzyme at different pH using buffers of varying pH in 0.1 M concentration (pH 3 - 10.6) mentioned above, for 30 minutes and the resdiual enzyme activity was assayed as descried under section 2.7.5.

#### 2.11.3.4.3 Temperature

Reaction mixture having the purified enzyme and the substrate was incubated at various incubation temperatures (21 to 57$^{o}$C) to examine the effect of temperature on enzyme activity. Enzyme assay was performed as mentioned under section 2.7.5.

#### 2.11.3.4.4 Thermostability

Thermostability of chitinase was determined by incubating the enzyme at 30, 40, 50 and 60$^{o}$ C for 60 minutes. Later the residual enzyme activity was determined as described under section 2.7.5.

#### 2.11.3.4.5 Metal ions

Effect of metal ions on enzyme activity was tested by incubating the reaction mixture with various metal ions ($Sn^{2+}$ $Ni^{2+}$ $Cd^{2+}$ $Co^{2+}$ $Hg^{2+}$ $Ag^{+}$ $Mg^{2+}$ $Na^{+}$ and $Ca^{2+}$) at a final concentration of $10^{-3}$ M. Enzyme activity was assayed as mentioned under section 2.7.5.

#### 2.11.3.4.6 Molecular weight

Molecular weight of the native enzyme protein was determined by calibrated gel filtration chromatography.

#### 2.11.3.4.6.1 Calibrated gel filtration chromatography

A gel filtration column of sephadex $G_{100}$ was prepared as described earlier (section, 2.11.3.2) was used for the determination of molecular weight. One ml of the purified enzyme preparation was loaded onto the sephadex $G_{100}$ column, pre-equilibrated with 0.05 M phosphate buffer (pH 8.0) and eluted isocratically with the same buffer at a flow rate of 28 ml per h. Fractions were analyzed for protein content, and the molecular weight of the enzyme was determined using the

data obtained from the calibration of the column with marker proteins of known molecular weight. A graph plot between the ratio of elution volume (Ve) to void volume (Vo), (Ve/Vo), and the logarithm of molecular weight of protein markers were used to estimate the molecular weight of the enzyme from its elution volume.

The molecular weight markers (Sigma, USA) used to calibrate the column were

1. Cytochrome C. Horse Heart - (M.Wt. - 12,400)
2. Carbonic Anhydrogenase - (" - 29,000)
   Bovine Erythrocytes
3. Albumin, Bovine Serum - (" - 66,000)
4. Alcohol Dehydrogenase, yeast - (" - 150,000)
5. β-Amylase, sweet potato - (" - 200,000)

**2.11.3.4.7 Subunit molecular weight**

Molecular weight of the subunits of the enzyme protein was determined following the method of Laemmli (1971) using sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE).

The relative mobility of the chitinase band subjected to denaturation with SDS in the loading buffer was compared to that of the standard molecular weight markers. The molecular weight was determined by a semi-logarithmic

curve plot between electrophoretic mobility (Rf) and log. molecular weight of marker proteins.

All the buffers used in the SDS - PAGE were similar to non-denaturing PAGE except for the inclusion of SDS (section 2.11.3.3.1). The staining and destaining procedures adapted for visualizing the protein band were similar to that of non-denaturing PAGE mentioned under section 2.11.3.3.3.

The gels for SDS - PAGE were prepared following the same procedures which were used for non-denaturing polyacrylamide gel electrophoresis, except for the addition of SDS into the buffers.

#### 2.11.3.4.7.1 Preparation of samples

Samples were prepared by mixing 1 ml of purified enzyme (1 mg/ ml) in citrate buffer (0.05 M) with sample buffer, containing a final concentration of 2% SDS, 5% 2-mercapto ethanol, 10% glycerol and 0.002% Bromophenol Blue, using concentrated stock solutions. The content was mixed well, heated in a boiling water bath for 3 minutes, and cooled down to room temperature. Insoluble materials in the mixture were removed by centrifugation.

#### 2.11.3.4.7.2 Sample loading and electrophoresis

Electrophoresis was conducted using a slab gel apparatus as described under section (2.11.3.3.2). 7.5% resolving gel of 1.5 mm thickness was prepared and attached to the electrophoresis unit. Electrode reservoir was filled with reservoir buffer. 20 $\mu$l of the samples, prepared as above, was applied on to the well of the gel. The electrophoresis was performed at room temperature, at a constant voltage of 75 V until the sample entered the resolving gel. Later the voltage was increased to 100-125 V.

#### 2.11.3.4.7.3 Staining and destaining

Staining and destaining of gels were performed as described earlier under section 2.11.3.3.3.

The molecular weight markers (Sigma, USA) used were:

Myosin, Rabbit Muscle (205,000), $\beta$-galactosidase, *E. coli* (116,000), phosphorylase b, Rabbit Muscle (97,000), Fructose-6-phosphate Kinase, Rabbit Muscle (84,000), Albumin, Bovine Liver, (55,000), Ovalbumin, Chicken Egg (45,000), Glyceraldehyde-3-phosphate Dehydrogenase, Rabbit Muscle (36,000), Carbonic Anhydrase, Bovine Erythrocytes (29,000), Trypsinogen, Bovine Pancreas (24,000), Trypsin Inhibitor, Soybean (20,000), $\alpha$-Lactalbumin, Bovine Milk (14,200), Aprotinin, Bovine Lung (6,500).

### 2.11.3.5 Substrate specificity

The substrate specificity of the purified enzyme was studied by following the liberation of reducing sugars from various substrate as detailed below.

#### 2.11.3.5.1 Chitinous substrate

Purified crab shell chitin (Sigma, USA) 5 mg, purified prawn shell chitin (CIFT, India) 5 mg, prawn shell powder (native chitin) 5 mg, chitotriose (Sigma, USA) 500 $\mu$g, chitobiose (Sigma, USA) 500 $\mu$g and colloidal chitin 5 mg in 0.1 M citrate buffer pH 6.0 were incubated with 1 ml of the purified enzyme, at 32$^{o}$C for 2 h. Chitosan powder > 80% deacetylated (CIFT, India) 5 mg in 0.1 M acetate buffer pH 6 was incubated with 1 ml of the enzyme at 32$^{o}$C for 10 minutes. The reaction was terminated by boiling the reaction mixture for 5 minutes. The undigested materials were removed by centrifugation (10,000 rpm, 10 minutes). The amount of reducing sugars liberated per ml of the reaction mixture was determined using DNS reagent as described under section 2.7.6.2, against two reference compounds, N-acetyl-D-glucosamine/ and glucose. Heat denatured enzyme with substrate was used as control.

#### 2.11.3.5.2 Non-chitinase substrate

Non-chitinous substrate CM -cellulose (HiMedia, India) and soluble laminarin (Sigma, USA) were used, and

activity was tested as described under sections 2.11.2.4 and 2.11.2.5 respectively for the substrate. Estimation of reducing sugars was as described under section 2.7.6.2. Glucose was used as a reference compound. Heat denatured enzyme with substrate was used as control.

#### 2.11.3.5.3 Bacterial cell wall lytic activity

Bacterial cell wall lytic activity of the purified enzyme was determined using freeze dried *Micrococcus lysodeikticus* cells as described under section 2.11.2.7 Heat denatured enzyme plus cell suspension was used as control. After incubation the absorbence of cell suspension was measured at 450 nm.

## 2.12 Fungal mycelial and yeast cell lytic activity studies

Fungal cell lytic activity of the crude enzyme was studied with phytopathogenic and non-phytopathogenic fungi and yeast, following the liberation of reducing sugars from whole cell preparation.

### 2.12.1 Enzyme source

Crude enzyme recovered from the fermented wheat bran medium (section 2.9.5) was used for the study.

### 2.12.2 Fungal strains Sources and maintenance

(i) *Rhizoctonia solani*

Obtained as a gift from Kerala Agricultural University. This was subcultured and maintained on potato dextrose agar (PDA) (HiMedia, India) slant and stored at 4°C.

(ii) *Aspergillus niger*

Obtained as a gift from Kerala Forest Research Institute, Peechi. This was subcultured and maintained on PDA slant and stored at 4°C.

(iii) *Mucor* sp.

A stock culture available in the culture collection of the Department of Biotechnology, CUSAT, originally isolated from soil. This was subcultured and maintained on PDA slants and maintaned at 4°C.

(iv) Yeast (*Saccharomyces cereveciae*)

Obtained from the culture collection of National Chemical Laboratory, Pune. This was subcultured and maintained on malt extract - glucose - yeast extract - peptone (MGYP) agar slant and stored at 4°C.

### 2.12.3 Preparation of fungal cells

#### 2.12.3.1 Media

All the filamentous fungi, *Rhizoctonia solani*, *Aspergillus niger* and *Mucor* sp. were cultivated on mycological broth (HiMedia, India) and yeast was cultivated on glucose-yeast extract-peptone (GYP) broth.

#### 2.12.3.2 Inoculum Preparation

Mycelial macerates were prepared by fragmenting the 10 day old agar slope culture, grown on PDA slant, in test-tube (25 ml capacity) in sterile physiological saline for *Rhizoctonia solani*.

Spore suspensions were used as inocula for *Aspergillus niger* and *Mucor* sp. This was prepared using the 10 day old agar slope culture grown on PDA slant in sterile distilled water with 0.1% Tween 80.

Inoculum for yeast was prepared as cell suspension prepared with 48 h old MGYP slant culture in sterile physiological saline.

#### 2.12.3.3 Cultivation of organisms

Both mycological broth and GYP broth prepared as 250 ml aliquot in 1000 ml conical flasks were sterilized, inoculated with the prepared inoculum as described above and

incubated on a rotary shaker (at 150 rpm) at room temperature $28 \pm 2^{o}C$.

### 2.12.3.4 Harvesting

After 48 hours of incubation all the cultures were harvested. The yeast cells were separated from the culture broth by centrifugation at 5,000 rpm for 10 minutes. The collected cells were washed repeatedly with sterile distilled water.

All other filamentous fungi were harvested by filtration, using filter paper, and washed repeatedly with excess sterile distilled water on filter paper. The washed mycelia was homogenized by a tissue homogenizer at 5,000 rpm for 5 minutes. The homogenate was washed again with sterile distilled water on filter paper.

A portion of the washed yeast cells and mycelial fragments were autoclaved. Both autoclaved and non-autoclaved cells/mycelial fragments were lyophylized and powdered using a mortor and pistil. The powdered cells/mycelial fragments were stored at $-20^{o}C$.

### 2.12.4 Dissolution of whole fungal cells by chitinase preparation

The ability of chitinase preparaion to lyse different fungal cells/yeast cells and thereby release

reducing sugars was tested in a reaction mixture containing 1 mg of powdered cells in 1 ml of McIlvaine's citrate buffer pH 6 (0.1 M) and 1 ml of appropriately diluted enzyme solution in test - tube. The tubes containing the reaction mixture were incubated for 2 h at 32$^{o}$C in a shaking water bath. Heat inactivated enzyme plus cells were used as controls. The reaction was terminated by boiling the content in the tube for 5 minutes and the undissolved materials were removed by centrifugation The amount of sugars liberated was measured as reducing sugars per ml of reaction mixture, with the dinitrosalicylic acid reagent method using glucose as a reference compound (Jones and Graniger, 1983) described under section 2.7.6.2.

# CHAPTER - THREE

# RESULTS

## 3.1 Isolation of chitinolytic fungi from marine environments

A total of 60 samples (34 sediment and 26 water) were screened for isolation of chitinolytic fungi. Quantitative distribution of chitinolytic fungi, on a seasonal basis, in the various marine environments were not studied since it was out of scope of the present study. However, in general, the population of chitinolytic fungi was more in the sediments compared to that in the water. A total of 120 strains were isolated from both sediment and water samples.

## 3.2 Selection of potential chitinolytic fungus

Selection of potential strains for chitinase production was carried out on the basis of qualitative and quantitative determination of chitinase production.

All the isolates were subjected to primary screening (qualitative) on agar plates. Based on the results obtained the 120 strains were ranked and the top ranked 22 strains were selected for secondary screening.

Secondary screening (quantitative) was based on their enzyme yield in solid substrate fermentation using wheat bran medium. Based on the enzyme yield the strains were ranked and the top ranked strain was selected for further

studies. The source of the selected strain was marine sediment.

## 3.3 Identification of the selected strains

All the strains isolated were identified upto their generic level. Interestingly it was observed that 99% of them belonged to the *Beauveria* sp. and the remaining 1% belong to the *Mucor* sp.

The strain selected for further studies, after secondary screening was identified as *Beauveria bassaiana* and designated as *Beauveria bassiana* BTMF S10 (plate 1, plate 2). This belonged to the subclass Hypomycetes of the class Deuteromycotina.

## 3.4 Growth studies

### 3.4.1 Optimization of growth conditions

**Temperature:**

The results presented in Figure 3 suggest that *B. bassiana* preferred on optimal temperature of 27°C for maximal growth. Significant levels of growth was also recorded at 22°C. Whereas temperatures 32°C and above did support only poor growth compared to that observed at 27°C.

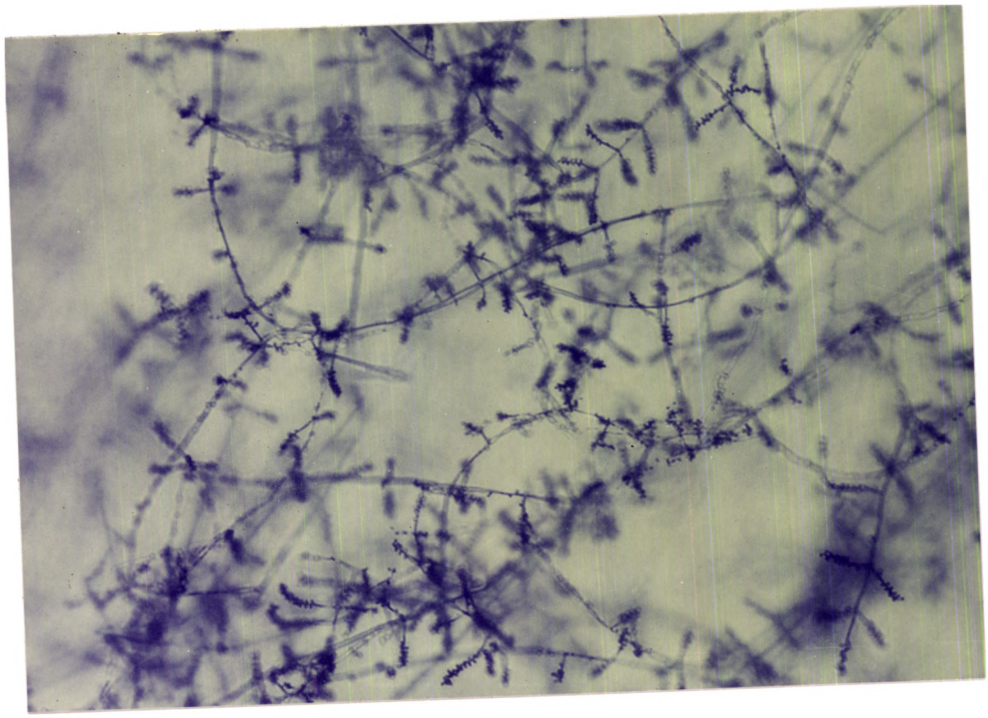
**Plate 1** *Beauveria bassiana* BTMF S10 grown on wheat bran

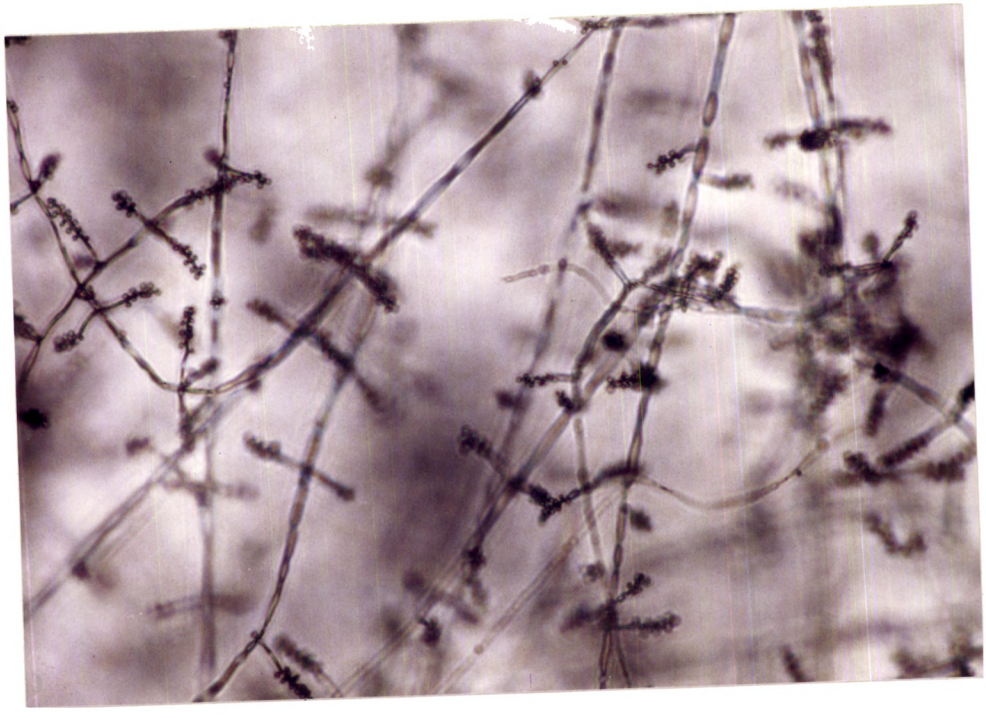

WB


WB

**Plate 2** *Beauveria bassiana* BTMF S10 grown on Bennet's agar plate

**Plate 3** Photomicrograph of *Beauveria bassiana* BTMF S10





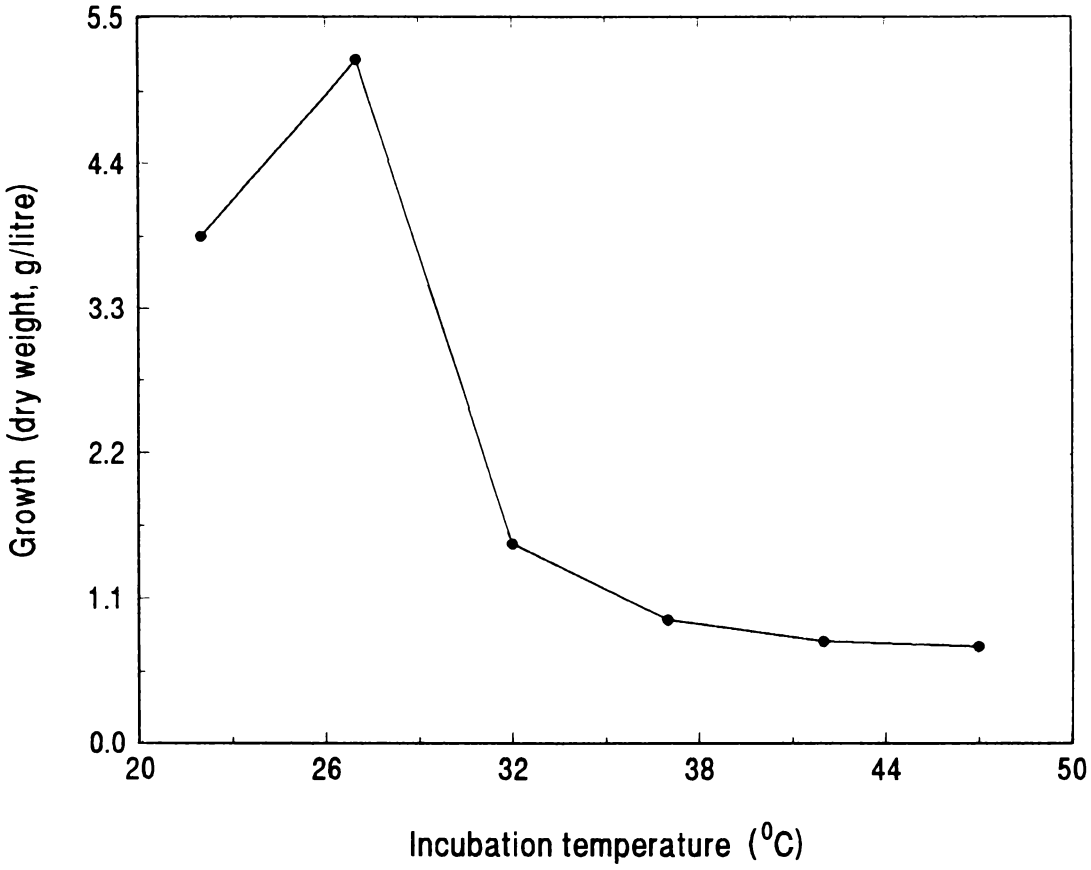



**Figure 3** Effect of incubation temperature on growth of *B. bassiana*

**pH:**

Variation in pH, over a wide range pH 5-11, did not affect the growth of *B. bassiana* significantly (Figure 4). In fact significant levels of growth could be obtained at all pH levels tested, recording two peak of growth. One small peak in the acidic range (6.0) and another, a large one, in the alkaline range (9.0) were recorded However, pH 9.0 was observed to be the optimum although very significant growth could be achieved over a pH range of 6-10.

**Sodium chloride**

From the results presented in Figure 5 it is inferred that 3-5% NaCl concentration supported maximal growth despite recording significant levels of growth over a wide range of NaCl concentration (0-12%). Interestingly this marine fungus could grew even in the absence of sodium chloride, recording significant growth.

**Colloidal chitin concentration**

From the results documented in Figure 6 it is evident that the fungus could grew to a maximum at 8% colloidal chitin as substrate, although significant levels of growth was recorded at concentrations ranging from 1-10%. The growth in the absence of colloidal chitin in the medium was only 10.5% and 23.3% of that observed with 8% and 1% of colloidal chitin respectively.

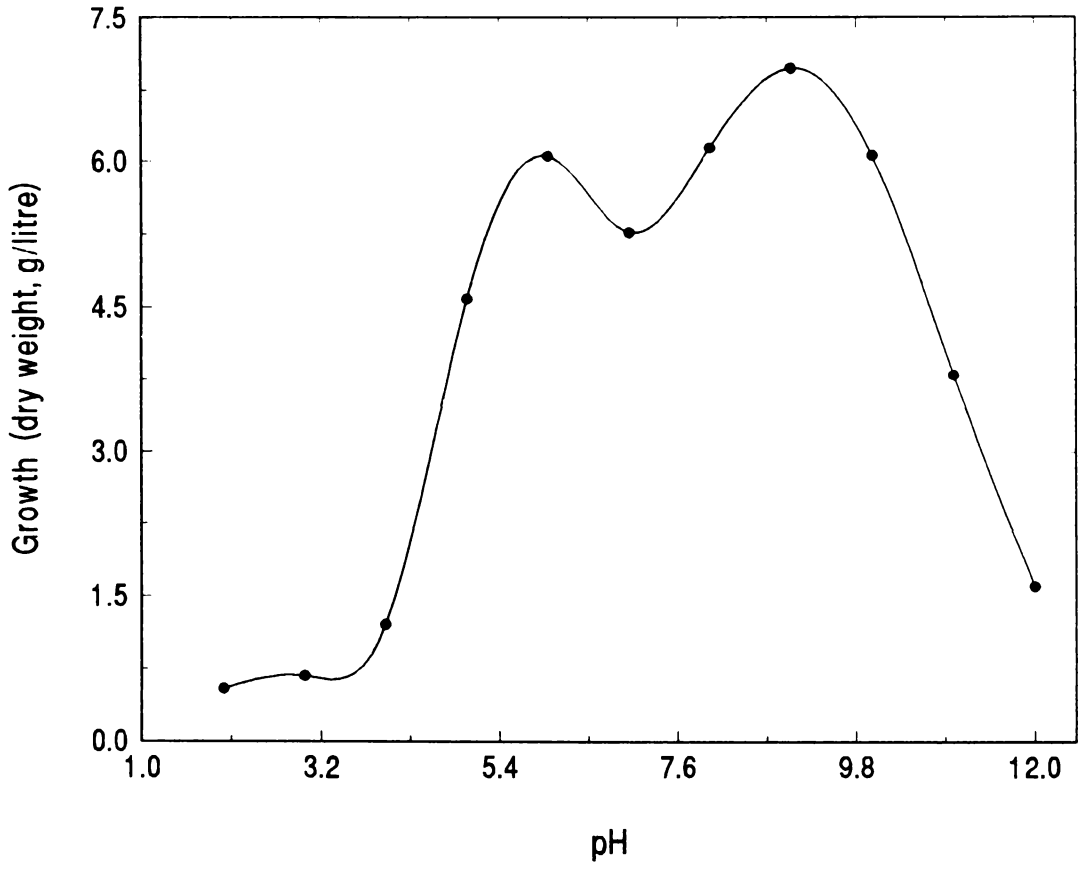



**Figure 4** Effect of pH on growth of *B. bassiana*

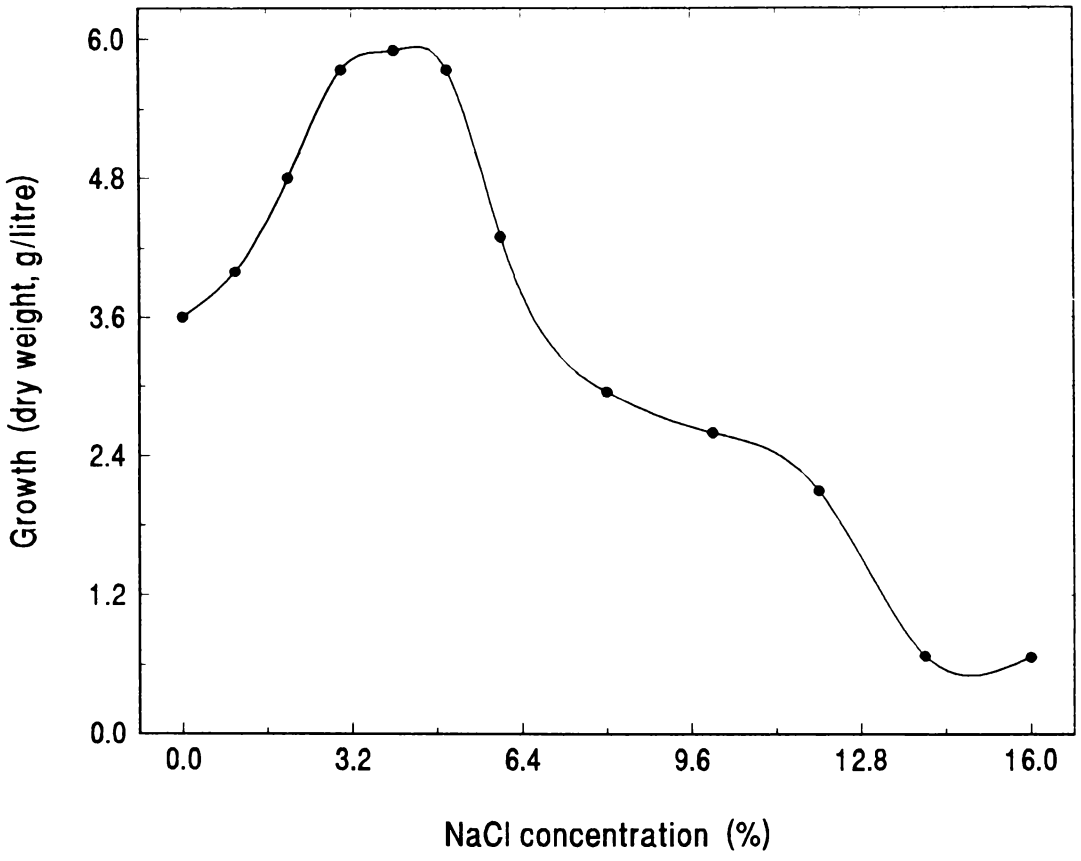



**Figure 5** Effect of sodium chloride concentration on growth of *B. bassiana*

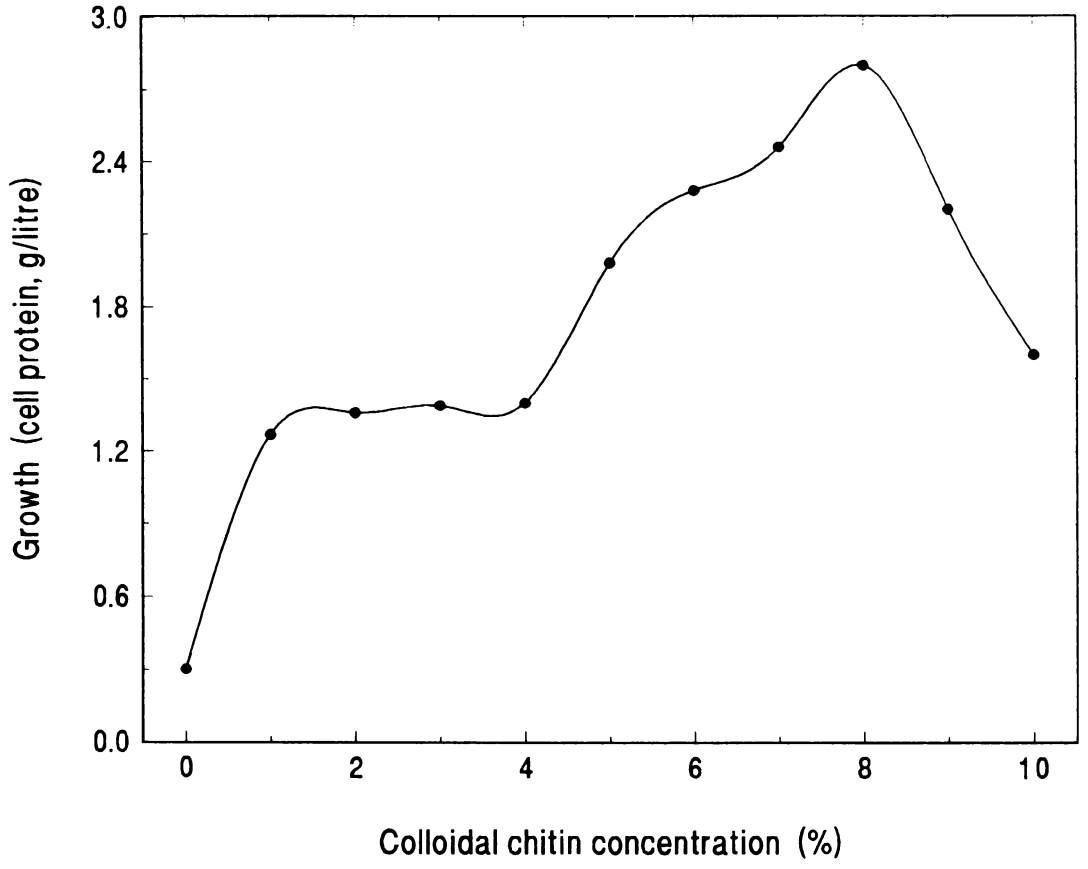



**Figure 6** Effect of colloidal chitin on growth of *B. bassiana*

**Carbon sources:**

The effect of carbon sources on growth was studied by incorporating different carbon sources in the cultivating medium and the results are presented in Figure 7 Maximal growth was registered with glucose. Among the other various carbohydrates tested, ribose and xylose inhibited growth, while lactose led to poor growth compared to the control (no carbon) Whereas all the other carbohydrates favoured significant growth of the fungus. On the other hand inorganic carbon sources, such as trisodiumcitrate led to minimal growth.

**Nitrogen sources:**

Effect of nitrogen sources on growth was determined by incorporating various nitrogen sources, including organic, inorganic and amino acids, in the cultivation medium and the results are shown in Figure 8. Of the different organic nitrogen sources tested, yeast extract supported maximal growth followed by beef extract, peptone, and liver extract. Whereas casein led to a minimal growth compared to control and malt extract inhibited the growth completely. Among the various inorganic nitrogen sources tested $NH_4Cl$ and $NaNO_3$ favoured significant growth. But $(NH_4)_2SO_4$ did not support enhanced growth. Of the different aminoacids tested glycine promoted maximal growth and all the other amino acids except tryptone, supported significant growth.

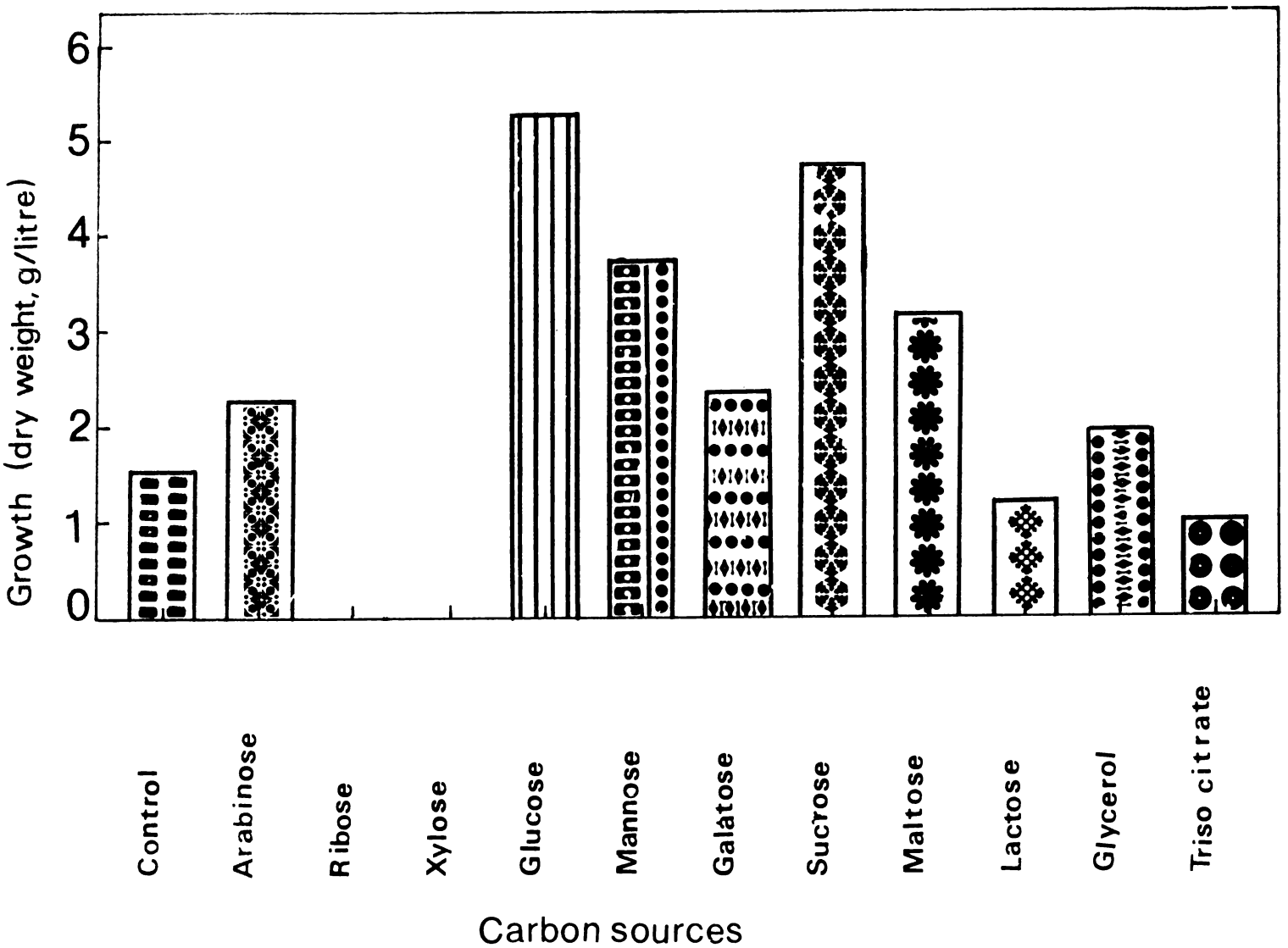



**Figure 7** Effect of carbon sources on growth of *B. bassiana*

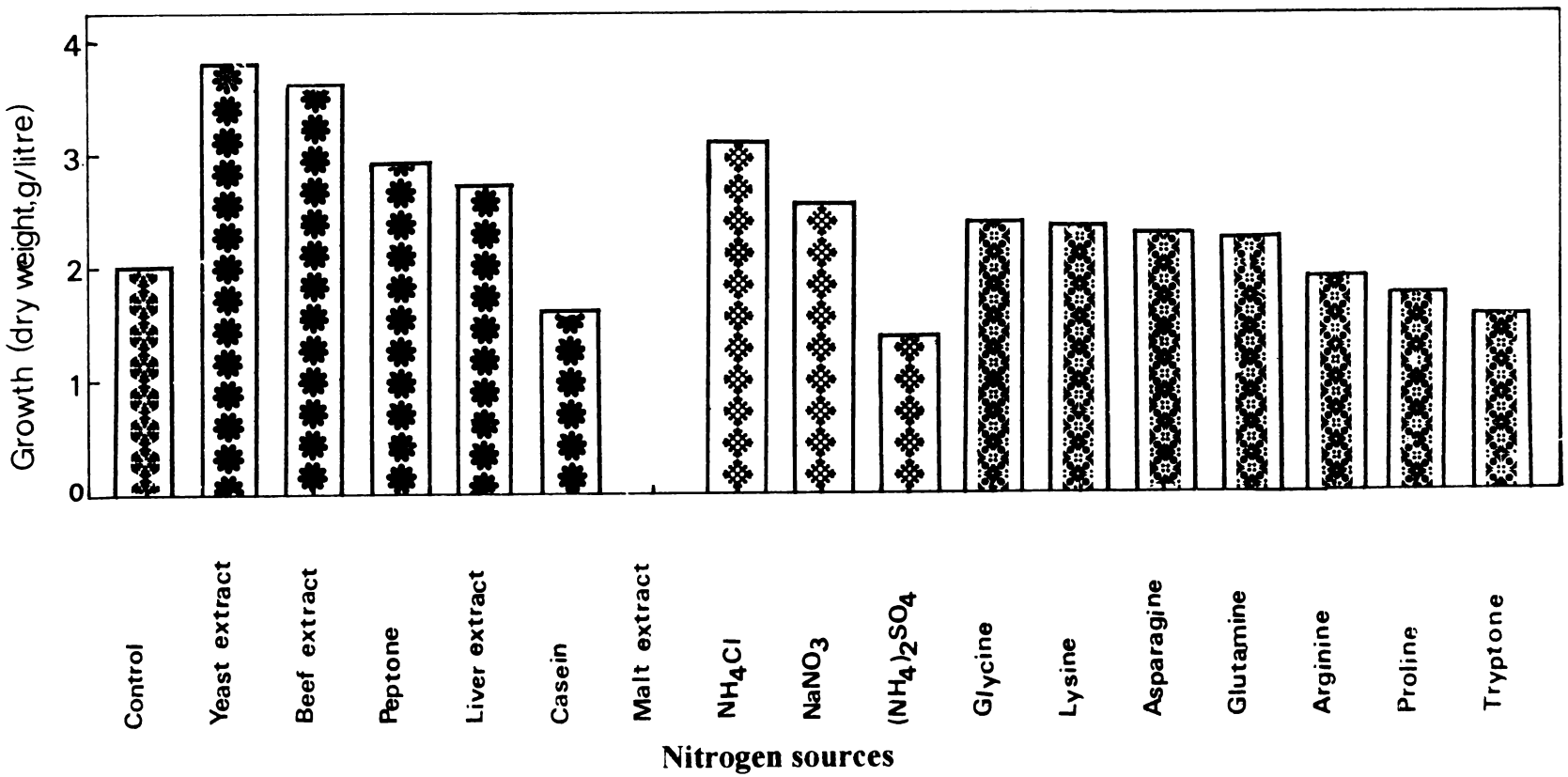



**Figure 8** Effect of nitrogen sources on growth of *B. bassiana*

**Glucose:**

Optimal level of glucose required for maximal growth was standardized, since it was observed to promote significant levels of growth. The results presented in Figure 9 indicate that significant levels of growth could be obtained with 3-18% of glucose concentration. However maximal level of growth was recorded with 12% glucose. The growth in the absence of glucose was only 6.9% of the maximal growth recorded at 12% glucose. Interestingly, even with 27% of glucose, about 26.4% of the maximal growth, could be noted.

**Effect of salinity:**

Effect of salinity of sea water on growth of the fungus was studied in both liquid medium and agar plates. The results presented in Figure 10 suggest that in liquid medium maximal growth was supported by 75% of aged sea water followed by 100%, 50%, 25%, DW + 5% NaCl and DW. It was noted that about 44% of the maximal growth could be recorded with distilled water. Addition of 5% NaCl to distilled water yielded about 59% fo the maximum growth recorded with 75% aged sea water.

Data presented in Figure 11 indicate that, the effect of salinity on growth of fungus on solid medium is the same as in the case of liquid medium (mentioned above), as the maximal growth was observed with the medium prepared with 75% aged sea water. The growth in distilled water was only 50% of

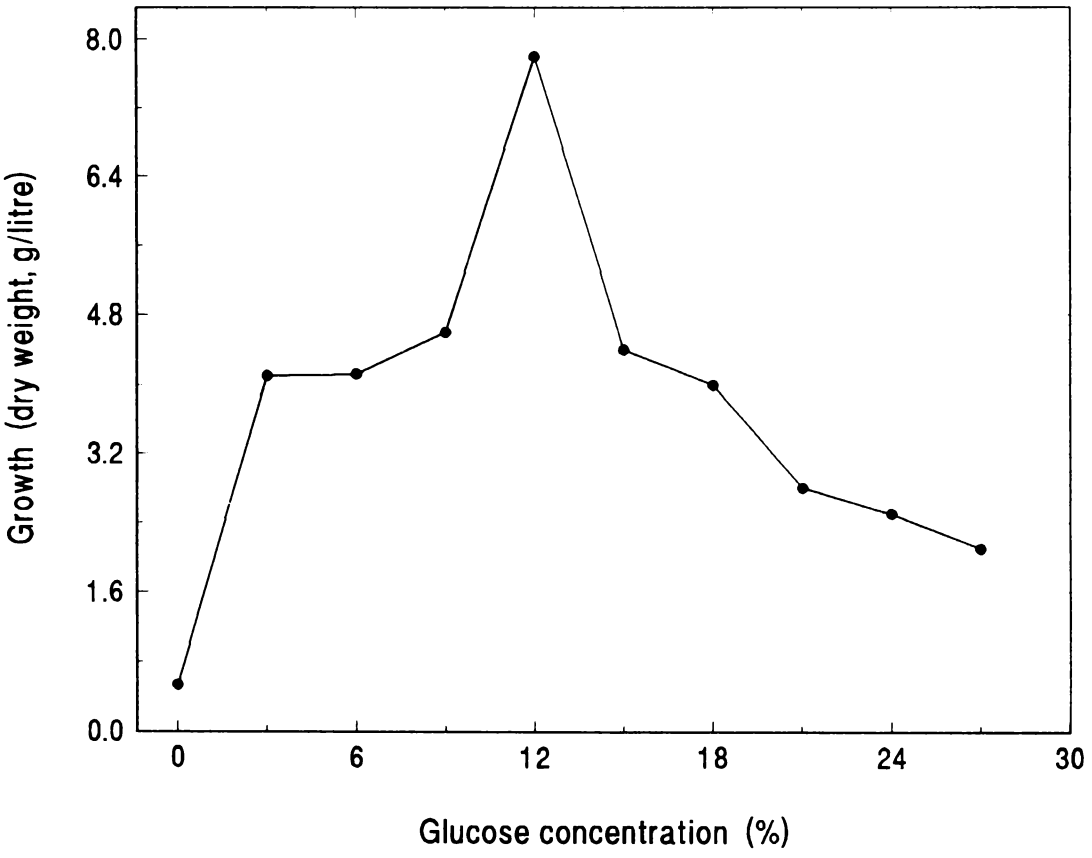



**Figure 9** Effect of glucose on growth of *B. bassiana*

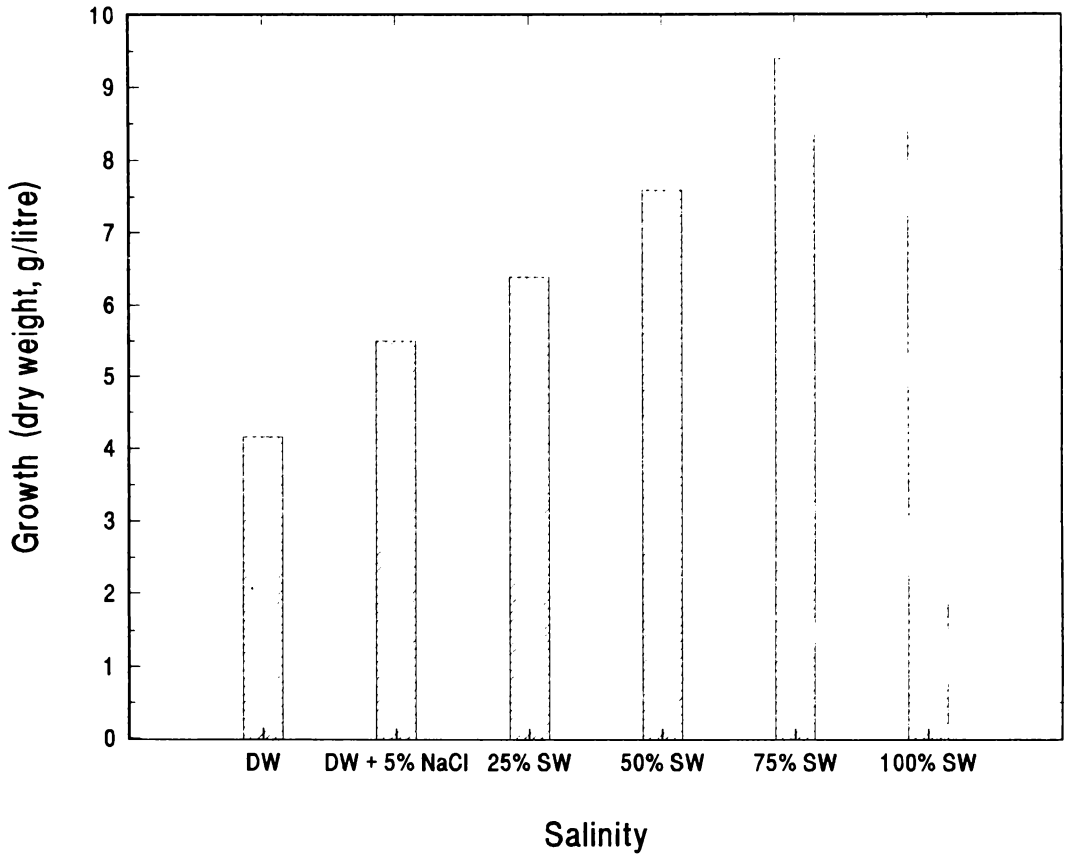



**Figure 10** Effect of salinity on growth of *B. bassiana* in liquid medium, DW - Distilled water, SW - Sea water

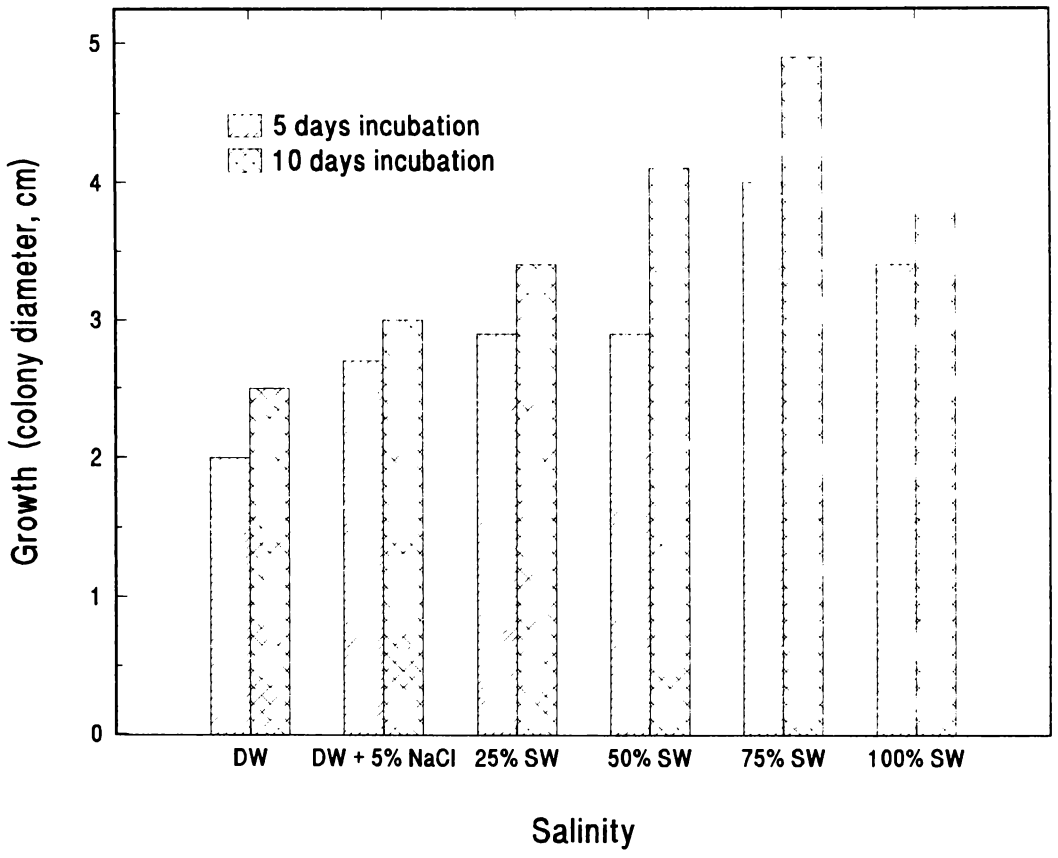



**Figure 11** Effect of salinity on growth of *B. bassiana* on solid medium
DW - Distilled water, SW - Sea water

that recorded with 75% aged sea water in agar medium, very similar to that of liquid medium.

**Incubation period**

Optimum incubation period that supports maximal growth was determined, at the optimised growth conditions of the organism. From the results presented in Figure 12 it is inferred that 60 h of incubation is necessary for attaining maximum growth. Further incubation over 60 h led to a decline in growth.

### 3.4.2 Growth curve:

Growth curve obtained for *Beauveria bassiana* is presented in Figure 12. From the graph it is clearly evident that the fungus entered into the logarithmic phase after 24 h and continued till 54 h. The generation time for the organism was recorded as 7.5 h.

## 3.5 Chitinase production by *B.bassiana* under submerged fermentation - shake flask culture

### 3.5.1 Carbon sources

It is evident from the results documented in Table 7 that presence of glucose as additional carbon source led to a maximal increase in enzyme titres 1.55 U/ml. Among the other carbon sources tested, dextrin promoted considerable levels of chitinase production (1.3 U/ml) followed by mannose and sorbitol. Whereas soluble starch, sucrose, maltose, and

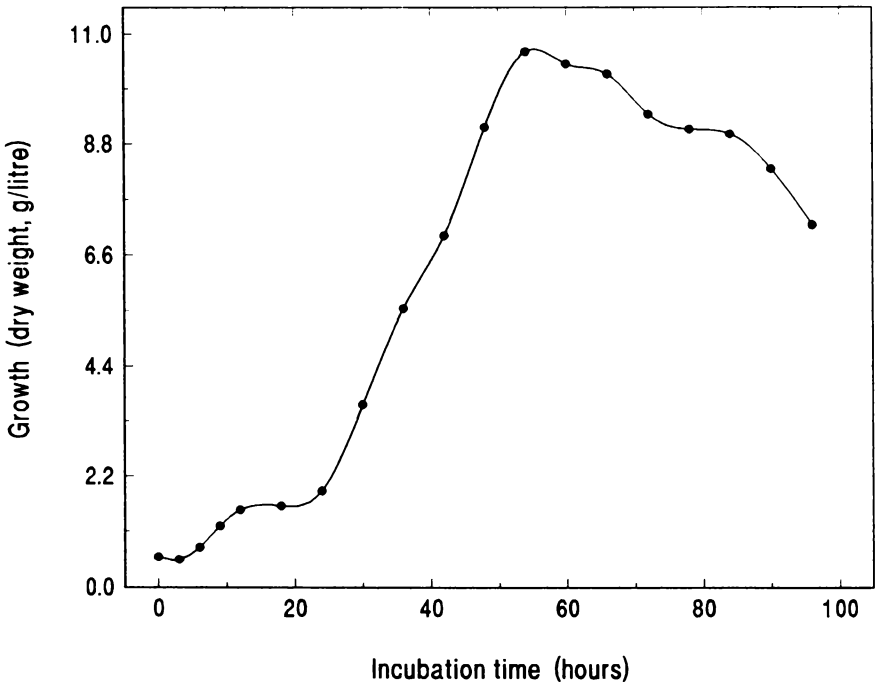



**Figure 12** Effect of incubation time on growth of *B. bassiana*

Table 7 Effect of additional carbon source on chitinase production by *B. bassiana* under SmF

| Carbon source % (w/v) | Enzyme activity (U/ml) | Reducing sugar (mg/ml) | pH of the medium after fermentation |
|---|---|---|---|
| No carbon source | 0.18 | 2.8 | 7.78 |
| colloidal chitin only | 0.62 | 2.2 | 6.64 |
| Colloidal chitin + organic carbon source (1%) | | | |
| glucose | 1.55 | 3.1 | 6.92 |
| dextrin | 1.30 | 2.8 | 6.33 |
| mannose | 0.98 | 3.2 | 6.99 |
| sorbitol | 0.86 | 2.9 | 6.89 |
| soluble starch | 0.51 | 2.2 | 6.92 |
| sucrose | 0.33 | 2.8 | 6.65 |
| maltose | 0.28 | 2.9 | 6.86 |
| mannitol | 0.21 | 2.7 | 7.69 |
| Colloidal chitin + inorganic carbon source (1%) | | | |
| trisodiumcitrate | 0.0 | 2.5 | 6.59 |

mannitol led to a reduction in enzyme yield. The inorganic carbon source, trisodiumcitrate, completely inhibited the enzyme synthesis.

Soluble protein content in the culture filtrate showed variation from 2.18 mg/ml to 3.18 mg/ml for the different carbon sources tested and irrespective of the enzyme yield.

### 3.5.2 Chitinous substrate

All chitinous substrates, excluding glucosamine, promoted similar levels of chitinase production, although a maximum was noticed with prawn waste (0.632 U/ml) followed by colloidal chitin (0.598 U/ml) and purified chitin powder (0.556 U/ml), chitosan (0.522 U/ml) (Table 8). Glucosamine could support only 0.224 U/ml of enzyme yield while without chitinous substrate the yield was 0.403 U/ml chitinase. Soluble protein content in the culture filtrate was maximum in the case of glucosamine, (12.11 mg/ml) followed by native chitin, colloidal chitin and chitin powder. Similarly reducing sugar content in the culture filtrate was maximum in the case of glucosamine (10.77 mg/ml), followed by native chitin. Whereas, colloidal chitin, chitosan and chitin powder recorded very low level of reducing sugar.

### 3.5.3 Prawn waste

Since prawn waste was found to promote maximal enzyme production, the optimal concentration of this substrate required for maximal levels of chitinase production was determined.

From the results presented in Table 9 it is seen that after 10 days of incubation chitinase yield was maximum with 10% followed by 5%, 3% and 1% substrate concentration. Infact the variation in total yield at concentrations 3-10% is insignificant compared to the difference observed between 1% and other concentrations. It was also observed that after 5 days of incubation the chitinase production was very low at 3-10% compared to the significant levels of enzyme yield observed with 1%. There was a 36% increase in enzyme titres with 10% prawn waste compared to that of 1% prawn waste. The increase in enzyme yield production was 20%, 79% and 99% levels respectively with 1%, 3% and 5% prawn waste.

The soluble protein content in the culture filtrate was found to increase linearly along with the increase in the enzyme titres and the concentration of prawn waste from 7.795 mg/ml to 17.3 mg/ml after 5 days and from 9.8 mg/ml to 29.1 mg/ml after 10 days of fermentation. There was a 40.6% increase in the soluble protein with 10% prawn waste after 10 days compared to that observed with 5 days of fermentation. However, the reducing sugar content in the

Table 8 Chitinase production using various chitinous substrate by *B.bassiana* under SmF

| Chitinous substrate 1% (w/v) | Enzyme activity (U/ml) | Soluble protein (mg/ml) | Reducing sugar (mg/ml) | pH of the medium after fermentation |
|---|---|---|---|---|
| Colloidal chittin | 0.598 | 6.086 | 0.691 | 7.03 |
| Purified chitin powder | 0.565 | 4.39 | 0.698 | 8.22 |
| Native chitin (Prawn shell powder) | 0.632 | 9.05 | 1.032 | 7.45 |
| Glucosamine | 0.224 | 12.11 | 10.72 | 6.01 |
| Chitosan | 0.522 | 5.12 | 0.836 | 7.2 |
| No chitinous substrate | 0.403 | 6.22 | 0.378 | 7.3 |

Table 9 Effect of various concentration of prawn waste on chitinase production by *B. bassiana* under SmF

| Concentration of prown waste %( w/v) | Enzyme activity (U/ml) Incubation time (days) | | Soluble protein (mg/ml) incubation time (days) | | Reducing sugar (mg/ml) Incubation time (days) | |
|---|---|---|---|---|---|---|
| | 5 | 10 | 5 | 10 | 5 | 10 |
| 1 | 0.735 | 0.925 | 7.81 | 9.73 | 0.38 | 0.53 |
| 3 | 0.290 | 1.38 | 11.32 | 14.66 | 0.66 | 0.33 |
| 5 | 0.015 | 1.445 | 14.1 | 18.06 | 0.57 | 0.81 |
| 10 | 0.004 | 1.455 | 17.3 | 29.10 | 0.75 | 0.03 |

culture filtrate varied between 0.38 mg/ml and 0.75 mg/ml after 5 days and from 0.265 mg/ml to 0.806 mg/ml after 10 days, irrespective of the enzyme yield and the concentration of prawn waste. Maximal concentration of reducing sugar was observed with 5% prawn waste followed by 1%, 3% and 10%, after 10 days of fermentation.

3.5.4. The physico-chemical parameters that influence chitinase production by *B. bassiana* during SmF under shake flask culture were optimised and the results obtained for the various parameters are detailed below.

**Incubation temperature**

From the results presented in Figure 13 it is inferred that incubation at 27°C promoted maximal chitinase production (0.33 U/ml). Nevertheless *B. bassiana* could record considerable levels of chitinase production at 22°C and 32°C. However incubation at temperatures above 32°C showed a decline in enzyme production (0.076 and 0.04 U/ml at 37°C and 42°C respectively).

Data for soluble protein content in the culture filtrate varied irrespective of the chitinase yield. Maximum level of soluble protein was recorded at 42°C compared to other temperatures. It was also noted that the pH of the culture broth, after fermentation, declined sharply from the

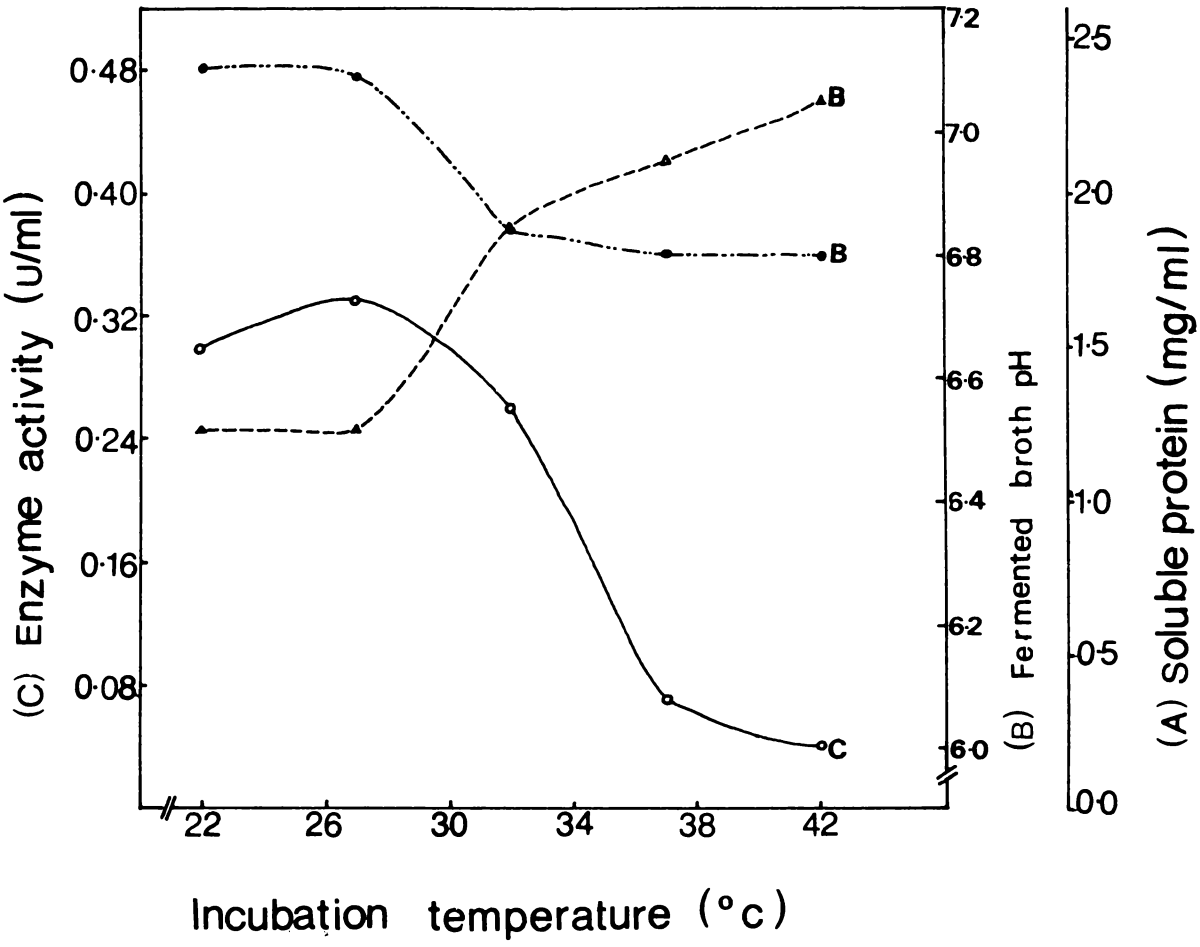



**Figure 13** Effect of incubation temperature on chitinase production by *B. bassiana* under SmF

initial pH 8.0 to 6.8 at temperatures 32-42°C and to 7.1 at 22 and 27°C.

pH

Data presented in Figure 14 suggest that *B. bassiana* could produce considerable levels of chitinase over a wide range of pH (6-11). Two peaks of optima, one at pH 6.0 and another at pH 9 were noted. In general the enzyme production was significant at pH 8 - 10 (0.42 - 0.50 U/ml). However the enzyme production declined sharply at pH levels less than pH 5.0 and above pH 11.0.

The soluble protein content in the culture filtrate, recorded a linear increase along with increase in the pH of the medium (1.8 mg/ml at pH 4 to 3.6 mg/ml at pH 12.0), irrespective of the enzyme yield.

pH of the medium was observed to undergo marked change during fermentation. The final pH of the fermented broth recorded significant raise to pH 6.42-6.57 for the initial pH 4-6.0. On the other hand for alkaline pH levels 9-12, there was significant change and the tendency was to decline down and prevail around pH 8.64-9.96.

**Substrate concentration (colloidal chitin)**

Optimal concentration of colloidal chitin required for chitinase production by *B.bassiana* was studied by

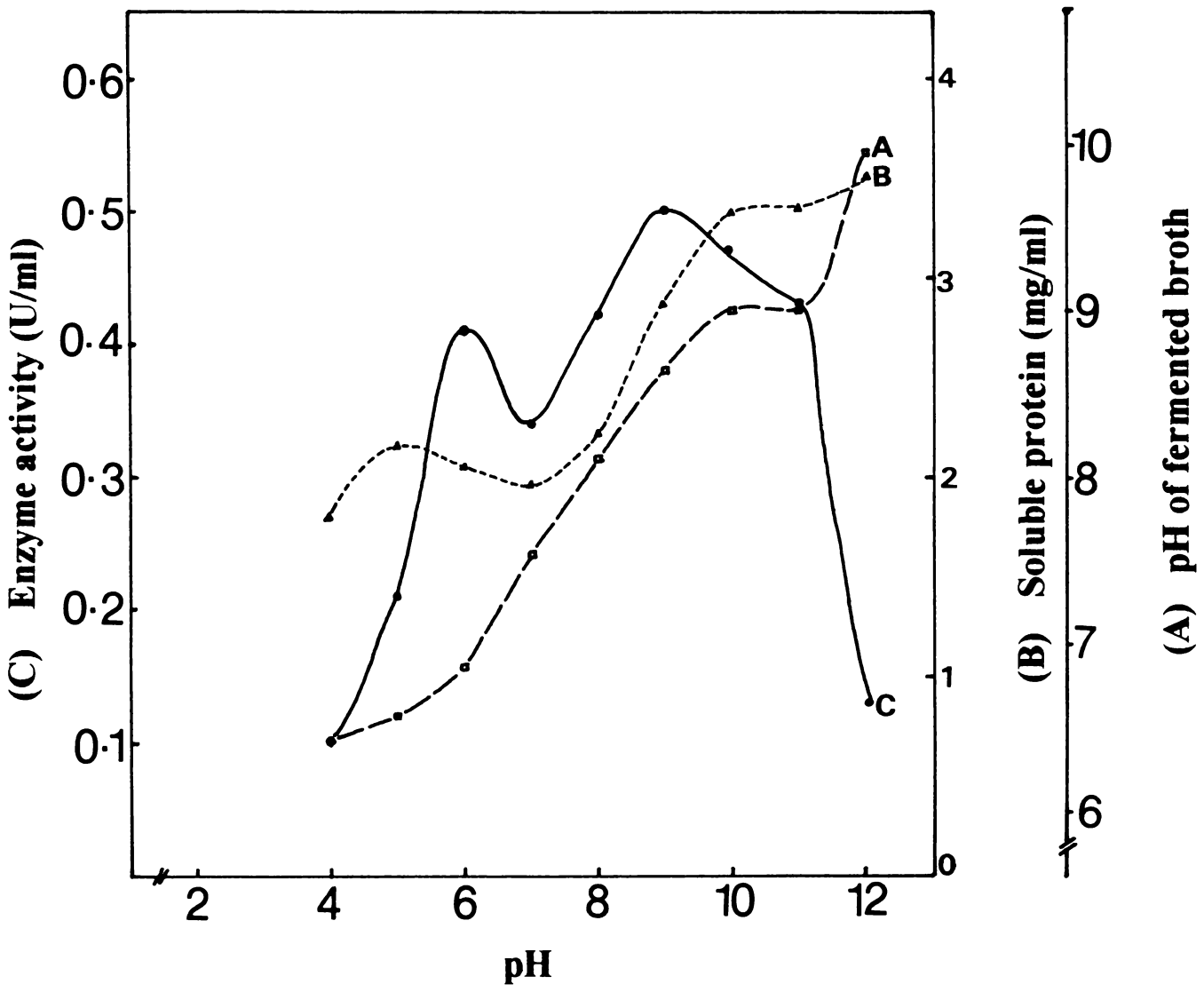



**Figure 14** Effect of pH on chitinase production by *B. bassiana* under SmF

incorporating colloidal chitin at different concentration in enzyme production medium and the results obtained are presented in Figure 15. Significant levels of enzyme yield could be obtained with the substrate concentration ranging from 1-10%, although maximum enzyme yield was recorded at 8% colloidal chitin (0.82 U/ml). This fungus could also yield chitinase in the absence of colloidal chitin, 20.7% of maximum yield that obtained with 8% colloidal chitin Soluble protein content in the culture filtrate showed a linear increase along with increase in the concentration of colloidal chitin, with the maximum amount of protein at 8%.

pH of the fermented broth, declined from the initial pH 9.0 to a minimum of pH 7.18 (0 % colloidal chitin) In general the pH of the fermented medium varied from pH 7.18 to 7.66 for the various concentration of colloidal chitin.

**Sodium chloride**

Data presented in Figure 16 indicate that maximal levels of chitinase was supported by the presence of 9% NaCl in the enzyme production medium (1.3 U/ml) Nevertheless significant levels of chitinase production was observed over a wide range of NaCl (1-11%) concentration in the medium. Absence of sodium chloride could also promote chitinase synthesis, but it was only 3.5% of the maximum observed with 9% NaCl. However addition of 1% NaCl led to a 63.8% of the enzyme yield recorded at 9% NaCl concentration.

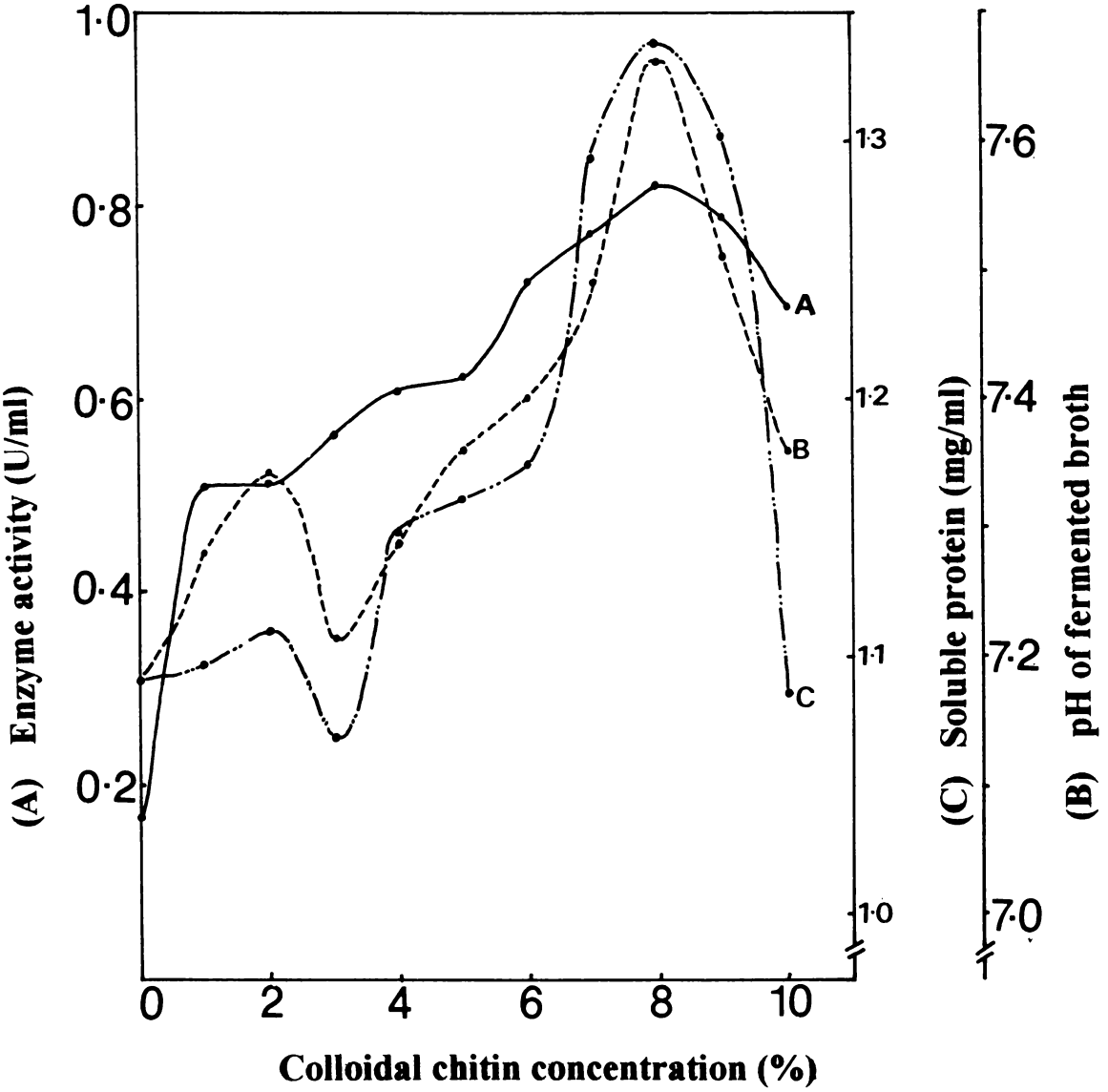



**Figure 15** Effect of colloidal chitin on chitinase production by *B. bassiana* under SmF

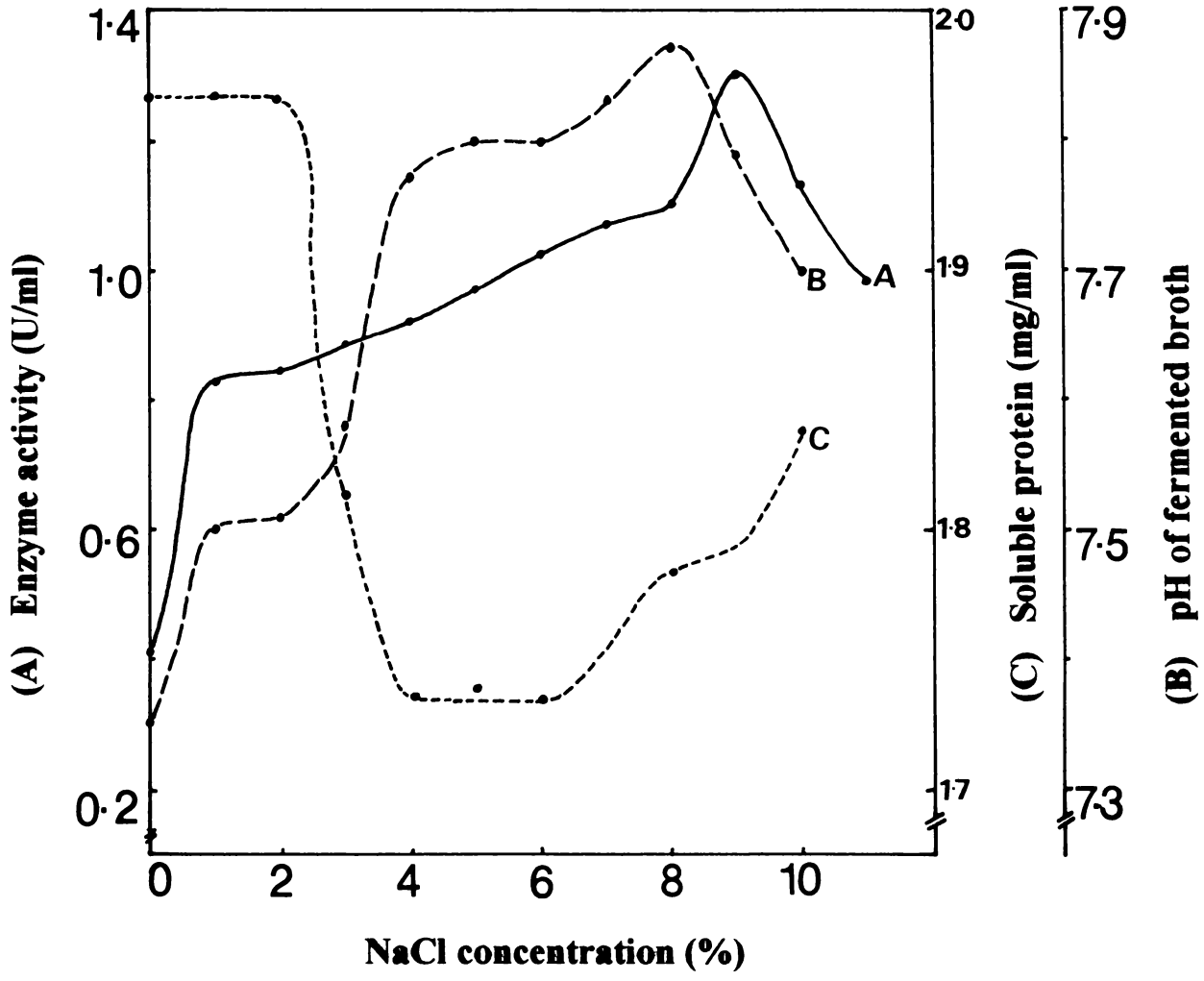



**Figure 16** Effect of sodium chloride on chitinase production by *B. bassiana* under SmF

Addition of NaCl upto 2% did not influence the level of soluble protein compared to the absence of NaCl. Further, the soluble protein content in the culture filtrate gradually decreased along with the increase in NaCl concentration from 3% to 11%. pH of the culture broth after fermentation, recorded a rapid decline from pH 9.0 to 7.35-7.87 Relatively reduction in the level of pH was higher at 0-3% NaCl concentration than at 4-10% NaCl concentration.

**Additional nitrogen sources**

Incorporation of various nitrogen sources in the enzyme production medium resulted in enhanced chitinase production at considerable levels (Table 10).

Among the various organic nitrogen sources tested, yeast extract promoted maximal chitinase production (2.97 U/ml) followed by tryptone (1.58 U/ml) peptone (1.45 U/ml) and beef extract (1.15 U/ml). The combinations of these sources, either peptone and yeast extract or peptone + beef extract + yeast extract, did not enhance enzyme production and instead led to a sharp decline in the enzyme yield compared to that recorded with yeast extract as additional nitrogen source.

Among the various inorganic nitrogen sources tested $NaNO_3$ promoted enhanced production of enzyme (1.3 U/ml) followed by $NH_4NO_3$ (1.27 U/ml). $(NH_4)_2SO_4$ (0.9 U/ml) and $NH_4Cl$ (0.73 U/ml) A combination of yeast extract +

Table 10 Effect of additional nitrogen source on chitinase production by *B.bassiana* under SmF

| Nitrogen source 1% (w/v) | Enzyme activity ((U/ml)) | Soluble protein (mg/ml) | pH of the medium after fermentation |
|---|---|---|---|
| Control | | | |
| No nitrogen source | 0.4 | 5.44 | 8.4 |
| Organic | | | |
| yeast extract | 2.97 | 11.1 | 8.1 |
| tryptone | 1.58 | 6.46 | 8.2 |
| peptone | 1.45 | 5.84 | 8.4 |
| beef extract | 1.17 | 6.46 | 8.1 |
| yeast extract + peptone | 2.96 | 11.39 | 8.4 |
| yeast extract + peptone+ beef extract | 1.14 | 12.53 | 8.1 |
| Inorganic | | | |
| $NaNO_3$ | 1.3 | 0.050 | 7.8 |
| $NH_4NO_3$ | 1.27 | 0.058 | 7.4 |
| $(NH_4)_2SO_4$ | 0.9 | 0.030 | 7.9 |
| $NH_4Cl$ | 0.73 | 0.030 | 7.81 |
| Organic + Inorganic | | | |
| yeast extract + peptone+ $(NH_4)_2SO_4$ | 1.8 | 14.17 | 8.2 |

$(NH_4)_2SO_4$, and yeast extract + peptone + $(NH_4)_2SO_4$, led to decreased enzyme production. In the absence of nitrogen sources the enzyme production was greatly reduced (6%) compared to that of yeast extract.

The soluble protein content in the culture filtrate varied for the different nitrogen sources tested The protein content was more in the case of organic nitrogen compared to that with inorganic sources (Table 10).

**Yeast extract concentration**

Since the yeast extract enhanced considerable levels of chitinase production, among the various nitrogen sources tested the optimum requirement of yeast extract for maximal level of chitinase production was determined. From the results, presented in Figure 17 it was inferred that maximal levels of enzyme production (13 U/ml) was recorded at 9% yeast extract concentration, despite the fact that significant levels of enzyme production could be observed with a wide range of yeast extract concentration (3-13%). However the enzyme synthesis sharply declined at concentrations above 9%.

**Inoculum concentration.**

Results presented in Figure 18 indicate that the *B. bassiana* could produce considerable levels of enzyme at all levels of inoculum tested although maximal enzyme yield (12.66 U/ml) was recorded with 124 $\mu$g dry weight equivalent of

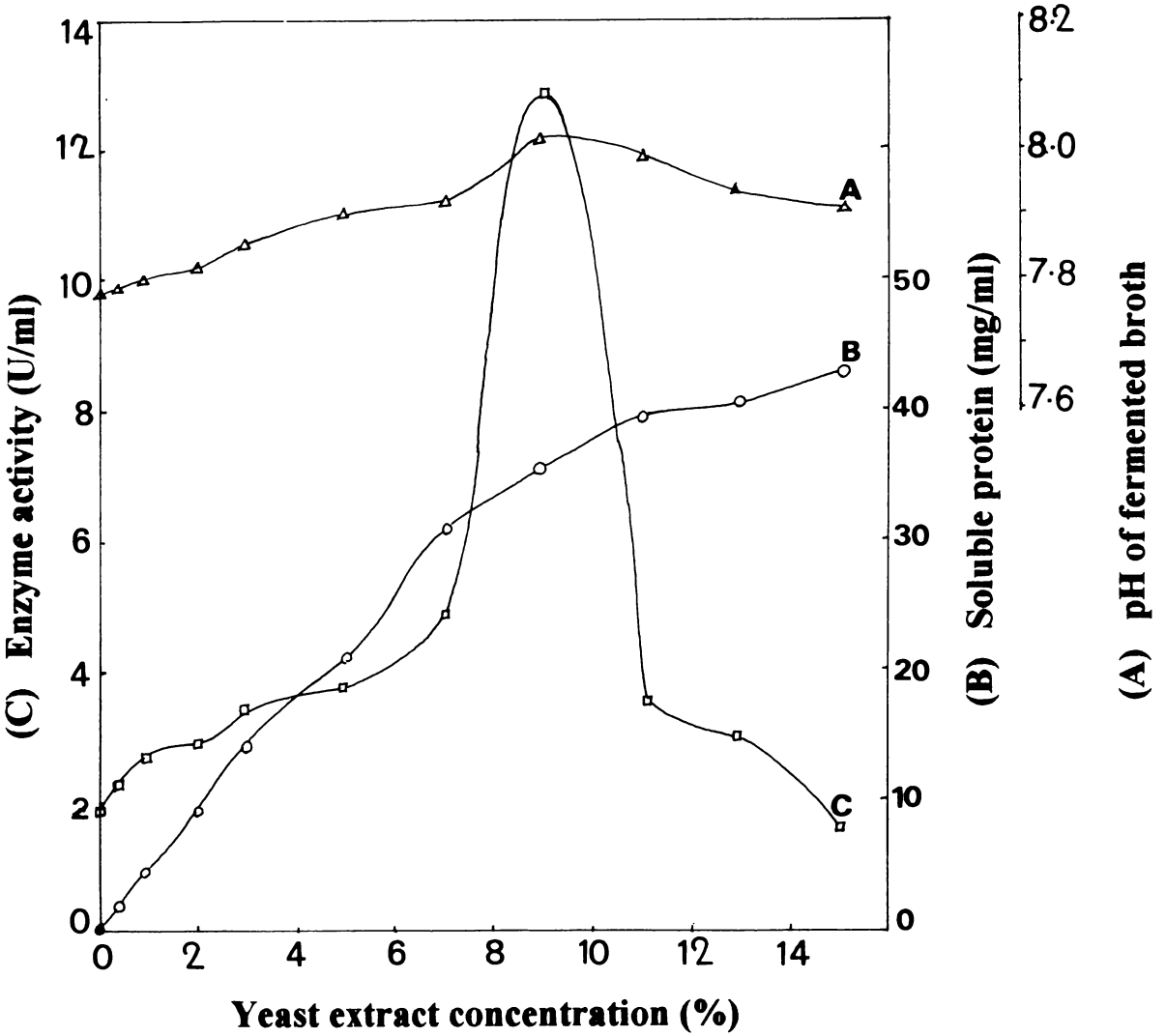



**Figure 17** Effect of yeast extract concentration on chitinase production by *B. bassiana* under SmF

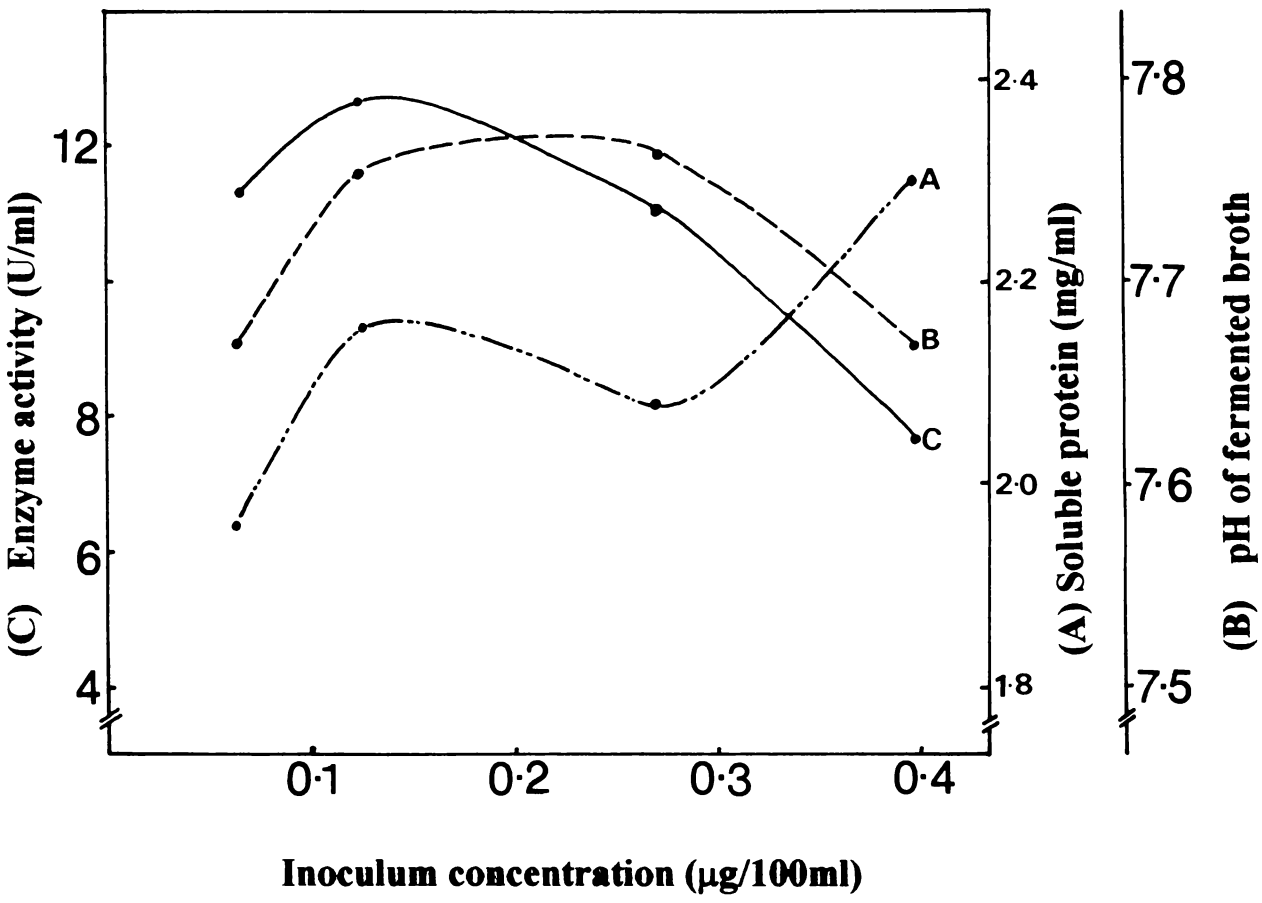



**Figure 18** Effect of inoculum concentration on chitinase production by *B. bassiana* under SmF

inoculum per 100 ml of medium. It was observed that, concentrations of inoculum above and below the optimum level led to a decline in the enzyme production. Soluble protein content in the culture filtrate varied between 1.818 mg/ml (124 μg inoculum) to 2.328 mg/ml (267 μg inoculum) irrespective of the enzyme titres.

**Incubation time**

Optimum incubation period, required for maximal chitinase production by *B. bassiana*, at optimal conditions, was recorded at 120 h (5 days) (13.34 U/ml) (Figure 19) Nevertheless, significant levels of enzyme titres could be recorded from 24 h of incubation (0.78 U/ml) on wards although incubation beyond 120 h. led to a decline in enzyme titres.

Soluble protein content in the culture filtrate varied between 28.228 mg/ml (12 h) and 32.108 mg/ml (120 h) irrespective of the incubation period. However, the concentration of reducing sugars in the culture filtrate generally increased from 12 h of incubation upto 96 h and fluctuated later irrespective of the incubation period. pH of the fermented broth was observed to decline sharply, from the initial level of pH 9.0, to a level of pH 8.2 at 96 h, in a gradual fashion. Interestingly, at later hours of incubation the pH raised slowly towards the initial pH 9.0. Altogether, a trough in the pH profile was recorded in the fermented broth during the course of incubation.

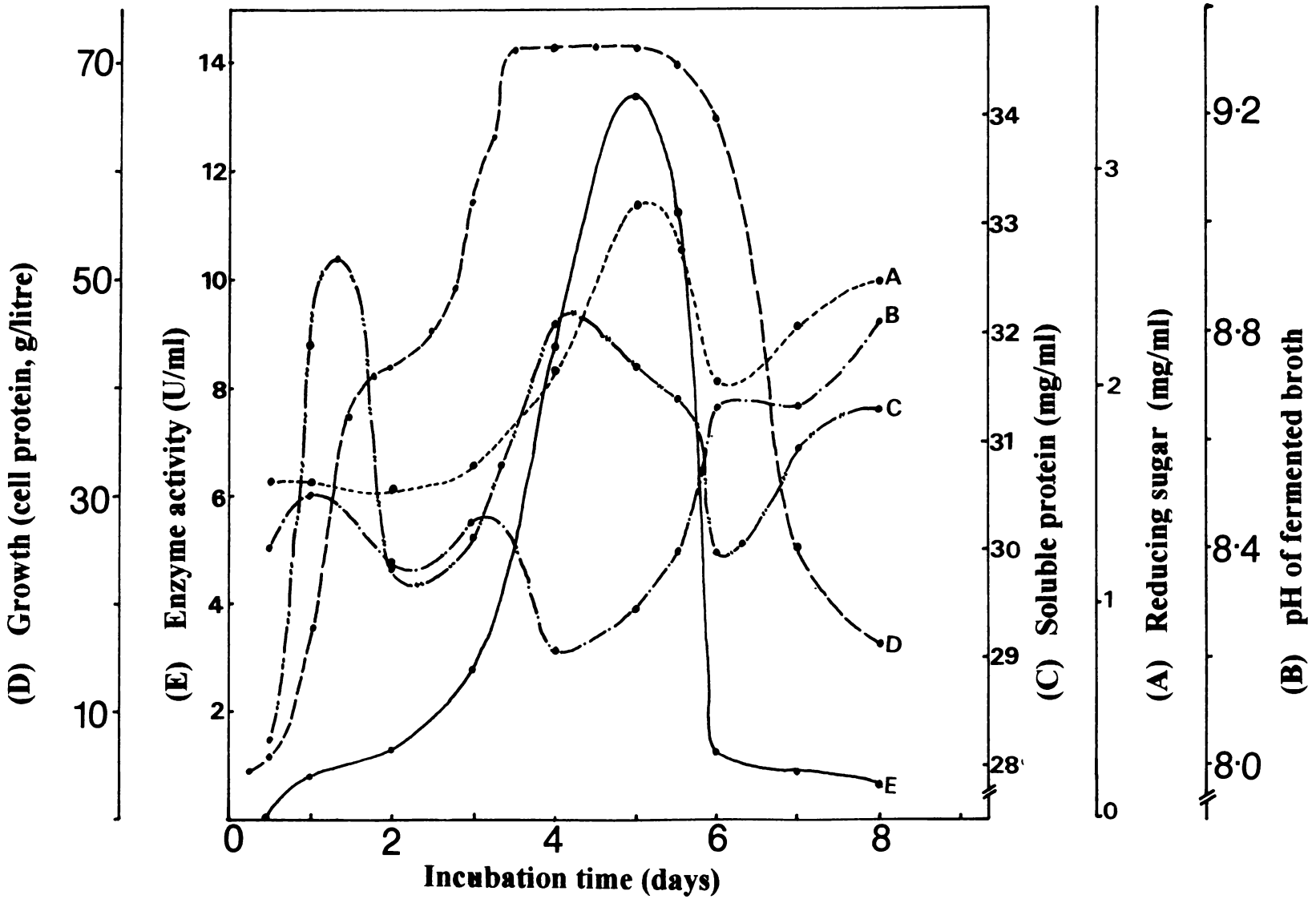



**Figure 19** Effect of incubation time on chitinase production by *B. bassiana* under SmF

**Effect of $MgSO_4$ concentration**

Results presented in Figure 20 indicate that 0.25% $MgSO_4$ is optimum for the maximal enzyme production (13.92 U/ml) inspite of recording significant levels of chitinase production over a range of 0-0.5% $MgSO_4$. It was noted that presence of $MgSO_4$ (0.25%) in the enzyme production medium contributed to a double fold increase in enzyme synthesis when compared to that in the absence of $MgSO_4$ (7.01 U/ml).

Soluble protein in the culture filtrate varied between 26.04 mg/ml and 28.992 mg/ml for the different concentration of $MgSO_4$ tested. pH of the culture broth remained more or less same, after fermentation, for the concentration of $MgSO_4$ varying from 0.05-1.0%.

**Effect of phosphate**

From the results presented in Table 11 it is evident that, except with 1 2% ratio of $K_2HPO_4$ and $KH_2PO_4$, at all other phosphate levels, there was considerable increase in the enzyme titres. However, maximal enzyme production was recorded with 2 0.5% ratio of $K_2HPO_4$ and $KH_2PO_4$ (35.85 U/ml) (78% of that recorded without phosphate) followed by 3 2% ratio (22.8 U/ml) and 0.5 0.5% ratio of $K_2HPO_4$ concentration (20.2 U/ml). Both soluble protein content and pH in the fermented broth recorded significant variation with respect to the various levels of phosphate concentration and enzyme yield. Thus soluble protein varied from 23.794 mg/ml to

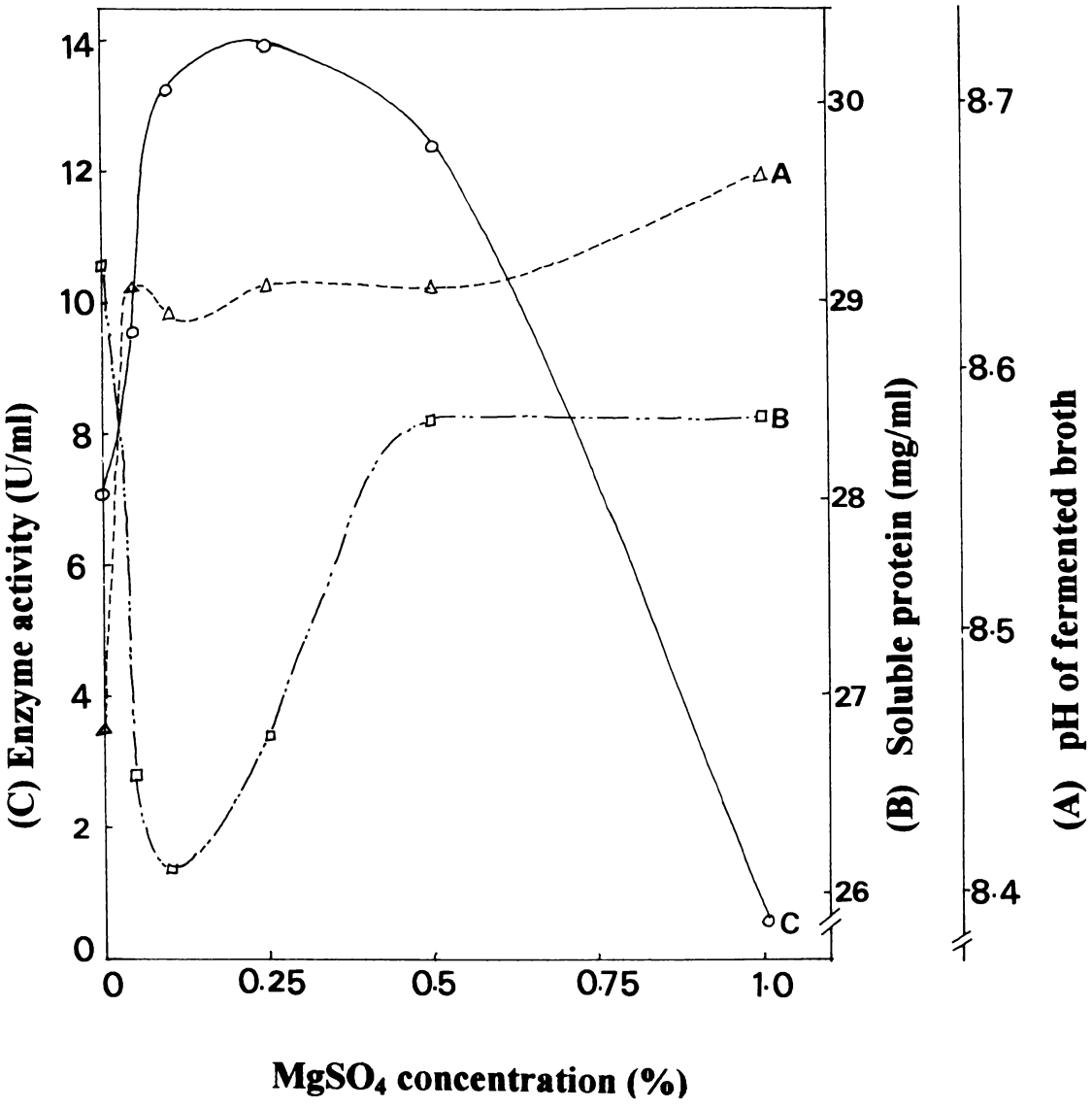



**Figure 20** Effect of $MgSO_4$ on chitinase production by *B. bassiana* under SmF

Table 11 Effect of phosphate on chitinase production by *B. bassiana* under SmF

| Phosphate concentration (mg/25 ml) | | Enzyme activity (U/ml) | Reducing sugar (mg/ml) | pH of the medium after fermentation |
|---|---|---|---|---|
| No phosphate | | 7.6 | 1.59 | 8.49 |
| $K_2HPO_4$ | | | | |
| 125 mg | | 12.5 | 1.2 | 8.45 |
| $KH_2PO_4$ | | | | |
| 125 mg | | 12.98 | 1.5 | 8.59 |
| $K_2HPO_4$ | $KH_2PO_4$ | | | |
| 125 | 125 | 20.2 | 1.30 | 8.05 |
| 250 | 250 | 15.6 | 0.81 | 8.62 |
| 250 | 500 | 5.0 | 1.2 | 8.65 |
| 500 | 125 | 35.9 | 1.2 | 8.68 |
| 750 | 500 | 22.8 | 1.1 | 8.7 |
| 500 | 750 | 11.5 | 1.3 | 8.60 |

30.184 mg/ml and final pH varied from 8.05 to 8.7 for the different level of phosphate.

### 3.6 Chitinase production under submerged fermentation - Static culture

The chitinase production by *B. bassiana* under static submerged condition recorded a linear increase along with increase in incubation period till 216 h (9 days), when maximum (6.64 U/ml) was recorded (Figure 21). Enzyme yield rapidly declined on further incubation. The rate of enzyme synthesis was very slow during the initial period of 72 h, and rapid secretion of enzyme was observed only after 5 days. Reducing sugar in the fermented broth did not show any significant increase over the period 48-168 h. (2-7 days). Whereas, during the later period (8-9 days), maximal levels of reducing sugars 2.694 mg/ml (9th day) was recorded, coinciding with maximal enzyme yield.

Results obtained for growth suggest that the fungus had spent more time under logarithmic period (48 h). Significant growth was observed at 96 h (4 days) and the fungus had remained under stationary phase for a day or two and entered declining phase. Results suggest that chitinase production is not associated with the logarithmic phase of fungus, but with the stationary phase, under static culture conditions.

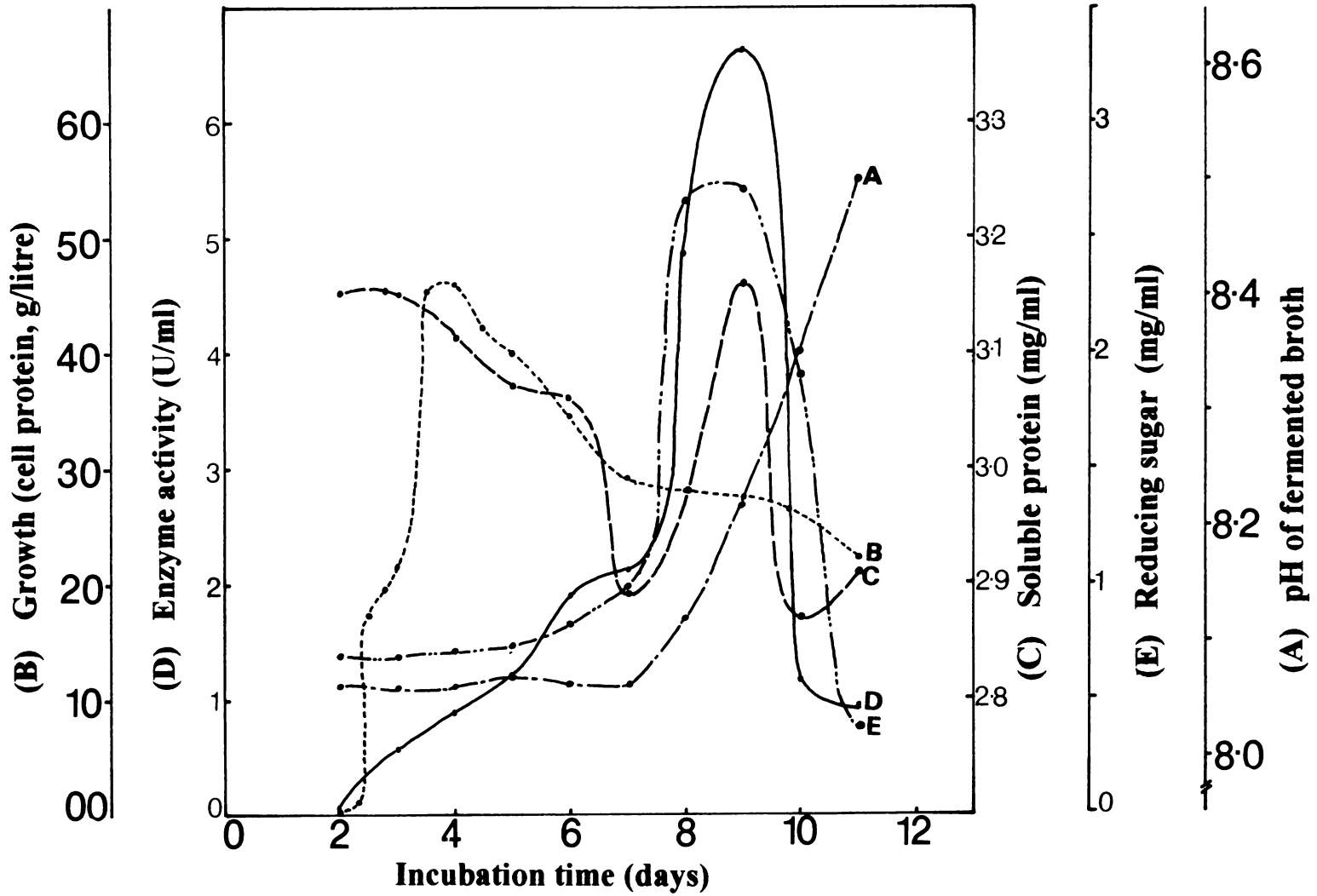



**Figure 21** Chitinase production by *B.bassiana* under submerged fermentation - Static culture

### 3.7 Effect of addition of N-acetyl D-glucosamine

Results presented in Figure 22 indicate that the chitinase synthesis of *B. bassiana* was catabolically repressed by N-acetyl-D-glucosamine The enzyme synthesis was sharply declined in the presence of N-acetyl-D-glucosamine at 1% levels (1.36 U/ml) compared to the control (35.41 U/ml) and completely repressed with 4% of N-acetyl-D-glucosamine.

### 3.8 Chitinase production by Bubble Column Bioreactor (BCB)

Data depicted in Figure 23, obtained for the studies on chitinase production under submerged fermentation in a bubble column reactor, indicate that significant levels of enzyme titres (22.5 U/ml) could be produced after incubation for 9 days Although the rate of enzyme synthesis was found to increase gradually during the initial period of 6 days, it increased significantly after 7 days and reached a maximum on the 9th day of fermentation. However, incubation beyond 9 days led to a rapid decline in the rate of enzyme production.

Soluble protein content in the culture filtrate showed variation, from 31.628 mg/ml (1st day ) to 37.062 mg/ml (2nd day) with the increase in the rate of enzyme synthesis and fermentation period. The maximal concentration of soluble protein was recorded at 11 days of fermentation, and further incubation above 11 days led to decline in the soluble protein.

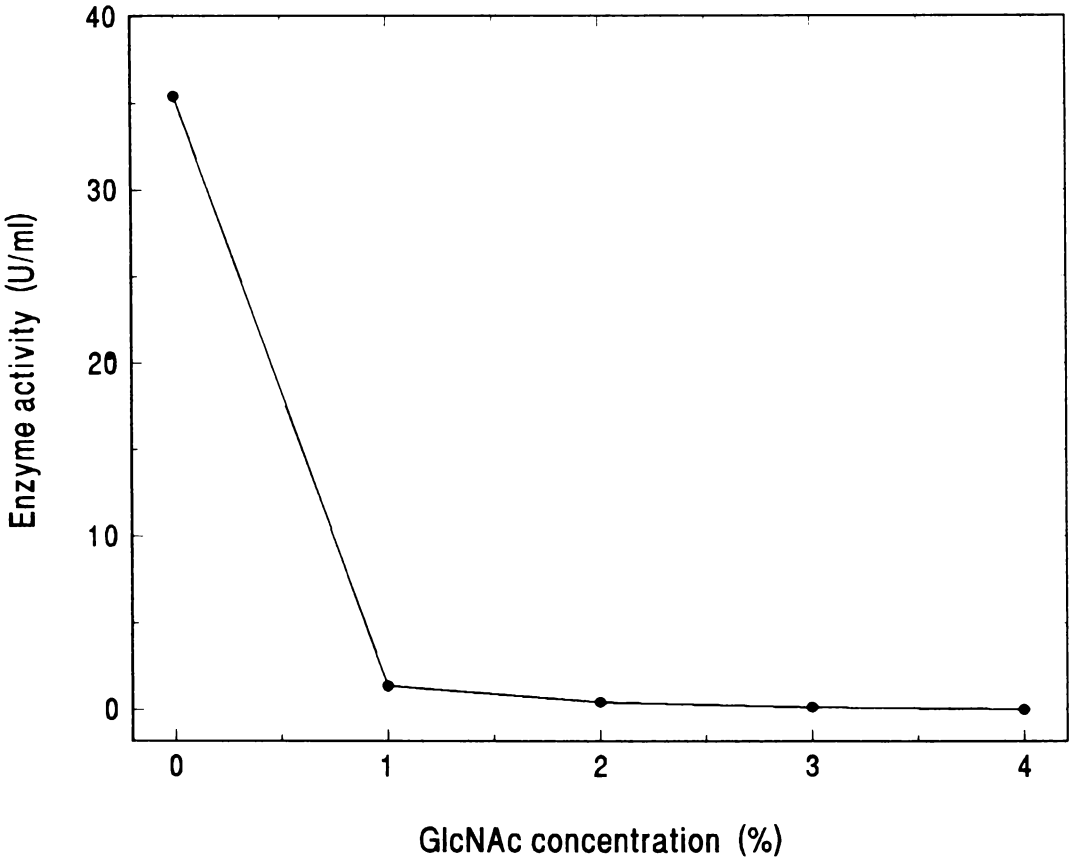



**Figure 22** Effect of addition of N-acetyl-D-glucosamine on chitinase production by *B. Bassiana* under SmF

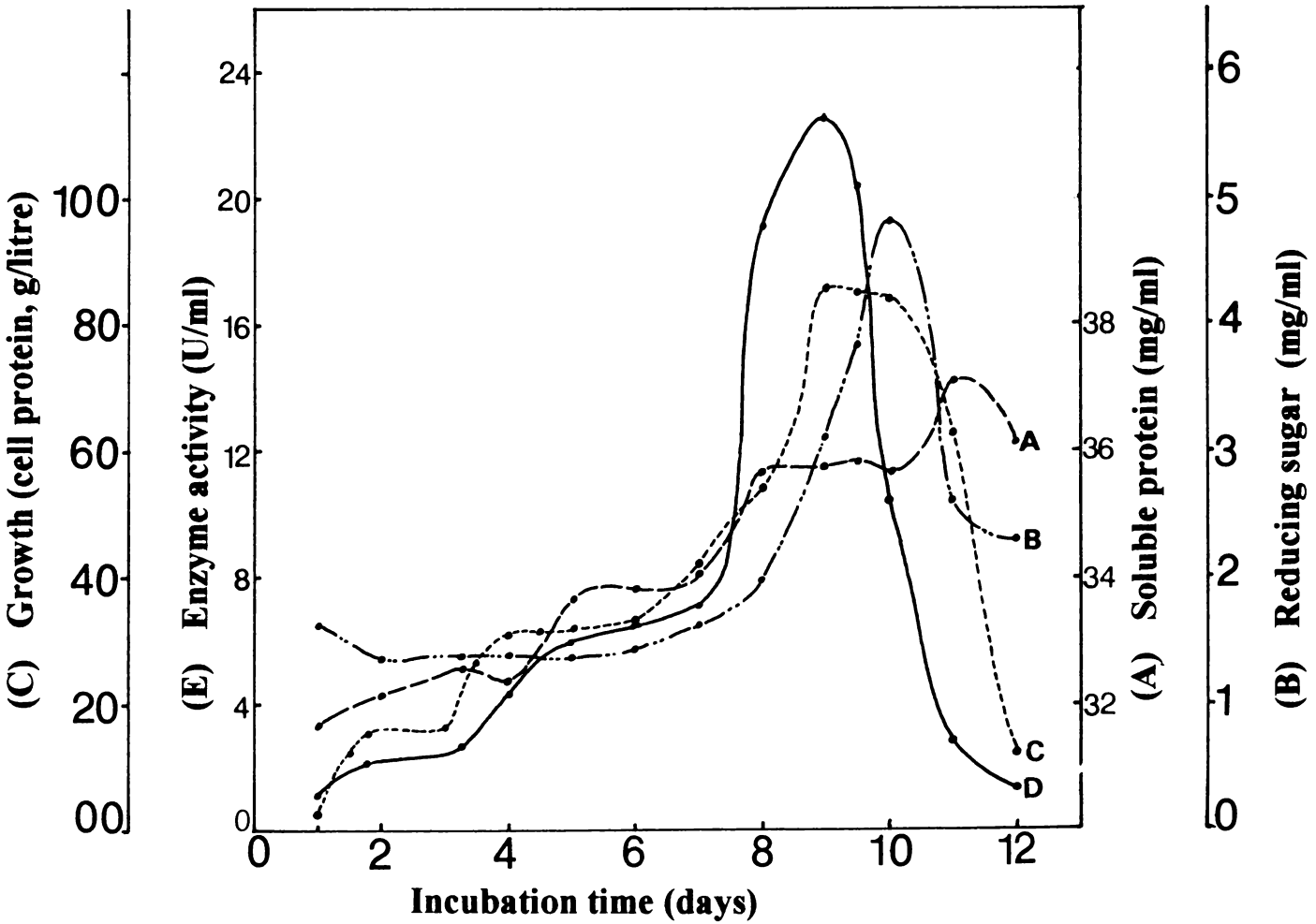



**Figure 23** Chitinase production by *B.bassiana* in a bubble column bioreactor

Reducing sugar concentration in the culture filtrate did not show any significant variation for 7 days, irrespective of the increase in the incubation period. However maximal reducing sugar concentration was observed after 10 days of fermentation.

Maximal growth was recorded after 9 days along with maximal enzyme titres, although significant growth of fungus was observed from 2 days of incubation onwards. It was also noted that rapid growth of fungus, in bubble column reactor, occurred after 7 days and was marked with rapid enzyme synthesis.

### 3.9 Comparative evaluation of chitinase production by *B.bassiana* under different submerged fermentation conditions

Chitinase production by *B. bassiana* under shake flask culture, static culture and bubble column reactor conditions of submerged fermentation was evaluated on a comparative basis. Results presented in Figure 24a, 24b, 24c very clearly indicates that the culture conditions influence the rate of chitinase synthesis. Thus it is seen that while the optimal incubation period for maximal enzyme production was 4-5 days under shake flask culture, (13.34 U/ml after 5 days) it was 8-9 days under static culture conditions and in bubble column reactor (6.4 U/ml after 9 days). Results also suggest that enzyme production has occurred during the stationary phase of growth under shake flask culture and

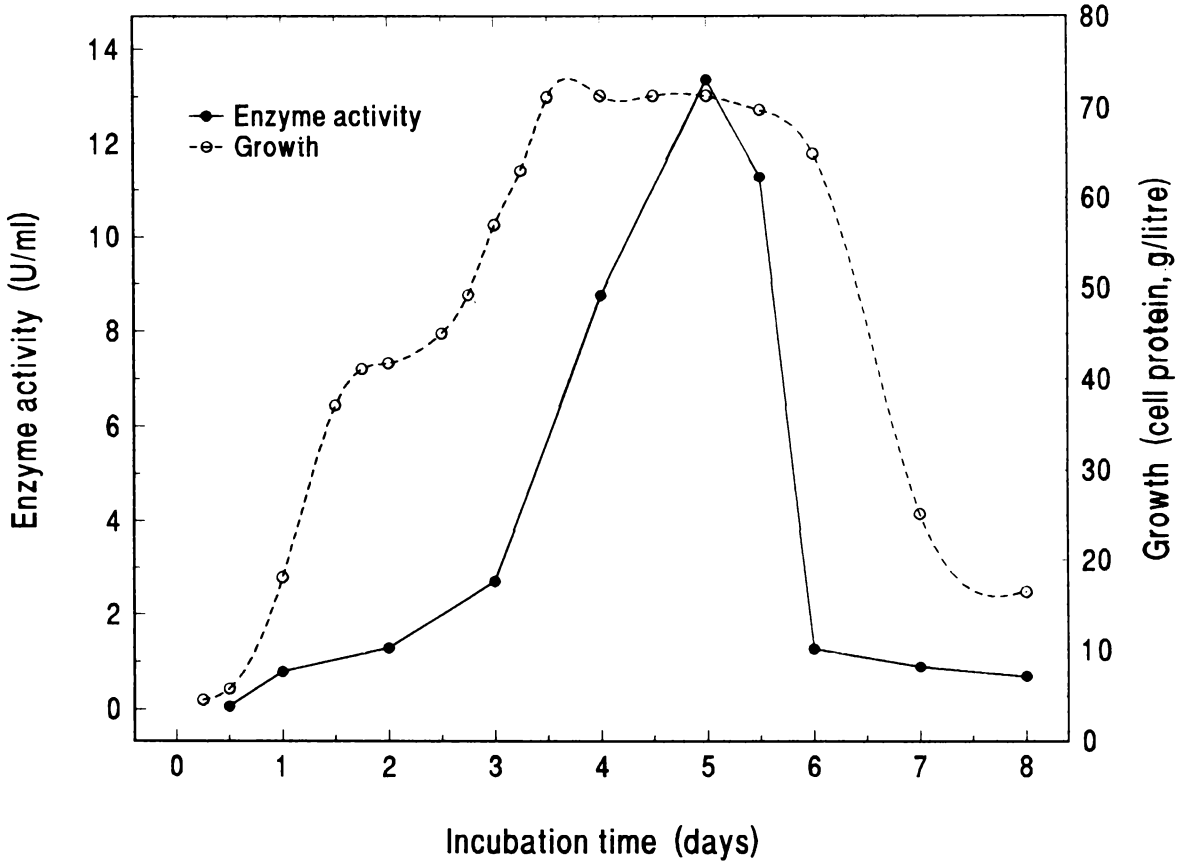



**Figure 24(a)** Comparative evaluation of chitinase production by *B. Bassiana* under different submerged fermentation conditions - Shake flask culture

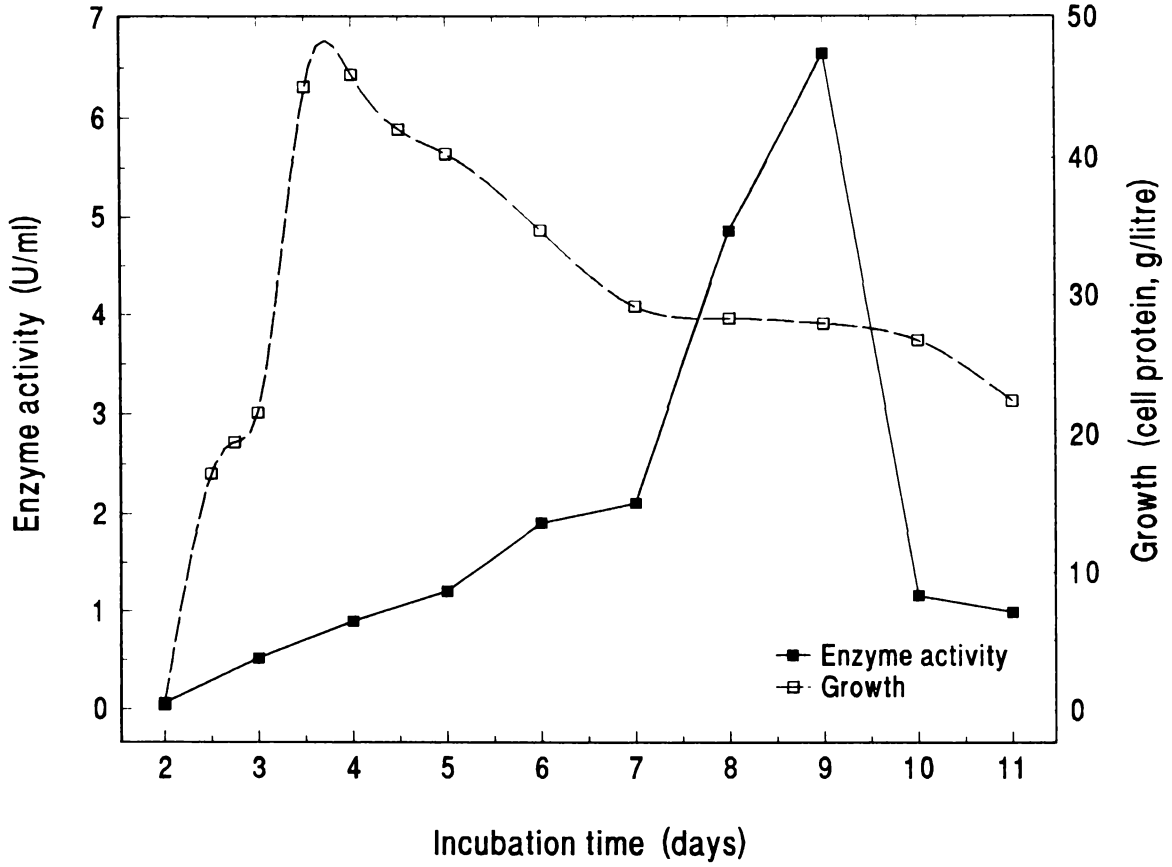



**Figure 24(b)** Comparative evaluation of chitinase production by *B. Bassiana* under different submerged fermentation conditions - Static culture

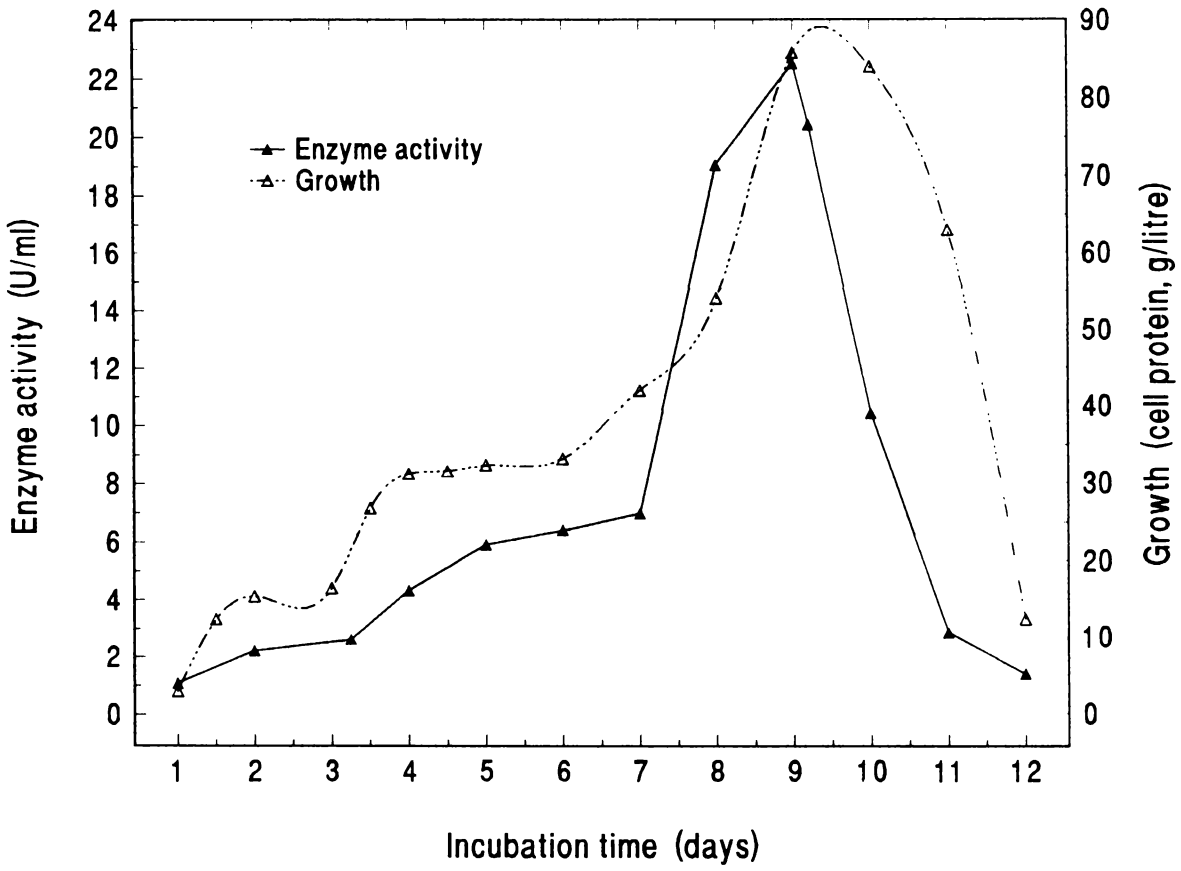



**Figure 24(c)** Comparative evaluation of chitinase production by *B. Bassiana* under different submerged fermentation conditions - Bubble column bioreactor

static culture and conditions. However, in bubble column reactor, the enzyme production was growth associated.

## 3.10 Chitinase production by *B. bassiana* under solid-substrate fermentation using wheat bran as solid substrate

Chitinase production through SSF was studied using wheat bran as solid substrate moistened with aged sea water supplemented with colloidal chitin. The process was optimized for enhanced enzyme production.

### Initial moisture content of the substrate

Impact of initial moisture content on rate of enzyme production was tested by altering the ratio of moistening medium to wheat bran medium. Maximal enzyme production was observed with 3:5 (v/w) ratio of the moistening medium to WB and after 3 days of fermentation (18.6 U/gIDS) (Table 12).

The increase in enzyme yield along with increase in the ratio (V:W) of the moistening medium to WB medium was observed upto 7:5 (13.6 U/gIDS), 3:5 (18.6 U/gIDS) and 2:5 (18.10 U/gIDS) rations after 2, 3 and 4 days of fermentation respectively. The results indicate that some limitation existed when the initial moisture content was higher than 3:5 ratio and consequently resulted in a marginal decrease in enzyme titres after 3 days of fermentation. Enzyme titres varied for the different ratios, above 3:5, and did not show any definite relationship. Whereas the enzyme yield recorded a

Table 12 Effect of initial moisture content of the medium on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Moisture content (v:w ratio) | Enzyme activity (U/gIDS) | | | Soluble protein (mg/gIDS) | | | Reducing sugar (mg/gIDS) | | | pH of the extract | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Incubation period (days) | | | Incubation period (days) | | | Incubation period (days) | | | Incubation period (days) | | |
| | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 3 | 4 | 2 | 3 | 4 |
| 0:5 | 0 | 0 | 3.4 | ND | 65 | 77.1 | ND | 105.7 | 122.0 | 5.51 | 5.53 | 5.6 |
| 0.5:5 | 0 | 3.6 | 6.6 | ND | 71 | 86.7 | ND | 126.8 | 162.0 | 5.5 | 5.54 | 5.64 |
| 1:5 | 0.13 | 8.3 | 10.6 | 72.0 | 71.8 | 79.6 | 70.1 | 131.6 | 178.0 | 5.42 | 5.52 | 5.8 |
| 2:5 | 0.31 | 9.8 | 18.1 | 72.0 | 73.5 | 82.1 | 78.1 | 171.9 | 203.7 | 5.49 | 5.57 | 5.89 |
| 3:5 | 0.39 | 18.6 | 17.9 | 73.7 | 75.3 | 74.7 | 78.44 | 176.1 | 110.83 | 5.57 | 5.64 | 6.35 |
| 4:5 | 0.55 | 13.02 | 14.5 | 79.2 | 73.3 | 74.4 | 75.82 | 139.1 | 98.8 | 5.55 | 5.9 | 6.58 |
| 5:5 | 0.98 | 6.1 | 5.4 | 82.2 | 74.4 | 70.1 | 92.25 | 155.7 | 84.4 | 5.49 | 5.74 | 6.42 |
| 6:5 | 1.36 | 4.6 | 3.9 | 83.7 | 76.4 | 60.9 | 93.78 | 165.1 | 39.5 | 5.51 | 5.97 | 6.54 |
| 7:5 | 1.01 | 3.9 | 1.8 | 82.9 | 75.3 | 70.2 | 91.55 | 131.4 | 56.6 | 5.58 | 6.8 | 6.51 |
| 8:5 | ND | 8.3 | 3.1 | ND | 66.3 | 62.3 | ND | 104.1 | 39.7 | ND | 6.06 | 7.0 |
| 10:5 | ND | 10.2 | 4.8 | ND | 68.3 | 67.3 | ND | 78.1 | 46.9 | ND | 6.06 | 7.05 |
| 12:5 | ND | 11.9 | 6.9 | ND | 69.4 | 63.8 | ND | 85.1 | 20.6 | ND | 6.05 | 7.07 |

ND Not determined

decline for the ratios above 4:5 after 4 days. However it recorded an increase in the case of ratios less than 3:5 after 3 days.

The pH of the enzyme extract in all the cases was increased with the increase in the initial moisture content of the substrate of the respective fermentation period, and showed a range of 5-5.8, 5.6-6.1, and 5.9-7.3 on 2nd day 3rd day, 4th day and 5th day of fermentation respectively. However pH was increased in all cases with increase in fermentation period (Table 12).

Soluble protein content in the enzyme extract showed variation irrespective of the enzyme yield and initial moisture content of the substrate (Table 12). In general the soluble protein varied from 65.0 mg/gIDS to 76.4 mg/gIDS for the various ratios tested after 3 days and from 60.9 mg/gIDS(6:5 ratio) to 82.1 mg/gIDS (2:5 ratio) after 4 days. The soluble protein content was found to record an increase alongwith increase in initial moisture content upto a ratio of 3:5 (v/w) after 3 days of fermentation while for the ratios 4.5-12.5, it recorded fluctuations, without demonstrating any definite pattern of relationship neither with moisture content nor with enzyme titres. A maximal protein was recorded with 2:5 ratio of moisture content coinciding with maximal enzyme yield. In general no direct relationship could be inferred

between enzyme titres and soluble protein values during the conduct of these experiments.

Data recorded for reducing sugar content in the enzyme extract showed a direct relationship with enzyme titres, by recording an increase along with increase in the initial moisture content of the WB medium. Thus concentration of reducing sugars in the enzyme extract was maximal along with maximal enzyme yield with the ratio of 3:5 (176.1 mg/gIDS) and 2:5 (203.7 mg/gIDS) respectively for 3rd and 4th days. In general the reducing sugar concentration demonstrated a declining trend along with increase in moisture content, for the ratios above 3:5, with same fluctuations.

**Incubation temperature**

Significant levels of enzyme could be produced over a wide range of temperature 22-32$^{o}$C with an optimum at 27$^{o}$C (Figure 25). It was noted that higher temperatures led to a decline in enzyme production. Thus there was a 44% decrease (10.6 U/gIDS) in enzyme yield at 37$^{o}$C compared with that recorded at 27$^{o}$C (18.9 U/gIDS).

The pH of the enzyme extract gradually decreased along with increase in the incubation temperature. However the variation was only marginal (6.1-6.6). Both soluble protein and reducing sugar content in the enzyme extract were maximal, 76.6 mg/gIDS and 182.97 mg/gIDS respectively for

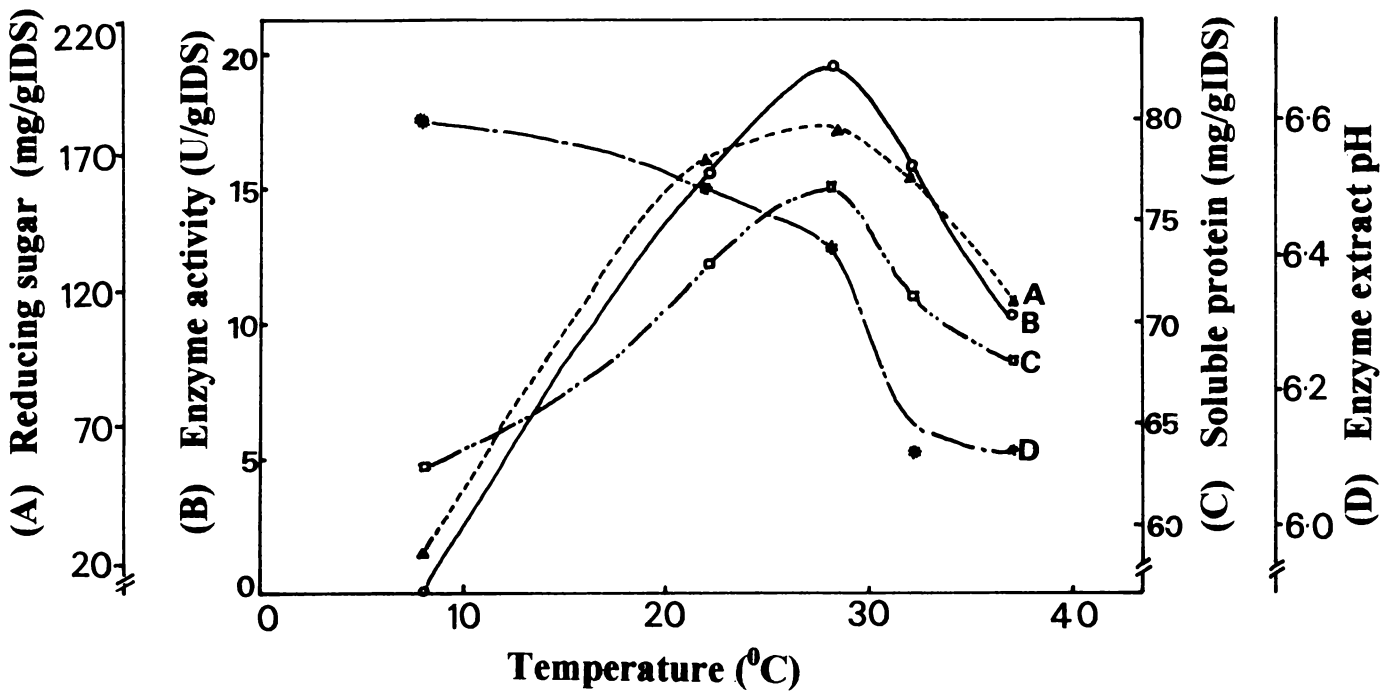



**Figure 25** Effect of incubation temperature on chitinase production by *B. bassiana* using wheat bran medium under SSF

soluble protein and reducing sugar at 27°C. Interestingly the enzyme yield, soluble protein and reducing sugar showed an increase in their values along with increase in incubation temperature upto 27°C and decrease gradually along with increase in incubation temperature above 27°C. pH of enzyme extract varied from pH 6.1 (37°C) to pH 6.6 (8°C).

**Initial pH of the substrate**

Data obtained for the studies conducted with optimization of initial pH of the solid substrate using wheat bran showed very interesting and usual observation (Table 13). When the sea water was adjusted to different levels of pH using NaOH solution for pH 8 - 12.9 and used for moistening the wheat bran, the pH of the wheat bran did not undergo the expected change to the desired levels and instead remained almost acidic varying from 6.08 to 6.73 for the expected pH 7.0 to pH 12.0. Only in the case of expected pH 12.9 the pH 10.73 was obtained in the wheat bran. Further it was observed that after autoclaving the WB medium there was further change in the pH of the WB medium and it was observed to vary between 5.49 to 6.2 for the pH 6.08 to 6.83. Very interestingly irrespective of the marginal variation in the pH of autoclaved WB, the enzyme yield varied between 5.9 U/gIDS (pH 5.71) to 41.2 U/gIDS (pH 6.2). An enzyme yield of 41.8 U/gIDS are also recorded at pH 9.2. However in general it was obseved that for the pH above 6.0 after autoclaving WB medium, there was higher yield of enzyme compared to the pH levels below 6.0.

Table 13 Effect of initial pH of the substrate on chitinase production by *B. bassiana* using wheat bran medium under SSF

| pH of the moistening medium (sea water) | pH of the substrate before autocla-ving | pH of the substrate after autocla-ving | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) | pH of the extract |
|---|---|---|---|---|---|---|
| 7.0 | 6.08 | 5.4 | 17.6 | 74.5 | 181.4 | 6.16 |
| 8.0 | 6.09 | 5.51 | 18.8 | 76.1 | 182.9 | 6.15 |
| 9.0 | 6.1 | 5.52 | 12.4 | 72.3 | 175.5 | 6.40 |
| 10.0 | 6.2 | 5.71 | 5.9 | 71.5 | 160.6 | 6.41 |
| 11.0 | 6.62 | 6.1 | 32.0 | 73.1 | 164.4 | 6.73 |
| 12.0 | 6.73 | 6.2 | 41.2 | 87.0 | 154.3 | 6.86 |
| 12.9 | 10.73 | 9.2 | 41.8 | 97.0 | 140.7 | 7.2 |

Both soluble protein and reducing sugars did not show significant variation unlike enzyme yield with respect to vaious pH levels of the autoclaved WB medium.

**Effect of NaOH**

Initially the impact of NaOH on the pH of the solid support was tested by pretreatment of the wheat bran with NaOH of different normalities. From the results presented in Table 14 it was inferred that the pH of the wheat bran medium added with NaOH undergoes drastic change before and after autoclaving. Thus the increase in normality of NaOH resulted in increase in pH, a maximum of 11.04 with 2 N NaOH before autoclaving. However after autoclaving there is a decline in pH. The decline in the pH was in the order of 0.81, 1.28, 1.33 and 1.44 respectively for 0.1, 0.5, 1.0 and 2.0 N NaOH after autoclaving.

Results also show that the pH after autoclaving and at the commencement of fermentation undergoes significant change. Thus the enzyme extract recovered after fermentation recorded an increase from pH 6.12 to 6.96 and 6.98 to 7.58 for the 0.1 and 0.5 N NaOH respectively, and decreased from pH 8.07 to 7.3 and pH 9.6 to 8.23 for the 1 N and 2 N respectively.

Results presented in Table 14 showed that NaOH solution significantly influenced chitinase production during

Table 14 Effect of NaOH solution on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Normality of NaoH solution | pH of WB medium before autocla-ving | pH of WB medium after autocla-ving | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) | pH of the extract |
|---|---|---|---|---|---|---|
| 0.1 | 6.93 | 6.12 | 50.4 | 69.2 | 164.2 | 6.96 |
| 0.5 | 8.26 | 6.98 | 45.6 | 81.9 | 182.7 | 7.58 |
| 1.0 | 9.4 | 8.07 | 23.3 | 109.8 | 205.6 | 7.30 |
| 2.0 | 11.04 | 9.6 | 74.3 | 153.1 | 176.8 | 8.23 |

SSF using wheat bran. It was observed that the enzyme yield was maximum (74.3 U/gIDs) in WB medium humidified with 2 N NaOH solution.

In general, increase in NaOH concentration above 0.1 N led to decrease in enzyme yield from 50.4 U/gIDS to 23.3 U/gIDS (1 N NaOH), although a maximum of 74.3 U/gIDS was recorded with 2 N NaOH. A contrasting trend was observed with reducing sugar, which recorded an increase along with increase in normality of NaOH upto 1 N, when a maximum of 205.6 mg/gIDS was recorded. Further increase in normality, 2 N NaOH, led to a decline. Whereas soluble protein recorded linear relationship of increase along with increase in normality of NaOH, with a maximum of 153.1 mg/gIDS (2 NaOH). Soluble protein and reducing sugar showed a linear relationship by recording an increase along with increase in the concentration of NaOH. Interestingly maximal soluble protein and reducing sugar concentrations were recorded along with low level of enzyme yield (Table 14) pH of the enzyme extract varied from 6.96 (0.1 N) to 8.23 (2 N).

**Effect of colloidal chitin concentration**

It is evident from the Table 15 that optimum concentration of colloidal chitin required for maximum chitinase production was 7.5% (w/w) (73.6 U/gIDS) Nevertheless significant levels of chitinase synthesis could be observed over a range of substrate concentrations varying

Table 15 Effect of colloidal chitin concentration on chitinase production by B.bassiana using wheat bran medium under SSF

| Concentration of colloidal chitin %(w/w) | Enzyme activity (U/gIDS) | | Soluble protein (mg/gIDS) | | Reducing sugar (mg/gIDS) | | pH of the extract | |
|---|---|---|---|---|---|---|---|---|
| | Moistening media | | Moistening media | | Moistening medium | | Moistening medium | |
| | sea water | 2 N NaOH solution | sea water | 2 N NaOH solution | sea water | 2 N NaOH solution | sea water | 2 N NaOH solution |
| 0 | 7.81 | 27.2 | 125.2 | 152.3 | 166 | 33.3 | 7.79 | 8.0 |
| 0.5 | 10.5 | 34.5 | 124.1 | 154.3 | 156.8 | 39.9 | 7.6 | 8.9 |
| 1 | 41.2 | 79.4 | 127.5 | 164.7 | 155.6 | 88.1 | 7.64 | 8.82 |
| 2 | 43.5 | 83.9 | 128.4 | 167.1 | 154.1 | 124.0 | 7.63 | 8.6 |
| 3 | 47.8 | 60.3 | 130.1 | 161.7 | 151.6 | 49.3 | 7.64 | 8.0 |
| 5 | 62.6 | 44.0 | 135.3 | 159.3 | 143.3 | 28.7 | 7.82 | 8.85 |
| 7.5 | 73.6 | 42.0 | 139.3 | 155.2 | 158.4 | 26.3 | 7.87 | 8.89 |
| 10 | 58.8 | 26.9 | 137.6 | 144.6 | 147.3 | 24.0 | 7.72 | 8.85 |
| 15 | 51.5 | 24.9 | 137.3 | 139.9 | 160.0 | 19.0 | 7.76 | 8.86 |

between 1-15% and 0.5-7.5% respectively with the moistening media, sea water and 2 N NaOH.

The chitinase production showed a linear increase along with the increase in colloidal chitin concentration upto 7.5% (w/w) levels in the case of sea water. However, further increase, above 7.8% (w/w), did not enhance the chitinase yield. Whereas, with respect to NaOH there was increase in enzyme yield along with increase in chitin concentration upto 2% and progressive decrease along with further increase in chitin concentrations above 2%.

The results exhibited an interesting trend, with respect to the rate of enzyme synthesis in response to sea water/NaOH moistening of wheat bran, and the varying concentration of colloidal chitin in the medium. The enzyme yield was higher, varying between 1.3 fold - 3.3 fold, in the case of NaOH along with increase in concentrations of chitin upto 3% compared to that of sea water. In contrast, for the chitin concentration above 3% the enzyme yield declined to the order of 0.5-0.7 fold with NaOH compared to sea water.

The pH of the enzyme extract showed insignificant variation irrespective of the concentration of colloidal chitin and enzyme titres, in both the cases of moistening media. Thus the pH of the enzyme extract varied from pH

7.6-7.9 and from pH 8.6-8.9 for the sea water and NaOH respectively (Table 15).

Soluble protein content in the enzyme extract varied (from 124.1 to 139.34 mg/gIDS) for the different concentration of chitin tested in the case of sea water compared to that observed with NaOH (139.9 to 167.1 mg/gIDS), (Table 15). However protein was more in the case of NaOH medium compared to sea water medium, irrespective of the concentrations of colloidal chitin tested.

The soluble protein showed an increase along with increase in chitin concentration upto 7.5, in the case of sea water. when a maximum of 139.34 mg/gIDS was recorded (Table 15). Further increase in chitin concentrations led to a marginal decline in protein. Whereas in the case of NaOH the increase in protein was linear along with increase in chitin concentration upto 2%, when a maximum of 167.1 mg/gIDS was recorded. Higher concentration of chitin, above 2%, led to a progressive decline in protein.

Data for the reducing sugars in the enzyme extract showed variation in their concentration irrespective of the concentration of colloidal chitin and enzyme yield (Table 15). The reducing sugar varied from 143.3 to 166.0 mg/gIDS and from 19.0 to 124.0 mg/lDS respectively in the case of wheat bran moistened with sea water and NaOH solution. It is seen that,

while the variation in reducing sugar was narrow in the case of sea water, it was so wide and significant in the case of NaOH solution with respect to the different concentrations of colloidal chitin. In the case of NaOH, in general the reducing sugar was significantly high only with the chitin concentration of 1 - 3% compared to other concentrations. pH of the enzyme extract varied from pH 7.6 to 7.96 and from 8.6 to 8.9 for the medium with sea water and NaOH solutions respectively.

**Effect of sodium chloride**

Results presented in Table 16 clearly indicate that chitinase production by *B. bassiana* increased in the presence of NaCl. However, the optimal concentration of NaCl, needed for maximal chitinase production varied with the choice of solvent used for humidifying the WB medium. With sea water as solvent for humidifying the WB medium, the maximal enzyme yield was obtained with 2.5% (w/w) NaCl concentration (98.8 U/gIDS) compared to that observed with 10% NaCl in the case of 2 N NaOH (118. U/gIDS). Results indicate that addition of NaCl could enhance yield only marginally upto 136.6% for the NaCl of 2.5% compared to that in NaOH where a maximal of 222.3% for 10% NaCl concentration was recorded.

Except for 1 and 2.5% NaCl, all other concentration of NaCl, the NaOH supported higher enzyme yield than sea water. It was also noted that even in the absence of NaCl,

Table 16 Effect of sodium chloride on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Concentration of sodium chloride %(w/w) | Enzyme activity (U/gIDS) | | Soluble protein (mg/gIDS) | | Reducing sugar (mg/gIDS) | | pH of the extract | |
|---|---|---|---|---|---|---|---|---|
| | Moistening medium | | Moistening medium | | Moistening medium | | Moistening medium | |
| | sea water | 2 N NaOH solutions | sea water | 2 N NaOH solution | sea water | 2 N NaOH solution | sea water | 2 N NaOH solution |
| 0 | 72.4 | 80.7 | 109.7 | 154.2 | 193.3 | 201.2 | 6.52 | 6.24 |
| 1 | 92.4 | 83.3 | 106.8 | 142.7 | 172.6 | 160.8 | 7.26 | 5.5 |
| 2.5 | 98.8 | 90.7 | 115.1 | 142.5 | 160.4 | 161.3 | 7.22 | 5.6 |
| 5 | 78.4 | 100.2 | 111.4 | 126.3 | 145.1 | 177.9 | 6.3 | 6.8 |
| 7.5 | 59.5 | 113.4 | 103.1 | 115.9 | 124.6 | 171.4 | 5.46 | 7.74 |
| 10 | 53.3 | 118.5 | 98.9 | 109.8 | 102.6 | 171.3 | 5.6 | 7.42 |
| 12.5 | 48.6 | 65.2 | 97.6 | 102.8 | 97.5 | 140.3 | 5.88 | 7.42 |
| 15 | 43.8 | 45.5 | 87.1 | 93.3 | 93.1 | 136.8 | 6.76 | 7.67 |
| 17.5 | 42 | 41.2 | 88.8 | 85.7 | 84.4 | 56.0 | 5.57 | 7.66 |
| 20 | 15.9 | 7.1 | 68.8 | 82.8 | 25.1 | 22.9 | 6.95 | 7.6 |
| 22.5 | 5.9 | 4.8 | 68.4 | 81.7 | 20.3 | 2.4 | 6.97 | 7.65 |

NaOH medium showed about 12% higher enzyme yield over sea water. While NaCl concentration above 2.5% led to decline in enzyme yield with sea water, only concentration above 10% NaCl resulted a decrease in enzyme yield in the case of NaOH.

Maximal protein was recorded with 2.5% NaCl concentration (115.02 mg/gIDS) in the case of sea water medium, and without NaCl in the case of NaOH solution medium (154.2 mg/gIDS) (Table 16). However, the soluble protein content declined gradually in a linear fashion, along with increase in NaCl concentration in the case of NaOH.

Reducing sugar concentration in the enzyme extract was maximal in the absence of NaCl in both the cases of WB medium moistened with sea water (193.3 mg/gIDS) as well as NaOH solution (201.2 mg/gIDS). The reducing sugar content in general, recorded a linear decline along with the increase in the concentration of NaCl.

The pH of the enzyme extract showed variation, from pH 5.46 to 7.26 and pH 5.5 to 7.76 respectively with sea water and NaOH, irrespective of the NaCl concentration and enzyme synthesis.

**Effect of phosphate**

Wheat bran medium without addition of either $K_2HPO_4$ or $KH_2PO_4$ yielded 96.4 U/gIDS of chitinase and 124.55 mg/gIDS

reducing sugar (Table 17). It was observed that addition of $K_2HPO_4$ led to a decline in enzyme yield and reducing sugar when compared to control. In the case of $KH_2PO_4$ addition, although reducing sugar got reduced than control, enzyme yield was higher than control when the concentration of phosphate was 5% (119.2 U/gIDS). Similarly, when both $K_2HPO_4$ and $KH_2PO_4$ were combined at various proportions and added to WB medium, except at ratios of 1:1 (97.4 U/gIDS) and 2.5:1 (119. 8 U/gIDS), in general the enzyme yield declined compared to control Reducing sugar also recorded a decline, compared to control at all ratios tested.

Results suggest that addition of $KH_2PO_4$ at 5% and a combination of $K_2HPO_4$ and $KH_2PO_4$ at a ratio of 2.5:1 could alone promote enzyme yield by 23.7% and 24.3% respectively.

When the proportion of $KH_2PO_4$ in the combinations was maintained at 1 and $K_2HPO_4$ altered, maximal enzymes yields were recorded (97.4, 119.8 and 82.2 U/gIDS for the ratios 1:1, 2.5:1 and 5:1 of $K_2HPO_4$ and $KH_2PO_4$ respectively) unlike when tested individually. A significant level of enzyme yield was also recorded with 1:2.5 ratio (88.7 U/gIDS) of $K_2HPO_4$ and $KH_2PO_4$. At other ratios the enzyme yield was very low. It was inferred that combinations with higher concentration of $K_2HPO_4$ and $KH_2PO_4$ led to decrease in enzyme yield.

Table 17 Effect of phosphate on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Phosphate concentration % (w/w) | Enzyme activity (U/gIDS) | Reducing sugar (mg/gIDS) | pH of the medium after fermentation |
|---|---|---|---|
| No phosphate | 96.4 | 124.6 | 7.15 |
| $K_2HPO_4$ | | | |
| 1 | 80.5 | 101.2 | 7.32 |
| 2.5 | 67.0 | 88.54 | 7.17 |
| 5 | 38.4 | 50.96 | 7.11 |
| $KH_2PO_4$ | | | |
| 1 | 38.6 | 80.4 | 7.15 |
| 2.5 | 74.5 | 113.8 | 7.21 |
| 5 | 119.2 | 97.8 | 7.36 |
| $K_2HPO_4$ $KH_2PO_4$ | | | |
| 1:1 | 97.4 | 116.1 | 7.63 |
| 1:2.5 | 88.7 | 46.1 | 7.60 |
| 1:5 | 6.5 | 4.2 | 7.62 |
| 2.5:1 | 119.8 | 115.2 | 7.35 |
| 2.5:5 | 35.2 | 30.2 | 7.80 |
| 5:1 | 82.2 | 96.9 | 7.74 |
| 5:2.5 | 25.4 | 4.1 | 7.71 |

pH of the enzyme extract obtained from control experiment was pH 7.15. Whereas the pH of the enzyme extract varied between 7.11 and 7.32; 7.15 and 7.36 and 7.6-7.8 respectively with the $K_2HPO_4$, $KH_2PO_4$, and both together.

**Inoculum type and concentration**

Both spore and vegetative inocula were tried at various concentrations. Enzyme production was high through out the course of fermentation with the spore inocula of 32 x $10^6$ per 5 g of wheat bran recording a maximum of 109.2 U/gIDS after 62 h of fermentation (Table 18). Further incubation above 62 h led to a decline in the enzyme yield. In the case of inocula concentrations less than 32 x $10^6$ number of spores, significant levels of enzyme titres could be observed after 80 h. It was also observed that increase in the concentration of spore inoculum led to an increase in enzyme yield irrespective of the incubation period.

The soluble protein in the enzyme extract showed a progressive increase along with increase in enzyme yield during the course of fermentation irrespective of the concentration of spore inoculum. The soluble protein content varied in general from 74.9 to 102.82 mg/gIDS during fermentation, irrespective of the inoculum concentration. With respect to high inoculum concentration (32 x $10^6$), the soluble protein varied from 78.2 to 88.8 mg/gIDS, recording an increase along with increase in enzyme yield. Relatively the

Table 18 Effect of inoculum type and inoculum concentration on chitinase production by B. bassiana using wheat bran medium under SSF

| Incubation periods (hour) | Spore inoculum | | | | | Vegetative inoculum | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Concentration of inoculum (number of spore/ 5 g substrate) | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) | pH of the extract | concentration of inoculum (mg dry weight/ 100 g substrate) | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) | pH of the extract |
| 30 | $3248x10^6$ | 31.4 | 78.2 | 8.9 | 7.07 | 952 | 47.8 | 83.6 | 192.1 | 7.22 |
| | $1624x10^6$ | 25.2 | 76.9 | 10.4 | 6.97 | 437 | 46.5 | 79.3 | 140.8 | 7.25 |
| | $812x10^6$ | 19.95 | 76.3 | 11.4 | 7.08 | 231 | 37.5 | 76.9 | 82.8 | 7.05 |
| | $406x10^6$ | 4.75 | 7.49 | 9.2 | 7.09 | 17 | 22 | 70.7 | 16.7 | 7.09 |
| 42 | $3248x10^6$ | 50.9 | 74.2 | 54.68 | 7.32 | 952 | 115.4 | 90.8 | 98.2 | 7.54 |
| | $1624x10^6$ | 37.6 | 76.8 | 30.3 | 7.12 | 437 | 67.2 | 85.2 | 101.1 | 7.45 |
| | $812x10^6$ | 25.3 | 78.3 | 10.4 | 7.02 | 231 | 43.8 | 82.1 | 88.9 | 7.43 |
| | $406x10^6$ | 6.4 | 76.9 | 11.32 | 6.98 | 17 | 30.2 | 74.0 | 89.3 | 7.26 |
| 62 | $3248x10^6$ | 109.2 | 88.8 | 95.7 | 7.44 | 952 | 117.2 | 104.7 | 92.7 | 7.4 |
| | $1624x10^6$ | 74.4 | 88.1 | 77.5 | 7.48 | 437 | 71.6 | 99.0 | 188.2 | 7.48 |
| | $812x10^6$ | 62.4 | 86.6 | 77.8 | 7.27 | 237 | 64.6 | 102.5 | 149.52 | 7.46 |
| | $406x10^6$ | 63.6 | 100.4 | 54.28 | 7.11 | 17 | 31.8 | 90.2 | 211.25 | 7.46 |
| 80 | $3248x10^6$ | 95.4 | 80.3 | 53.6 | 7.31 | 952 | 64.8 | 106.6 | 118.6 | 7.58 |
| | $1624x10^6$ | 78.3 | 103.5 | 60.1 | 7.45 | 437 | 67.2 | 101.8 | 91.1 | 7.53 |
| | $812x10^6$ | 68.8 | 102.8 | 48.8 | 7.41 | 237 | 76.4 | 103.5 | 99.3 | 7.51 |
| | $406x10^6$ | 69.4 | 94.94 | 53.4 | 7.49 | 17 | 43.2 | 102.4 | 98.6 | 7.4 |

increase in protein was slightly higher during the later hours of fermentation (Table 18).

Reducing sugar content in the enzyme extract recorded a gradual increase, irrespective of the inoculum concentration upto 62 h of fermentation. However, maximal concentration of reducing sugar was recorded with the inoculum concentration of 32 x $10^6$ spores for 5 g WB medium after 62 h. (95.7 mg/gIDS). Further incubation above 62 h led to a reduction in reducing sugar content in the enzyme extract. Similar trend was observed for all the concentration of inoculum tested (Table 18).

pH of the enzyme extract also increased along with the increase in the fermentation period, irrespective of the inoculum concentration, upto 80 h. However, there was a marginal decline in the pH, after 80 h, for the higher inoculum concentration of 32 x $10^6$ and 16 x $10^6$ Inspite of the increase, the variation in the pH was not significant as it varied from 6.97 to 7.48 irrespective of the inoculum concentration and incubation period.

The data presented in Table 18, for the enzyme titres obtained with vegetative inoculum, indicate that enzyme production was maximum with 952 mg dry weight equivalent of mycelia per 100 g of wheat bran after 62 h. (117.2 U/gIDS). Further incubation above 62 h resulted in a decline in the

enzyme titres. Whereas in the case of lower inocula concentrations (231 and 17 mg) the enzyme production continued to increase till 80 h. Results suggest that 42 h of incubation could be optimal for the inoculum concentration of 952 mg since 115.4 U/gIDS could be recorded when compared to 117.2 U/gIDS recorded after 62 h. The increase incurred after additional 20 h of incubation is marginal. Relatively, the enzyme yield recorded for other inoculum concentration was less.

The data for soluble protein content in the enzyme extract showed a linear relationship with enzyme production and inoculum concentration during the course of fermentation (Table 18). In general the protein varied from 70.04 mg/gIDS to 106.53 mg/gIDS for the various inocula sizes tested.

The reducing sugar content in the enzyme extract was observed to vary irrespective of the inoculum concentration from 16.7 mg/gIDS to 211.28 mg/gIDS during the course of fermentation for 80 h.

The pH of the enzyme extract was observed to vary between 7.09 and 7.58 irrespective of the inoculum concentration and incubation period. In general the pH was around pH 7.4 - 7.6 after 42 h for the various inoculum concentration in response to increase or decrease in enzyme yield.

An overall assessment of the results obtained for spore and vegetative inoculum suggest that vegetative inoculum is preferable for obtaining maximal yield of enzyme, since almost about 2.3 fold enhanced yield could be obtained after 42 h.

**Particle size and inoculum type**

The data presented in **Table** 19 show that the maximal enzyme production was promoted by the WB medium prepared with < 425 micron particle size, irrespective of the type of inoculum used. A maximum of 175.4 U/gIDS and 185.8 U/gIDS was recorded after 48 h and 72 h of fermentation with spore and vegetative inocula respectively, indicating that particle size < 425 micron was the optimum particle size of wheat bran substrate. The enzyme yield decreased along with increase in particle size of the substrate, during the course of fermentation, irrespective of the types of inoculum used. There was a 22.4% (185.8 U/gIDS) increase in enzyme yield with vegetative inoculum compared to that with spore inoculum (151.8 U/gIDS) in the medium with particle size of < 425 micron after 48 h. The rate of enzyme synthesis was very rapid in the case of vegetative inoculum, which reached a maximum after 48 h compared to spore inoculum, where 72 h was required to reach the maximum. However further incubation above 48 h led to a sharp decline in the rate of enzyme synthesis. Similarly in the case of spore inoculum, the rate

Table 19 Effect of particle size of the substrate and inoculum type on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Incubation period (days) | Particle size of the substrate (micron) | Enzyme activity (U/gIDS) | | Soluble protein (mg/gIDS) | | Reducing sugar (mg/gIDpH of the extract | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Inoculum type | | Inoculum type | | Inoculum type | | Inoculum type | |
| | | spore | vegetative | spore | vegetative | spore | vegetative | spore | vegetative |
| I | < 425 | 45.0 | 97.4 | 101.6 | 104.3 | 8.68 | 97.3 | 7.3 | 7.19 |
| | 425-600 | 40.1 | 60.2 | 88.9 | 90.0 | 7.82 | 84.6 | 7.39 | 7.58 |
| | 600 -1000 | 27.3 | 52.6 | 81.0 | 103.8 | 2.96 | 74.1 | 7.4 | 7.48 |
| | 1000 -1400 | ND | ND | ND | ND | ND | ND | ND | ND |
| II | < 425 | 151.8 | 185.8 | 102.6 | 108 | 85.4 | 133.2 | 7.89 | 7.55 |
| | 425-600 | 113.2 | 102.6 | 114.7 | 119.1 | 71.4 | 79.3 | 7.60 | 7.71 |
| | 600-1000 | 71.6 | 95.4 | 104.3 | 116.5 | 43.1 | 76.5 | 7.55 | 7.60 |
| | 1000-14000 | ND | 65.3 | ND | 124.9 | ND | 64.4 | ND | 7.65 |
| III | < 425 | 175.4 | 67.2 | 117.0 | 120.3 | 44.9 | 120.3 | 8.43 | 7.41 |
| | 425-600 | 82.1 | 37.6 | 130.3 | 135.1 | 25.2 | 135.1 | 7.93 | 7.71 |
| | 600-1000 | 37.6 | 31.2 | 124.1 | 128.2 | 24.7 | 128.2 | 7.98 | 7.61 |
| | 1000-1400 | 17.2 | ND | 132.4 | ND | 22.4 | ND | 8.05 | ND |
| IV | < 425 | 158.6 | 34.6 | 111.8 | 120.7 | 34.7 | 120.7 | 8.64 | 7.72 |
| | 425-600 | 38.4 | 37.2 | 136.6 | 135.0 | 51.4 | 136.0 | 7.96 | 7.96 |
| | 600-1000 | 27.6 | 23.6 | 136.7 | 134.0 | 40.9 | 134.0 | 7.95 | 7.94 |
| | 1000-1400 | ND | ND | ND | ND | ND | ND | ND | ND |

ND- Not determined

of enzyme synthesis was found to get declined after 72 h of incubation.

Data presented in Table 19 on the soluble protein content in the enzyme extract showed irregular variation irrespective of the incubation period. However maximal concentration of protein (137 mg/gIDS) was observed with the substrate having the particle size between 425-600 micron during the course of fermentation except with particle size < 425 micron. where the maximum (104.3 mg/gIDS) was observed at 24 h. Nevertheless maximal protein values were recorded after 48 h of incubation irrespective of the type of inocula.

The reducing sugar content in the enzyme extract recorded a linear positive relationship with enzyme titres, and observed maximal concentration (143.3 mg/gIDS) in substrate having the particle size < 425 micron upto 72 h of fermentation. Reducing sugar decreased along with increase in particle size of the wheat bran substrate, irrespective of the incubation period upto 72 h. However the reduction in reducing sugar was rapid after 72 h for all the particle size tested (Table 19).

pH profile of the enzyme extract, obtained with respect to the various particle size of WB tested, showed significant variation during the course of fermentation. Thus it varied from pH 7.3 (24 h) to 8.64 (96 h) and from 7.19 (24 h to 7.94 (96 h) for the spore inoculum and vegetative

inoculum respectively. Although there was a gradual increase in pH along with increase in incubation period (Table 19) no definite relationship could be drawn between the pH and the particle size or inocula type. Although there was a gradual increase in pH along with increase in incubation period (Table 19).

**Effect of cooking cum autoclaving**

Enzyme yield increased, linearly, along with the increase in the autoclaving time, and a maximal yield of 138.8 U/gIDS was observed with the wheat bran medium autoclaved for 60 minutes (Table 20). However 42.9% and 86% of the maximal yield could be recorded respectively, in the WB medium autoclaved for 20 and 40 minutes indicating the impact of cooking of the wheat bran medium on enzyme production by *B. bassiana*.

Soluble protein content of the enzyme extract showed a positive relationship with enzyme yield and autoclaving time by recording an increase along with the increase in the autoclaving time. Maximal protein content was observed in the extract obtained from the medium autoclaved for 60 minutes. However, the reducing sugar content in the enzyme extract showed variation irrespective of the cooking time of the wheat bran medium and enzyme titres. Although maximal concentration was observed in the medium autoclaved for 20 minutes followed by 60 minutes and 40 minutes, it varied between 84.3 and

**Table 20** Effect of cooking cum autoclaving on chitinase production by *B.bassiana* using wheat bran medium under SSF

| Cooking time (minutes) | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) | pH of the extract |
|---|---|---|---|---|
| 20 | 59.6 | 82.1 | 88.6 | 7.55 |
| 40 | 119.4 | 89.8 | 84.3 | 7.59 |
| 60 | 138.8 | 97.3 | 87.42 | 7.97 |

88.62 mg/gIDS. pH of the enzyme extract recorded a marginal increase from 7.55 to 7.97 along with increase in the autoclaving time, and a high pH was observed in the extract obtained from the medium autoclaved for 60 minutes.

Incubation period

Enzyme production was maximum in the WB medium inoculated with vegetative inoculum after 48 h. (182.6 U/gIDS) and with spore inoculum after 72 h. (175.7 U/gIDS) of fermentation (Table 21). The rate of enzyme synthesis was rapid with vegetative inoculum, when compared to spore inoculum. The enzyme titres with vegetative inoculum, however decreased sharply when the fermentation time was above 48 h. Wheress in the case of spore inoculum also the enzyme titres decreased marginally when incubated beyond 72 h. Vegetative inoculum showed a higher yields of enzyme (96.4 U/gIDS, 182.6 U/gIDS reespectively after 24 h and 48 h) compared to spore inoculum (45.7 U/gIDS, 154.8 U/gIDS respectively after 24 h and 48 h) indicating their suitability for use towards enhanced enzyme production.

Soluble protein content in the enzyme extract increased along with increase in the fermentation period and a maximal concentration was recorded after 72 h irrespective of the inoculum type (Table 21). The protein varied between 102.78 and 118.15 mg/gIDS and between 103.16 and 121.18 mg/gIDS respectively for the spore and vegetative inocula.

Table 21 Effect of incubation time on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Incubation period (hour) | Type of inoculum | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) | pH of the extract |
|---|---|---|---|---|---|
| 24 | spore | 45.7 | 102.3 | 8.5 | 7.29 |
| | vegetative | 96.4 | 103.2 | 98.0 | 7.2 |
| 44 | spore | 154.8 | 102.7 | 86.3 | 7.86 |
| | vegetative | 182.6 | 109.1 | 132.0 | 7.46 |
| 72 | spore | 175.7 | 118.2 | 43.8 | 8.39 |
| | vegetative | 77.9 | 121.0 | 143.3 | 7.73 |
| 96 | spore | 158.8 | 113.7 | 34.6 | 8.65 |
| | vegetative | 48.9 | 121.1 | 38.0 | 7.42 |

The reducing sugar content in the enzyme extract showed variation irrespective of the fermentation period and enzyme titres. However, maximal reducing sugar was recorded after 98.0 h (86.31 mg/gIDS) and 72 h (143.26 mg/gIDS) respectively for the spore and vegetative inocula tested. Comparatively the reducing sugar concentration was at higher levels with vegetative inoculum than spore inoculum.

pH of the enzyme extract obtained from WB medium inoculated with spore inoculum and vegetative inoculum showed significant variation during 96 h fermentation. While the pH increased, from 7.29 to 8.65, gradually along with increase in incubation period with spore inoculum, in the case of vegetative inoculum, the increase in pH was marginal from 7.2 to 7.73. Maximal pH was recorded after 96 h in both the cases of inocula.

**Additional nitrogen sources**

Yeast extract, supplemented in the WB medium at 1% level, resulted 127.5% increase in the enzyme titres (222.8 U/gIDS) when compared to control, (174.8 U/gIDS) (Table 22) Further increase in yeast extract above 1% affected the synthesis of enzyme adversely resulting in significant decrease in enzyme yield.

The soluble protein content in the enzyme extract however increased along with increase in the concentration

Table 22 Effect of additional nitrogen sources on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Nitrogen source | Concentration of nitrogen source % (w/w) | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) | pH of the extract |
|---|---|---|---|---|---|
| Yeast extract | 0 | 174.8 | 93.2 | 113.9 | 7.7 |
| | 1 | 222.8 | 97.1 | 94.0 | 7.72 |
| | 2 | 123.2 | 102.7 | 106.1 | 7.20 |
| | 3 | 86.2 | 124.4 | 41.6 | 6.98 |
| | 4 | 65.2 | 100.5 | 44.0 | 7.29 |
| $NH_4Cl$ | 0 | 174.8 | 93.2 | 113.9 | 7.7 |
| | 1 | 94.6 | 93.9 | 53.3 | 6.95 |
| | 2 | 24.0 | 103.2 | 10.6 | 6.4 |
| | 3 | 21.0 | 101.1 | 11.2 | 6.38 |
| | 4 | 11.4 | 110.1 | 2.9 | 6.27 |

of yeast extract upto 3% (124.42 mg/gIDS) (Table 22) Whereas the reducing sugar content showed a decrease in response to addition of yeast extract irrespective of the concentrations tested compared to the control.

The pH of the enzyme extract also recorded a decline from pH 7.7 (control) to 6.98 on addition of increased concentration of yeast extract, except with 1% where the pH remained almost same as that of the control.

The data presented in Table 22 also indicate that addition of $NH_4Cl$, as nitrogen source, in the fermentation medium, adversely affected the chitinase synthesis, resulting in a sharp decrease in enzyme yield. The enzyme synthesis was reduced, at 1% $NH_4Cl$ concentration, to 54.1% of that of the control (without $NH_4Cl$) (174.8 U/gIDS). Further addition of $NH_4Cl$ led to sharp decline in enzyme titres to a minimum of 11.4 U/gIDS.

Interestingly soluble protein concentration in the enzyme extract showed gradual increase along with increase in the concentration of $NH_4Cl$ from 93.2 mg/gIDS to 110.1 mg/gIDS. However, reducing sugar content in the enzyme extract showed gradual decline (from 113.94 mg/gIDS to 2.9 mg/gIDS) along with the increase in concentration of $NH_4Cl$. pH profile of enzyme extract showed gradual decrease from 7.7 to 6.27 along with the increase in the concentration of $NH_4Cl$.

**Effect of culture vessel volume to medium volume**

Results presented in Table 23 showed that enzyme production was maximum in petriplates containing 5 g WB medium (232.6 U/gIDS) compared to 10 g WB medium (163.2 U/gIDS). However, results for the studies conducted with conical flasks clearly evidence the fact that the volume of the reactor vessel influence the rate of enzyme synthesis. Thus a sharp increase in enzyme yield along with the increase in the volume of conical flasks was recorded with the maximal production of chitinase in 500 ml conical flask (241.5 U/gIDS) The enzyme synthesis was much faster in the 500 ml flask compared to that in the 100 ml conical flask (92.6 U/gIDS). Whereas there was only a 3.8% increase in enzyme yield in the 500 ml conical flasks when compared to that in petriplates using 5 g of WB medium.

Soluble protein and reducing sugar concentration in the enzyme extract obtained from fermented wheat bran from plates showed a decline when WB quantity was increased from 5 g to 10 g. Whereas in the case of conical flasks a linear increase in protein along with increase in the enzyme extract, was observed irrespective of the volume of the culture vessel. Nevertheless maximal reducing sugar concentration was observed

with 100 ml conical flask (89.4 mg/gIDS) followed by 500 ml, 250 ml and 150 ml conical flasks.

**Effect of sea water concentration and salinity**

Enzyme production was maximum (246.6 U/gIDS) in WB medium humidified with 75% aged sea water and salinities above (100%) and below (50%) respectively resulted in enzyme yields of 94.1% and 77.8% of the maximum (Table 24). In the case of wheat bran moistented with tap water and distilled water enzyme yield was lesser than that observed with various concentration of aged sea water.

The data for the soluble protein content in the enzyme extract showed variation irrespective of the enzyme titres. Nevertheless maximal concentration was recorded in the medium humidified with 75% aged sea water (175.91 mg/gIDS). The reducing sugar content in the enzyme extract also showed variation, irrespective of the enzyme titres, but recorded the maximum with 75% aged sea water (45.32 mg/gIDS).

In general the results suggest the use of 75% aged sea water for enhanced enzyme production.

Table 23 Effect of culture vessel volume to medium volume on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Culture vessel | Bran quality (g) | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) |
|---|---|---|---|---|
| Petri dish Size (10 x 2 cm) | 5 | 232.6 | 131.7 | 85.6 |
| | 10 | 163.2 | 104.5 | 79.1 |
| Conical flask volume (ml) | | | | |
| 100 | 5 | 92.6 | 56.2 | 89.4 |
| 150 | 5 | 147.8 | 59.1 | 84.9 |
| 250 | 5 | 216.2 | 74.2 | 85.3 |
| 500 | 5 | 241.5 | 87.4 | 85.9 |

Table 24 Effect of sea water concentration and salinity on chitinase production by *B. bassiana* using wheat bran medium under SSF

| Moistening medium | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) |
|---|---|---|---|
| 50% aged sea water | 194.4 | 161.1 | 87.9 |
| 75% aged sea water | 246.6 | 175.9 | 95.3 |
| 100% aged sea water | 233.8 | 131.6 | 85.7 |
| Tap water | 172.6 | 170.0 | 92.6 |
| Distilled water | 157.2 | 157.8 | 86.3 |

## 3.11 Chitinase production by solid substrate fermentation using prawn waste as solid substrate

### Effect of initial moisture content of the substrate

The enzyme yield, with respect to different levels of initial moisture content was evaluated every day for a total period of 6 days. Since interesting results were observed only on the 4th, 5th and 6th days, data recorded for these days alone are presented in Table 25. Enzyme production was maximum (22.1 U/gIDS) in PW medium with 1:5 ratios (v:w, sea water: prawn waste) of initial moisture content and on the 4th day. It was interesting to note that the rate of enzyme synthesis was rapid with lower initial moisture content levels when compared with higher initial moisture content of the substrate. For the moisture content less than 3:5 the enzyme yield declined on the 5th day after recording a maximum on the 4th day. Whereas for the higher moisture contents, (3:5-7:5) enzyme titres recorded their maximum on the 5th day only. It was also noted that the increase in enzyme titres from the 4th day to 5th day was rapid for the higher initial moisture content levels. Although a maximum of 37.0 U/gIDS was obtained with the moisture content of 6:5 (v/w), there was more free water at this condition which led to handling problems.

Soluble protein content in the enzyme extract showed a marginal variation irrespective of the initial moisture content of 1:5 (v:w) when maximal enzyme titre was recorded

Table 25 Effect of initial moisture content on chitinase production by *B. bassiana* using prawn waste medium under SSF

| Initial moisture content (v:w ratio) | Enzyme activity (U/gIDS) | | | Soluble protein (mg/gIDS) | | | Reducing sugar (mg/gIDS) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Incubation period (days) | | | Incubation period (days) | | | Incubation period (days) | | |
| | 4 | 5 | 6 | 4 | 5 | 6 | 4 | 5 | 6 |
| 0.5:5 | 1.42 | 8.6 | 0.0 | 111.1 | 105.0 | 112.1 | 5.9 | 3.3 | 3.3 |
| 1:5 | 22.1 | 14.1 | 2.6 | 103.7 | 104.1 | 109.3 | 6.8 | 3.2 | 5.6 |
| 2:5 | 6.0 | 4.5 | 0.1 | 107.7 | 105.2 | 112.8 | 2.2 | 5.0 | 4.5 |
| 3:5 | 5.4 | 6.6 | 11.3 | 106.2 | 105.8 | 103.6 | 2.1 | 2.2 | 4.2 |
| 4:5 | 0.3 | 8.0 | 21.4 | 94.2 | 101.3 | 106.4. | 0.6 | 4.7 | 1.3 |
| 5:5 | 3.9 | 23.4 | 14.5 | 96.4 | 102.6 | 104.2 | 0.8 | 8.2 | 5.4 |
| 6:5 | 5.8 | 37.0 | 26.1 | 110.6 | 101.3 | 96.6 | 3.6 | 4.0 | 2.4 |
| 7:5 | 17.7 | 33.6 | 2.5 | 102.8 | 99.8 | 93.3 | 2.0 | 1.3 | 1.3 |

(4th day). Very similar to enzyme yield, reducing sugars also recorded, on the 5th day, a decline with the low moisture contents, and a maximum with higher moisture content levels. However a maximum of 8.2 mg/gIDS was recorded with the moisture content of 5:5 (v:w) on the 5th day.

In general, the results of this experiment recommend the use of low initial moisture content (1:5 ratio of moistening media (sea water to substrate) and 4 days of incubation as ideal condition for maximal enzyme yield. Hence these conditions were employed in the subsequent experiments.

**Effect of pH of the substrate**

Enzyme production was maximum in the prawn waste medium humidified with sea water with an initial pH 10 (Figure 26) Two peaks of enzyme activity, a small peak at pH 5.0 and a large peak at pH 10 were observed in spite of significant levels of enzyme production over a wide range of pH (5-13), which varied between 14.7-31.4 U/gIDS.

Interestingly the pH of the fermented substrate, after fermentation for 4 days, varied between pH 8.0 (initial pH 11.0) and 8.26 (initial pH 6.0) irrespective of the initial pH of the substrate. While in the case of initial acidic pH the pH raised to alkaline conditions (pH 8.02), with respect to initial alkaline conditions (pH 13-9.0) the pH declined towards pH 8.0, after fermentation.

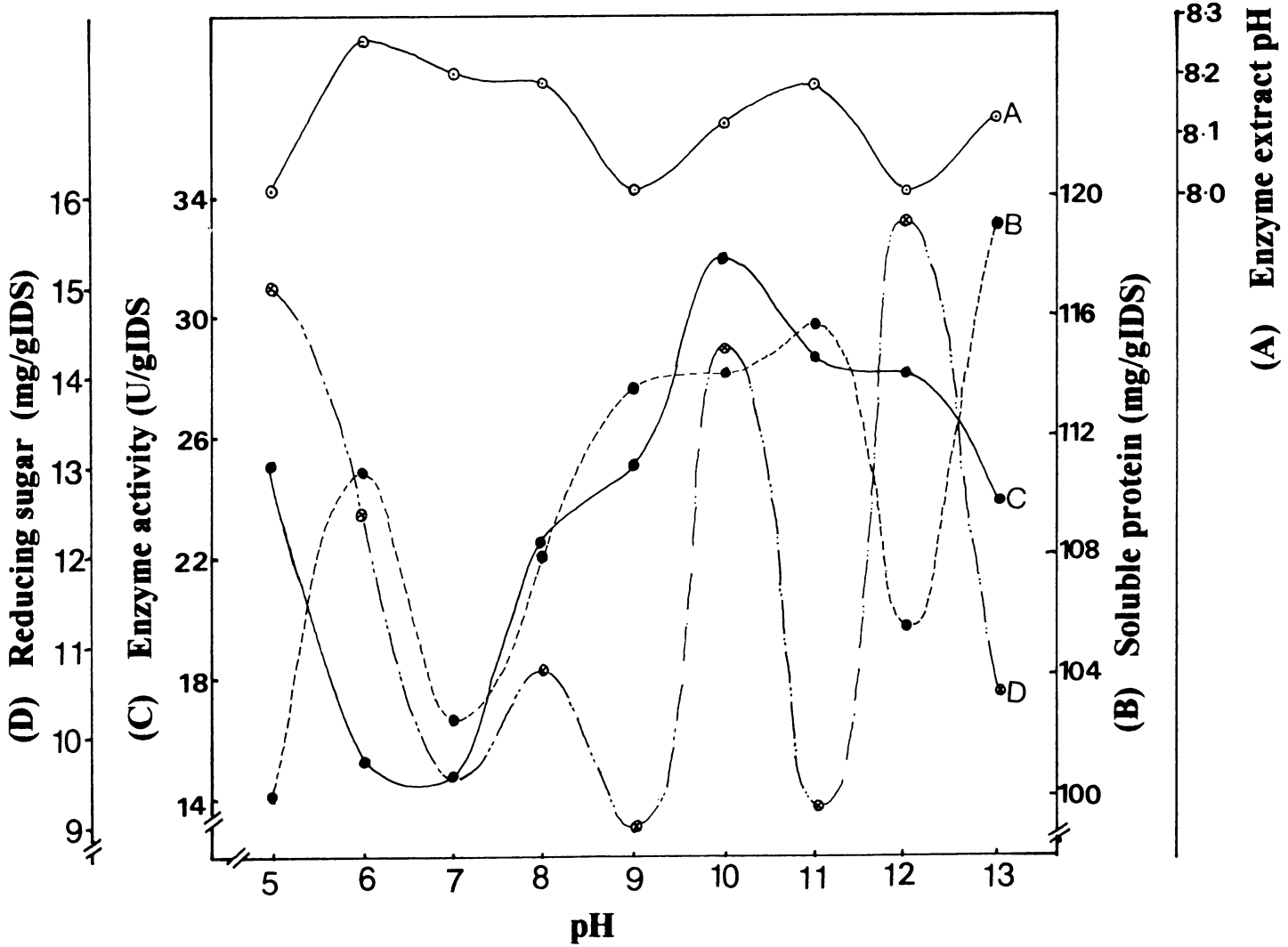



**Figure 26** Effect of initial pH of the substrate on chitinase production by *B.bassiana* using prawn waste medium under SSF

The data presented in the Figure 26 suggest that the soluble protein content in the enzyme extract varied, irregularly, irrespective of the initial pH of the substrate and the level of enzyme yield. Reducing sugar content also varied irrespective of the pH levels tested. However, there was some relationship with enzyme yield, as maximal concentration of reducing sugar was incurred in the medium at the initial pH of 5.0 and 13.0.

**Effect of sodium chloride**

Maximal yield of chitinase could be produced when NaCl was added to the sea water prawn waste medium at 1% level (Figure 27), although considerable levels of enzyme yield could be recorded at all concentrations of NaCl tested. The enzyme titres in general varied from 11.4 U/gIDS (15.0%) to 56.0 U/gIDS (1% NaCl). 178.3% and 116 % of enzyme yield could be obtained by the addition of 1% and 2.5 NaCl respectively to the medium. Further increase in NaCl concentration led to a decline in enzyme yield when compared with the control.

Interestingly, unlike in the case of other instances, the soluble protein recorded increase and decrease along with the increase and decrease of enzyme titres. It was noted that a significant raise in reducing sugar (259%) could be effected in response to addition of 1% NaCl. When the concentration of NaCl was raised above 1%, the reducing sugar,

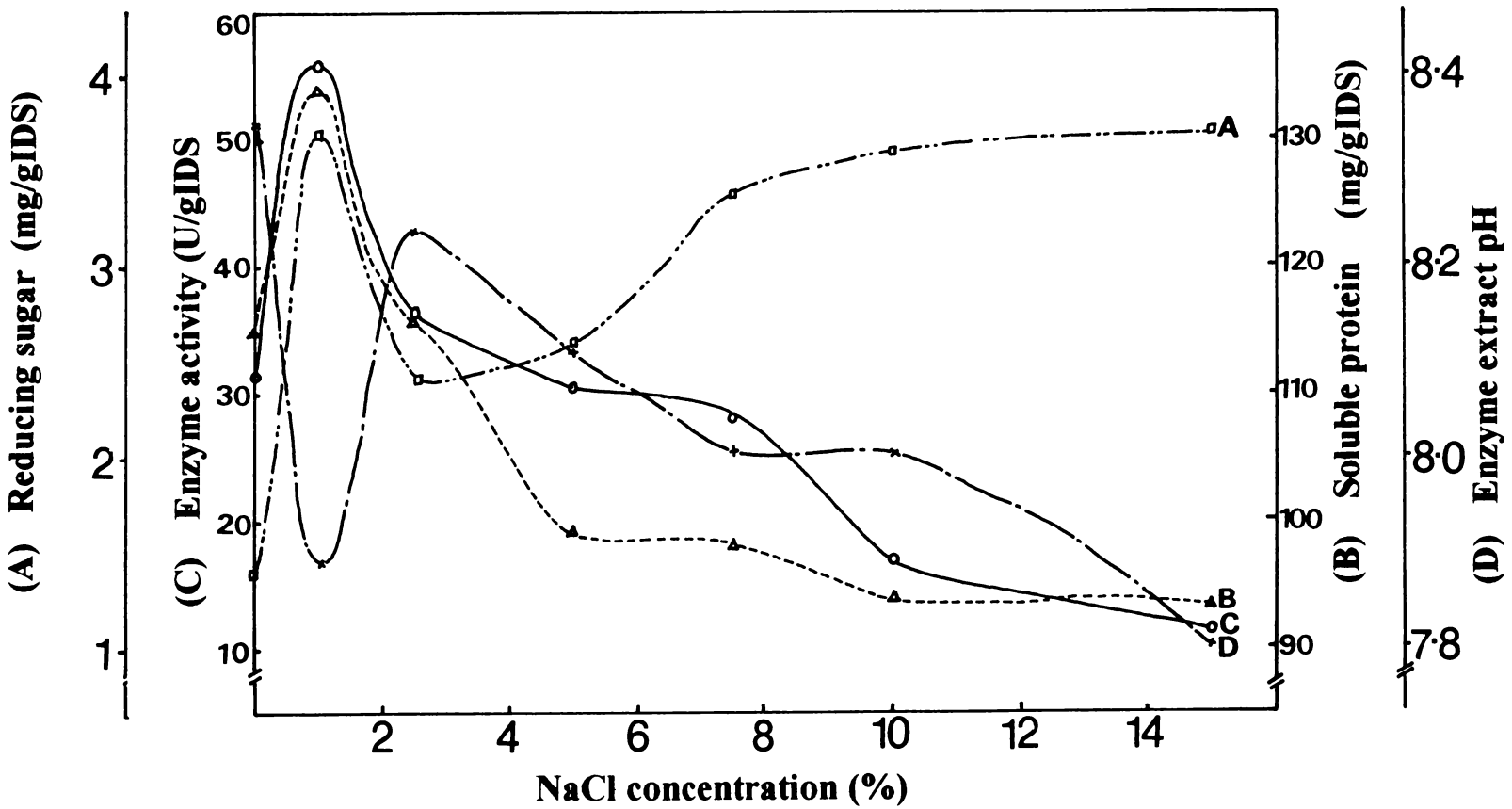



**Figure 27** Effect of sodium chloride on chitinase production by *B.bassiana* using prawn waste medium under SSF

after declining at 2.5% NaCl, showed gradual increase along with the increase in NaCl concentration.

The pH of the enzyme extract showed a sharp decline (pH 7.89) from that of control (pH 8.35), with 1% NaCl and the recorded an increase with 2.5% NaCl(8.24) However for NaCl concentrations above 2.5%, the pH of the enzyme extract, showed gradual decline along with the increase in the concentration of NaCl after fermentation.

**Effect of phosphate**

Phosphate concentrations in the medium considerably influenced the rate of chitinase synthesis when prawn waste was used as solid substrate (Table 26).

Chitinase production increased along with an increase in the concentration of $K_2HPO_4$. Thus there was a 160% increase in enzyme titres at 5% (w/w) of $K_2HPO_4$ compared to that without phosphate. The soluble protein and reducing sugar and pH of the enzyme extract showed a linear increase along with increase in enzyme yield and increase in the concentrations of $K_2HPO_4$.

Whereas, in the case of $KH_2PO_4$, maximum enzyme titres (117.0 U/gIDS), 211% increase, was recorded with 2.5% (w/w) and significant levels of enzyme titres was observed at other concentration of $KH_2PO_4$. Nevertheless there was 101.4%

Table 26 Effect of phosphate on chitinase production by *B. bassiana* using prawn waste medium under SSF

| Phosphate concentration % (w/w) | Enzyme activity (U/gIDS) | Soluble protein (mg/gIDS) | Reducing sugar (mg/gIDS) | pH of the extract |
|---|---|---|---|---|
| Control | | | | |
| No phosphate | 55.3 | 81.8 | 3.9 | 7.9 |
| $K_2HPO_4$ | | | | |
| 1 | 61.2 | 90.1 | 4.0 | 7.9 |
| 2.5 | 77.4 | 102.9 | 3.7 | 8.3 |
| 5.0 | 89.0 | 115.6 | 4.1 | 8.4 |
| $KH_2PO_4$ | | | | |
| 1 | 56.1 | 97.5 | 0.61 | 8.02 |
| 2.5 | 117.0 | 111.8 | 0.82 | 8.2 |
| 5.0 | 83.4 | 111.3 | 4.0 | 8.7 |
| $K_2HPO_4$ $KH_2PO_4$ | | | | |
| 1:1 | 25.1 | 109.4 | 2.4 | 8.3 |
| 1:2.5 | 107.0 | 114.8 | 0.46 | 8.54 |
| 1:5 | 87.1 | 122.4 | 2.98 | 8.72 |
| 2:1 | 100.4 | 118.0 | 0.21 | 8.32 |
| 2:5 | 108.2 | 123.0 | 2.4 | 8.58 |
| 3:1 | 75.4 | 113.3 | 1.14 | 8.34 |
| 3:2.5 | 49.2 | 127.4 | 3.1 | 8.42 |
| 3:5 | 33.4 | 132.3 | 0.8 | 8.75 |
| 5:1 | 62.1 | 130.0 | 0.6 | 8.78 |
| 5:2.5 | 40.2 | 130.4 | 1.74 | 8.61 |
| 5:5 | 24.3 | 136.0 | 1.19 | 8.81 |

and 150.8% increase with 1% and 5% $KH_2PO_4$. The soluble protein content, reducing sugar and pH of the enzyme extract showed a linear increase along with increase in the concentration of $KH_2PO_4$. There was a significant change in the pH of the enzyme extract compared to control, from pH 7.9 to 8.7

The data for the enzyme production in the presence of $K_2HPO_4$ and $KH_2PO_4$ at various combinations (ratios) are presented in the Table 26. Enzyme yield declined drastically, compared to control experiments (55.3 U/gIDS) for the ratios 1:1, 3:2.5, 3:5, 2:2.5 and 5:5 and recorded enzyme yield varying between 24.3 U/gIDS-49.2 U/gIDS. Enzyme yields with 1:1 and 5:5 ratios were almost identical (25.1 and 24.3 U/gIDS respectively) recording only 44% of the yield obtained, when the salts were mixed at equal proportions. Interestingly, when $K_2HPO_4$ and $KH_2PO_4$ were mixed at a ratio of 1:2.5 and 2:5, the enzyme yield was almost similar (107.0 and 108.2 U/gIDS respectively) recording an 193.4-195.7% increase in enzyme yield. In these cases higher proportion of $KH_2PO_4$ was found to influence enzyme yield. However, 181.6% enzyme yield could also be obtained at a ratio of 2:1, when $K_2HPO_4$ was added at higher proportion. Comparatively, other ratios 3:1 and 5:1, where $K_2HPO_4$ was at higher proportion yielded only 130.3% and 112.3% increase respectively. On the other hand, $KH_2PO_4$, at higher proportion (1:5 and 1:2.5) boosted enzyme yield. In general, from the results it is inferred that addition of

phosphate as $KH_2PO_4$ individually or in combination, at higher proportion, with $K_2HPO_4$, led to enhanced (double fold) enzyme yield, compared to $K_2HPO_4$.

The final pH of the fermented substrate varied in general between 8.32 - 8.81, without much significance for the various ratios of phosphates tested. However, it was higher (0.4-0.9) than the control experiments.

**Particle size, inoculum type and incubation time**

Data presented in Table 27 indicate that inoculum type influenced preference of particle size with respect to maximal chitinase synthesis. Of course incubation period also influenced rate of enzyme synthesis despite recording maximal levels after 5 days of incubation irrespective of the inoculum type.

In general, particle size of 425-600 micron supported maximal enzyme yield for the spore inoculum throughout the period of incubation, recording a maximum of 248.0 U/gIDS compared to other particle size. < 425 micron supported maximal enzyme yield on the 2nd and 4th day of incubation compared to 425-600 micron particle size for the spore inoculum. Nevertheless, irrespective of the particle size of the substrate, the enzyme yield varied between 116.0 to 248.0 U/gIDS after 5 days with spore inoculum. Whereas in the case of vegetative inoculum, although maximal enzyme yield

Table 27 Effect of particle size, inoculum type, incubation period on chitinase production by *B. bassiana* using prawn waste medium under SSF

| Incubation period | particle size | Enzyme activity (U/gIDS)) | | Soluble protein (mg/gIDS) | | Reducing sugar (mg/gIDS) | | pH of the extract | |
|---|---|---|---|---|---|---|---|---|---|
| | | Inoculum type | | Inoculum type | | Inoculum type | | Inoculum type | |
| (days) | (micron) | spore | veg. | spore | veg. | spore | veg. | spore | veg. |
| 1 | < 425 | 18.2 | 4.8 | 90.4 | 86.6 | 0.25 | 0.63 | 8.1 | 7.98 |
| | 425-600 | 25.4 | 12.6 | 80.7 | 85.2 | 0.86 | 0.28 | 8.21 | 8.01 |
| | 600-1000 | 6.2 | 7.1 | 91.4 | 86.1 | 0.52 | 0.1 | 8.19 | 8.1 |
| | 1000-1400 | ND | ND | ND | ND | ND | ND | ND | ND |
| | 425-1400 | 6.5 | 9.5 | 83.14 | 83.3 | 0.49 | 0.73 | 8.04 | 8.05 |
| 2 | < 425 | 60.0 | 33.6 | 85.0 | 98.3 | 0.92 | 0.81 | 8.2 | 8.22 |
| | 425-600 | 31.4 | 68.4 | 93.2 | 92.4 | 0.38 | 0.41 | 8.15 | 8.13 |
| | 600-1000 | 63.7 | 53.7 | 87.0 | 91.1 | 0.67 | 0.31 | 8.23 | 8.3 |
| | 1000-1400 | ND | ND | ND | ND | ND | ND | ND | ND |
| | 425-1400 | 33.0 | 43.2 | 89.0 | 95.2 | 1.2 | 0.95 | 8.15 | 8.2 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 3 | < 425 | 102.4 | 59.3 | 89.1 | 96.1 | 1.89 | 2.1 | 8.31 | 8.34 |
| | 425-600 | 70.1 | 79.8 | 93.1 | 93.1 | 2.14 | 3.1 | 8.29 | 8.22 |
| | 600-1000 | 72.3 | 108.6 | 86.2 | 90.4 | 1.3 | 4.2 | 8.34 | 8.42 |
| | 1000-1400 | 82.5 | 31.4 | 96.3 | 82.1 | 2.89 | 2.1 | 8.39 | 8.31 |
| | 425-1400 | 94.3 | 56.4 | 90.4 | 96.2 | 3.1 | 3.2 | 8.26 | 8.35 |
| 4 | < 425 | 160.4 | 89.2 | 93.0 | 88.2 | 84.2 | 2.1 | 8.44 | 8.4 |
| | 425-600 | 90.3 | 98.6 | 93.0 | 95.4 | 2.1 | 4.2 | 8.45 | 8.49 |
| | 600-1000 | 86.2 | 121.4 | 85.0 | 87.2 | 2.36 | 3.6 | 8.48 | 8.49 |
| | 1000-1400 | 79.8 | 57.3 | 78.2 | 75.6 | 2.56 | 4.1 | 8.44 | 8.44 |
| | 425-1400 | 118.5 | 65.1 | 92.2 | 97.4 | 5.58 | 3.2 | 8.41 | 8.4 |
| 5 | < 425 | 130.2 | 160.1 | 86.3 | 91.4 | 5.1 | 3.52 | 8.51 | 8.53 |
| | 425-600 | 248.0 | 92.0 | 90.4 | 94.5 | 7.86 | 26.0 | 8.46 | 8.51 |
| | 600-1000 | 211.0 | 151.8 | 91.2 | 93.6 | 6.21 | 3.2 | 8.48 | 8.52 |
| | 1000-1400 | 120.1 | ND | 79.5 | ND | 3.26 | ND | 8.45 | ND |
| | 425-1400 | 160.0 | 172.6 | 89.2 | 91.2 | 3.86 | 4.1 | 8.53 | 8.52 |

ND - Not determined
Veg. - Vegetative

was recorded after 5 days with a mixed particle size of > 425 micron (172.6 U/gIDS), and particle size of 425-600 micron supported enhanced enzyme yield during the initial days.

Comparatively, spore inoculum yielded higher levels of enzyme (248.0 U/gIDS) than vegetative inoculum (172.6 U/gIDS) after 5 days of incubation.

Soluble protein in the enzyme extract did not show significant variation although varied between 78.2 mg/gIDS to 93.2 mg/gIDS and 75 mg/gIDS and 78 mg/gIDS respectively for the spore and vegetative inoculum irrespective of the different particle sizes of the substrates tested.

Similarly pH of the enzyme extract varied from 8.04 to 8.52 and from 7.98 to 8.53 for the spore inoculum and vegetative inoculum respectively indicating insignificant variation with respect to different particle size.

**Effect of additional nitrogen source (yeast extract)**

Results presented in Figure 28 clearly indicate that addition of yeast extract in the prawn waste medium led to reduction in chitinase synthesis from 163.2 U/gIDS to 90.8 U/gIDS under SSF significantly. Increase in concentration of yeast extract drastically inhibited enzyme synthesis.

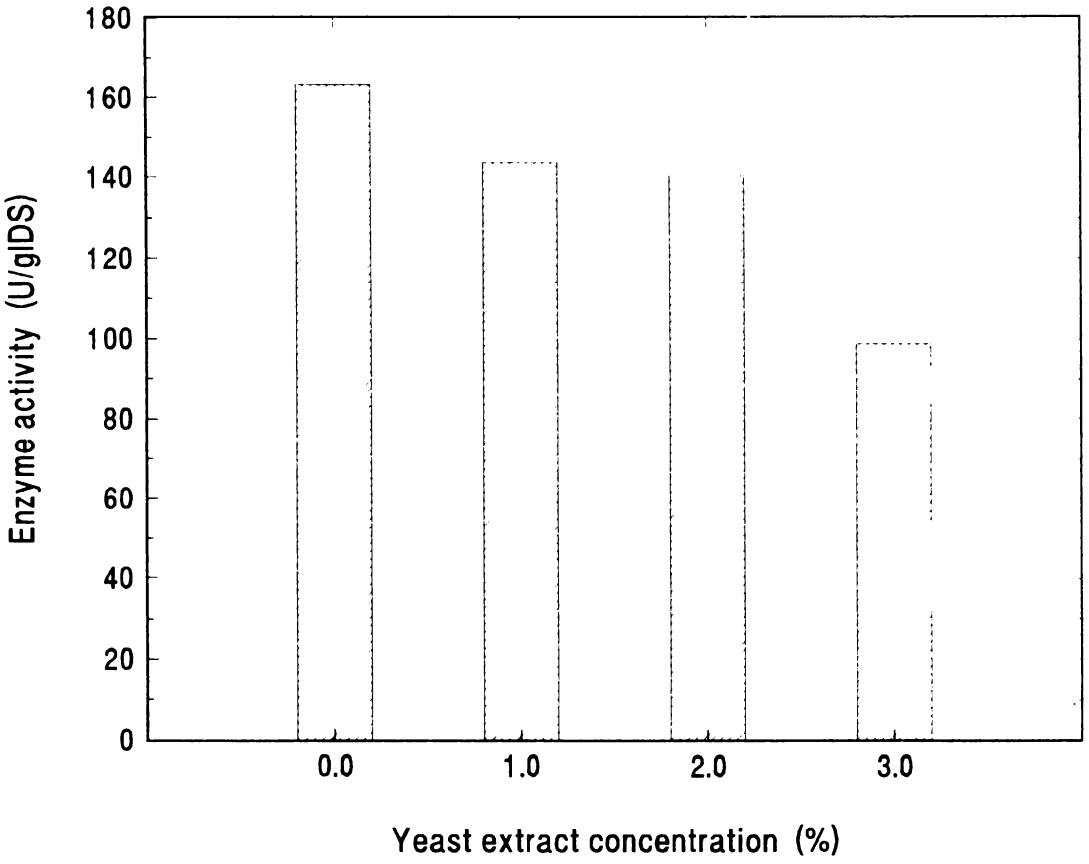



**Figure 28** Effect of additional nitrogen source (yeast extract) on chitinase production by *B.bassiana* using prawn waste medium under SSF

## 3.12 Enzyme studies

### 3.12.1 Enzyme profile of the crude enzyme preparation of chitinase produced under solid substrate fermentation

The crude enzyme preparation of *B. bassiana* obtained through solid substrate fermentation using WB medium showed the presence of chitinase (152.6 U/gIDS), β-1,4-N-acetyl-D-glucosaminidase/chitobiase (0.238 U/gIDS), amylase (174.3 U/gIDS), CM cellulase (28.5 U/gIDS), laminarinase (69.21 U/gIDS), protease (527.8 U/gIDS), chitosanase (8.77 U/gIDS) and muramidase (4800 U/gIDS) (Table 28).

### 3.12.2 Purification of chitinase produced by solid substrate fermentation using wheat bran medium

Chitinase from *B. bassiana* produced under solid substrate fermentation using wheat bran medium was purified by a procedure involving ammonium sulphate fractionation, dialysis and gel filtration chromatography using Sephadex $G_{100}$ (Figure 29) and Sephadex $G_{200}$ (Figure 30).

A 24.2 fold purification was achieved for chitinase with 22.3% recovery (Table 29). The specific activity of the chitinase, increased, during the course of purification, from 418.0 U/mg of protein after ammonium sulphate fractionation to 940 units and 1126 units per mg of protein after gel filtration with Sephadex $G_{100}$ and Sephadex $G_{200}$ respectively.

Table 28 Enzyme profile of crude chitinase preparation of *B. bassiana* produced using wheat bran medium under SSF

| Enzyme | Enzyme activity (U/gIDS) |
|---|---|
| chitinase | 152.6 |
| N-acetyl-D-glucosaminidase/ chitobiase | 0.238 |
| chitosanase | 8.77 |
| CM cellulase | 28.5 |
| protease | 527.8 |
| laminarinase | 69.21 |
| muramidase | 4800.0 |

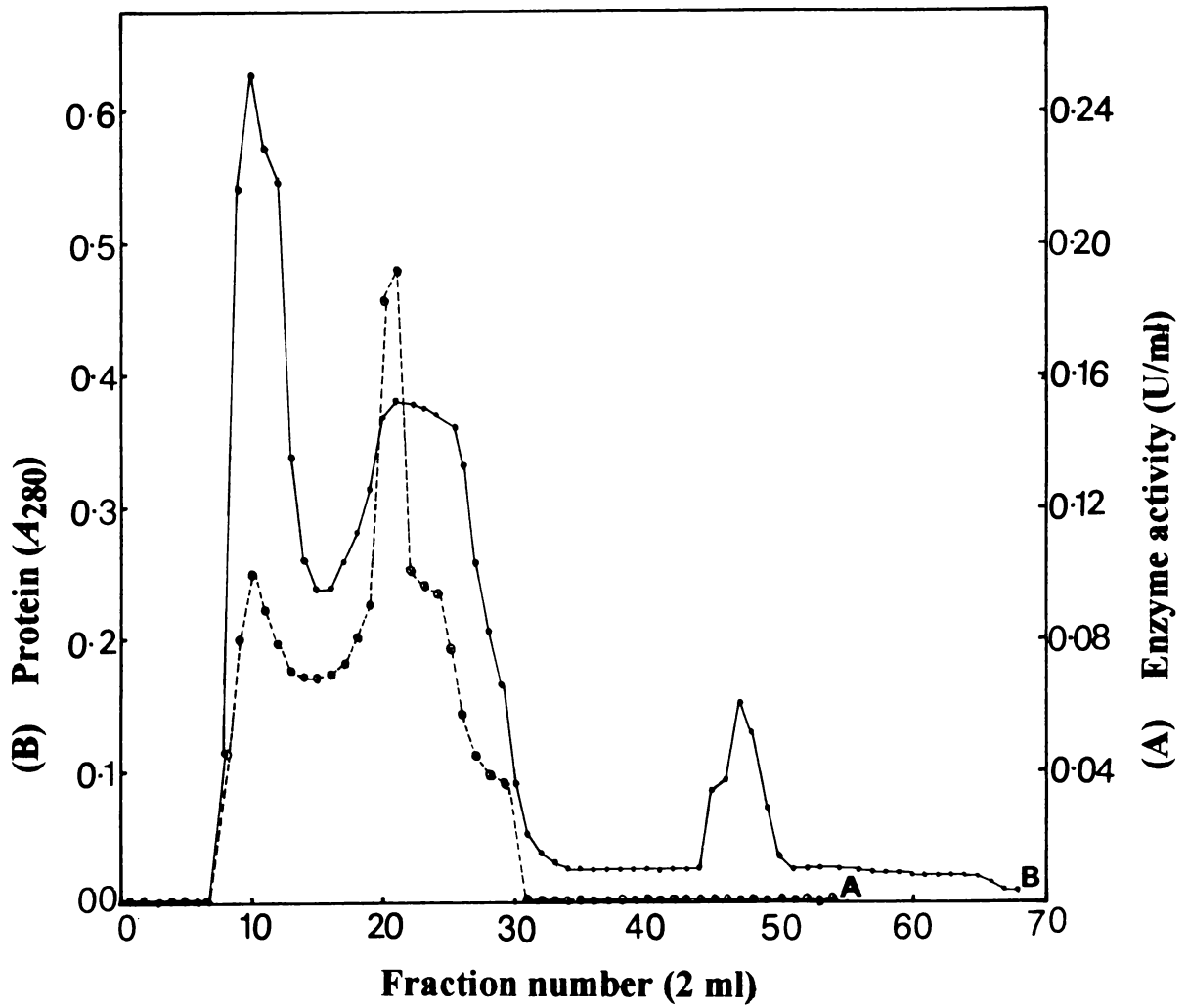



**Figure 29** Gel filtration chromatography on Sephadex $G_{100}$ of the chitinase produced by *B.bassiana* using wheat bran medium under SSF

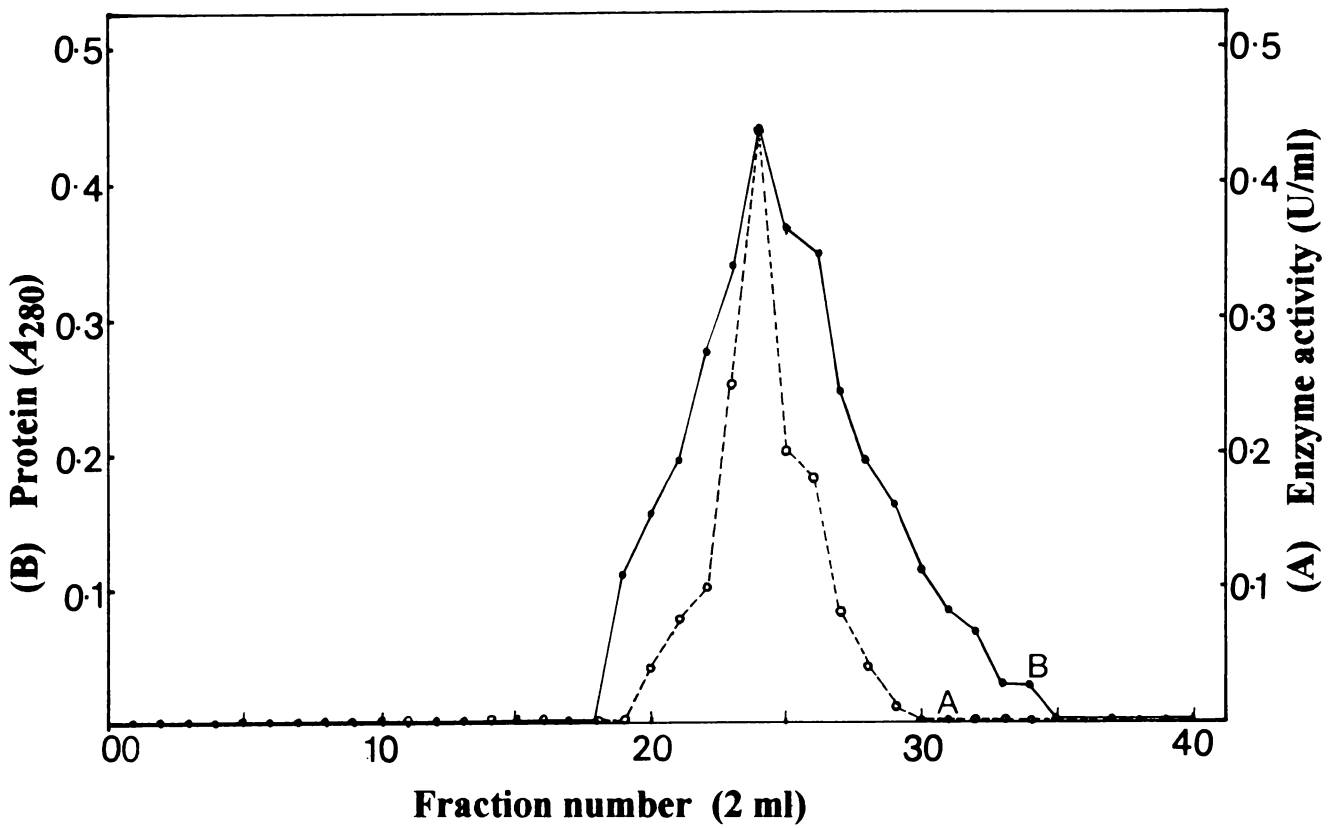



**Figure 30** Gel filtration chromatography on Sephadex $G_{200}$ of the chitinase produced by *B.bassiana* using wheat bran medium under SSF

Table 29 Summary of purification process of the chitinase produced by *B. bassiana* using wheat bran medium under SSF

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U/mg protein) | Purification fold | Recovery (%) |
|---|---|---|---|---|---|
| Crude extract | 4196.0 | $197 \times 10^3$ | 47 | 1.0 | 100.0 |
| $NH_4SO_4$ precipitate (50-80%) | 306.0 | $120 \times 10^3$ | 418 | 1.15 | 65.0 |
| Gel filtration | | | | | |
| Sephadex $G_{100}$ | 70.0 | 65810 | 940 | 20.0 | 33.4 |
| Sephadex $G_{200}$ | 39.0 | 43931 | 1126 | 24.0 | 22.3 |

Electrophoresis of the purified enzyme on polyacrylamide gel recorded a single band when stained for protein (plate 4).

## 3.13 Properties of chitinase

### pH

It was observed that the enzyme was considerably active over a wide range of pH (5-6 to 9.6) (Figure 31). The enzyme activity showed two peaks, a small peak at pH 6.0, and a large peak at pH 9.0. However it was inferred that the optimum pH for this enzyme is pH 9.0.

### pH stability

The enzyme was observed to be stable over the pH range 5-10 (Figure 32). However, the enzyme lost only 56% and 74% of its activity at pH 4.0 and pH 11.0 respectively.

### Temperature

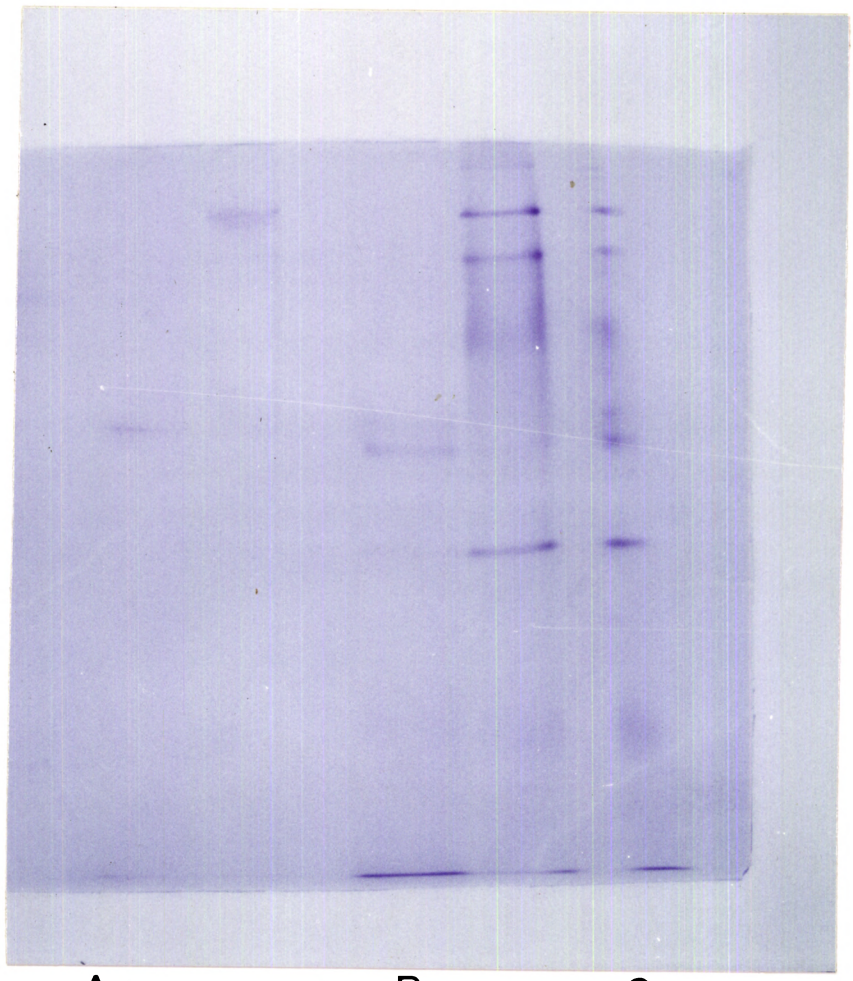
Optimum temperature for chitinase activity of *B. bassiana* was recorded as 32° C (Figure 33), while a considerable level of activity could be recorded upto 47°C. A sharp decline in enzyme activity was observed at temperatures above 47°C.

**Plate 4** Non-denaturing polyacrylamide gel electrophoresis of chitinase of *Beauveria bassiana*

Lane A - Purified enzyme after gel filtration on Sephadex $G_{200}$

B - Purified enzyme after gel filtration on Sephadex $G_{100}$

C - Purified enzyme after ammonium sulphate precipitation


A
B
C

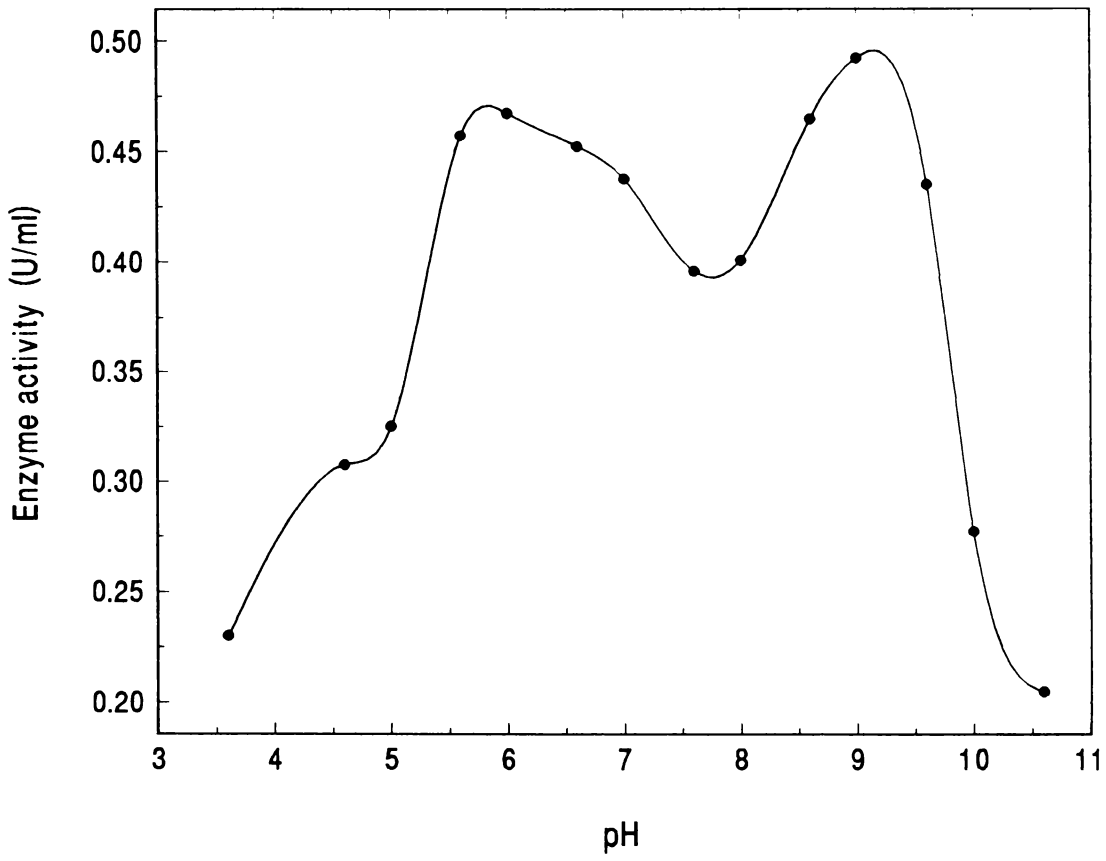



**Figure 31** Effect of pH on activity of chitinase produced by *B.bassiana*

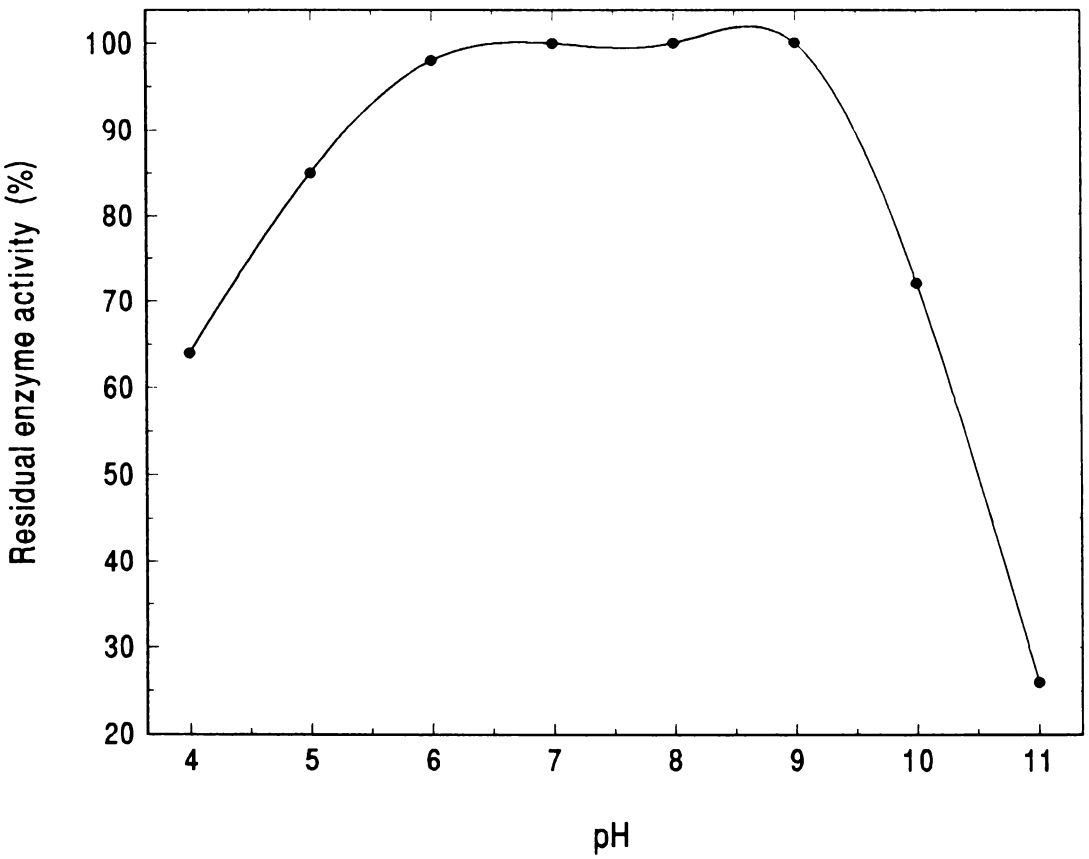



**Figure 32** Effect of pH on stability of chitinase produced by *B.bassiana*

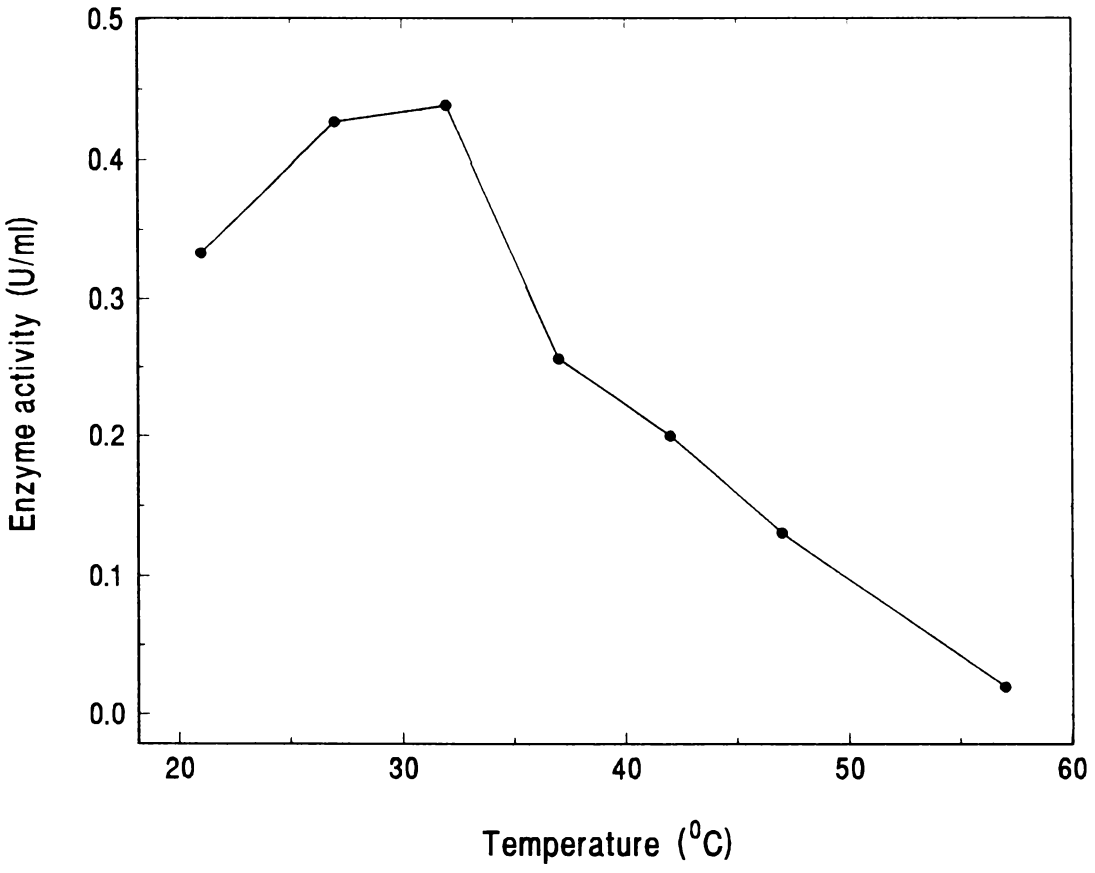



**Figure 33** Effect of incubation temperature on activity of chitinase produced by *B. bassiana*

**Thermostability**

Results presented in Figure 34 indicate that the enzyme was heat stable upto at 50°C. A rapid loss of activity was, however observed at temperatures above 50°C and the enzyme lost its activity at 70°C.

**Metal ions**

Various metal ions were examined for their effect on the activity of chitinase. Results presented in Figure 35 suggested that addition of $Ni^{2+}$, $Cd^{2+}$, $Co^{2+}$ and $Ca^{2+}$ led to 71%, 62%, 63% and 21% reduction in enzyme activity respectively. However addition of $Sn^{2+}$ $Hg^{2+}$ $Ag^{+}$ and $Mg^{2+}$ did not inhibit the enzyme activity.

**Molecular weight**

Molecular weight was determined as 66,000$\pm$ 2000 by gel filtration chromatography on Sephadex $G_{100}$.

**Subunit molecular weight**

Subunit molecular weight of chitinase was estimated by SDS (sodium dodecyl sulphate) polyacrylamide gel electrophoresis was 22,000 $\pm$ 500 with single band (plate 5).

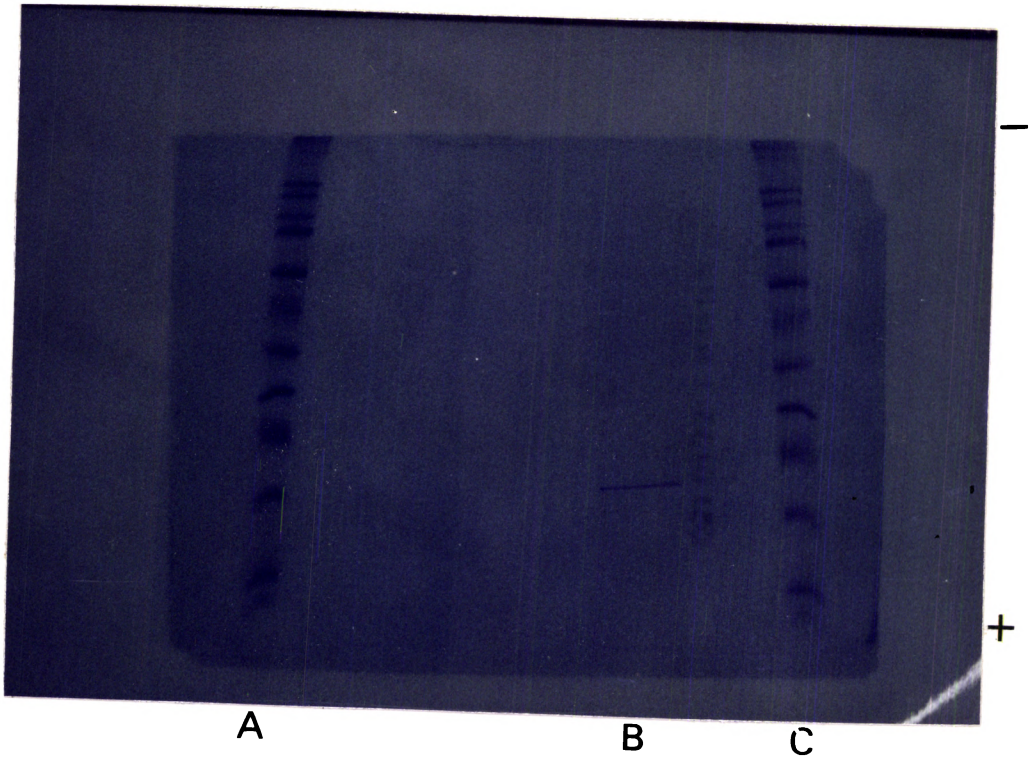
**Substrate specificity**

The results presented in Table 30 indicate that the enzyme was active against a wide range of substrates from highly purified crab shell chitin to very crude prawn shell

Plate 5 SDS-Polyacrylamide gel electrophoresis of chitinase of *B. bassiana*

Lane A and C - Molecular weight markers (Section 2.11.3.4.7.3)

B - Chitinase of *B. bassiana*


−
+
A
B
C

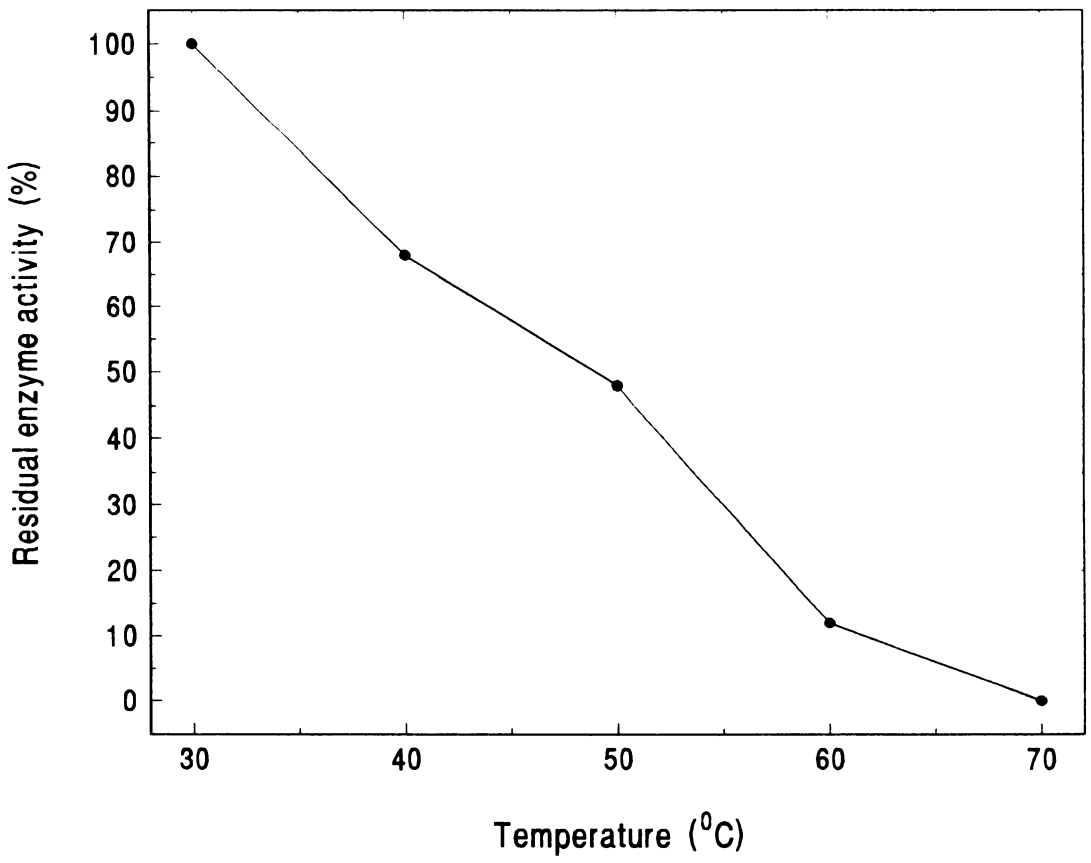



**Figure 34** Effect of temperature on stability of chitinase produced by *B. bassiana*

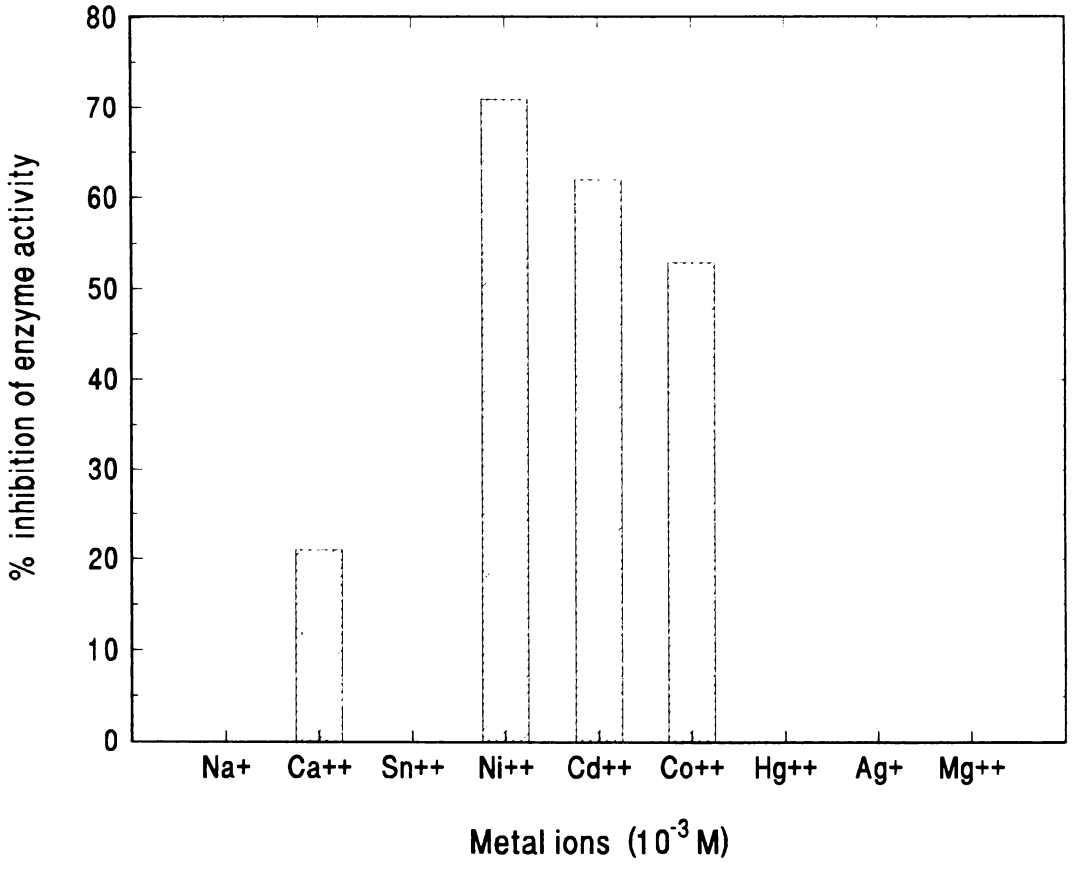



**Figure 35** Effect of metal ions on activity of chitinase produced by *B.bassiana*

powder. The purified chitinase was observed to have a substrate specificity in the following order of preference prawn shell powder (native chitin) colloidal chitin, purified crab shell chitin and purified prawn shell chitin. The enzyme also showed considerable activity on chitotriose, and chitosan powder and no activity on chitobiose.

The purified enzyme preparation was also observed to have the hydrolytic activity for CM cellulose, laminarin, and *Micrococcus lysodeikticus* cells (Table 30)

### 3.14 Fungal mycelial and Yeast cell dissolution

Incubation of crude enzyme with living and non-living (non-autoclaved and autoclaved) freeze dried mycelium of *Rhizoctonia solani*, *Aspergillus niger* and *Mucor* sp. and cells of *Saccharomyces cereviciae* resulted in the release of reducing sugars (as glucose) ( **Table** 31) Reducing sugars released from yeast cells was significantly higher than that from mycelium of *R. solani*, *A. niger and Mucor sp.* The amount of reducing sugars released from the phytopathogenic fungus *R. solani* mycelium was very negligible when compared to the other filamentous non-phytopathogenic fungi tested.

Table 30 Substrate specificity of purified chitinase produced by *B. bassiana* using wheat bran medium under SSF

| Substrates | Reducing sugar as N-acetyl-D-glucosamine (mg) | Reducing sugar as glucose (mg) | As decrease in optical density (A 430) |
|---|---|---|---|
| Chitinous | | | |
| Chitin flakes crab | 0.0686 | 0.1174 | ND |
| prawn | 0.0152 | 0.0151 | ND |
| Prawn shell powder (Native chitin) | 0.0798 | 0.1424 | ND |
| Colloidal chitin | 0.0286 | 0.0320 | ND |
| Chitotriose | 0·0310 | 0·0610 | ND |
| Chitobiose | 0 | 0 | ND |
| Chitosan | ND | 0.1676 | ND |
| Non-chitinous | | | |
| CM Cellulose | ND | 0.0326 | ND |
| Laminarin | ND | 0.1294 | ND |
| *M. lysodeiktieus* cells | ND | ND | ++ |

Table 31 Liberation of reducing sugar from the fungal whole cell wall with crude chitinase preparation of *B. bassiana* produced using wheat bran medium under SSF

| Fungal strain | Reducing sugar liberated | |
|---|---|---|
| | autoclaved cells | non-autoclaved cells |
| *Rhizoctonia solani* | + | + |
| *Aspergillus niger* | + + + + | + + + + |
| *Mucor* sp. | + + + + | + + + + |
| *Saccharomyces cerevicea* | + + + + + + | + + + + + + |

# CHAPTER - FOUR

# DISCUSSION

Chitinase producing fungi were present at significant levels both in water and sediments of marine environments. Results obtained in the present study very clearly indicate that marine environments are potential sources of chitinase producing fungi.

Marine environments, in general, are unique by virtue of their salinity, wide range of mineral content and well knitted ecosystem when compared to terrestrial environments which is constantly disturbed by human activities The marine fungi have not been experimentally tried for their potential in many of the human endeavours in which they would have a major role to play, for example in the field of pharmaceuticals, food, industry etc.

In the present study, chitinolytic fungal populations were more abundant in marine sediments than in sea water. This observation is a clear testimony to the role of chitinoclastic fungi and other chitinolytic microorganism in the secondary productivity of sea, particulary in the process of biodegradation of chitinous exoskeletons (shells) of marine invertebrates and in the cycling of the organic carbon and consequently in the maintenance of fertility of sea (Zobell, 1946) Generally it is known that the west coast of India is more rich in organic content and nutrients than the east coast

(Kurien and Sebastian, 1976). The presence of mud banks particularly in the offshore area of Kerala has contributed to the enormous crustacean fishery resources of India. Especially prawn fishery resources are abundant in the Kerala coast (CMFRI, 1969). Hence it is natural that chitinolytic fungal populations are abundant in the sediments than in water of the marine environments which play a key role in the mineralization of chitinous shells of dead and decaying shrimps in the locality. Except for the study on the distribution of chitinolytic bacteria in the Kerala coast (Ivy Thomas, 1982) no information is available on other chitinolytic microorganisms. In this context, the present results clearly suggest the possiblility of obtaining potential chitinolytic miroorganisms from marine sediments in the context of growing importance for this group, in the light of biotechnological evolution that is unfolding today,

Large quantities of chitin are produced in the oceans of the world each year. Most of the this is probably utilized by biological agents because little accumulates in marine sediments and moreover, if it were not decomposed, it would soon become a serious drain upon carbon and nitrogen in the cycle of these elements (Zobell and Rittenberg, 1938; Compbell and Williams, 1951). It is generally agreed that its degradation in nature is largely due to microbial action (Compbell and Williams, 1951).

The qualitative distribution of chitinase producing fungi was rather limited to very few genera that included species of *Beauveria* and *Mucor*. Since no other reports are available on qualitative distribution of fungi in marine environments of Cochin no comparison could be made. Most interesting observation, made in the present investigation, was the complete dominance of *Beauveria* sp. among the chitinoclastic fungi, isolated from marine environments.

Fermentation of certain microorganisms such as the bacterium *Streptomyces antibioticus*, the fungus *Beauveria* sp. and others is the process used by manufacturers tc obtain large quantities of chitianse (Muzzarelli, 1977). *Beauveria*, which is known in general as entamopathogenic (Steinhaus, 1967), is common in soil, and is known to be used for the large scale production of chitinase in industry (Muzzarelli, 1977). Hence it is quite obvious that this genera dominated the chitinolytic fungal population in marine sediments that is loaded with sedimented invertebrate exoskeletons rich in chitin content.

Claus (1961) reported that chitinase is an extracellular enzyme in *B. bassiana*. Other investigators have also reported that chitinase is produced as an exocellular enzyme in this fungus (Grula *et al.*, 1978) 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). In the present study also *B. bassiana* was observed to produce chitinase extracellularly.

Potential strains for chitinase production was selected for further studies mainly based on their ability to produce enhanced levels of chitinase in an economic medium supplemented with colloidal chitin. The objectives of using the sea water medium added with colloidal chitin for selection was to identify the organism that can produce chitinase in larger quantity, probably as an induced enzyme in a simple medium. Among the strains screened the top ranked potential strain, selected after screening, was identified as *Beauveria bassiana*. This species is already known to produce chitinase abundantly and used in the industrial production of chitinase in some countries like china (Mussey and Tinsley, 1981) USSR (Lisansky and Hall, 1987). Infact, chitinase preparation from *B. bassiana* is available as a commercial product in the name of Boverin in USSR and other preparations, produced by various companies are being tested in the USA primarly to control colorado beetle although other uses are also being sought (Liansky and Hall, 1987).

Initially, the optimum levels of the different environmental variables namely temperature, pH, NaCl

concentration colloidal chitin, additional carbon and nitrogen sources, salinity of sea water, and period of incubation were determined for attaining maximal growth of the selected strain. A sea water complex medium, GPYS medium, suggested by Molitoris and Schaumann (1986) and a mineral salt solution proposed by Hankin and Angnostakis (1975) were used for the growth studies under submerged culture conditions.

*B. bassiana* showed optimal requirement of 27°C for maximal growth although it could record significant growth at 22°C also. Temperatures above 32°C led to poor growth. Results of Barghoorn and Linder (1944) indicated that marine fungi have high temperature requirements (25-30°C) but the results of Tubaki (1969) and Jones and Irvine (1972b) suggest that they have a lower temperature requirement. Byrne (1971) has shown that temperature affects the germination of marine fungi, especially at low temperature. The two imperfect fungi *Asteromyces cruciatus* (F et Mme Moreau) ex Hennbert and *Zalerion maritimum* (Linder). *Anastasiou* prefered higher temperatures for conidial germination. Similar results have been reported by Kohlmeyer and Kohlmeyer (1966) for *Halorphaeria medioretigera* and Kohlmeyer (1968) stated that ascorpores of *Halosphaeria quadricornuta* Cribb et Cribb will not generate at 20°C but do so at 28°C The present results are in agreement with these reports.

Barnghoorn and Linder (1944) showed that some of the higher marine fungi exhibited a double pH peak for growth, e.g. *Microthelia maritima* (Linder) Kohlm. (4.4 and 8.4) and *Luloworthia opaca* (Linder) Cribb et Cribb (5.2 and 8.4). Jones and Irvine (1972) found a similar response in the fungi they tested, one pH peak occurring in the acidic range (6.0-6.6) and the other in the neutral to alkaline range (7.0 - 8.0) In the present study *B. bassiana*, also showed two peaks one at 6.0 and another at pH 9.0 although the optimal pH for maximal growth was pH 9.0.

*B. bassiana* was observed to grow significantly over a wide range of NaCl concentrations varying from 0-12% although maximal growth could be recorded at 3-5% NaCl concentration. When tested in sea water, diluted to various dilutions, maximal growth was recorded with 75% aged sea water, both in liquid and solid media. This fungus could only record about 50% of the optimal growth, in distilled water medium, indicating the requirement of salinity or NaCl for its maximal growth and biomass accumulation.

The maximum salinity for growth varied from 10% sea water for *Dendryphiella salina* (Jones, 1963 a) to 100% sea water for *Lulwarthia floridana* (Janes, 1963 b), *Orbimyces spectabilis* Linder, *Varicosparina ramulosa* (Meyers and Hoyo, 1966), *Lindra thalassiana* (Meyers and Simms, 1965) and

*Crollospora maritima* (Meyers and Scott, 1967; Byrne, 1971). Three isolates of *D. salina* used by Byrne (1971) exhibited optimum growth at relatively high salinities 60-70% sea water. Optimum salinity for growth was 80% sea water for *Aspergillus flavus* Link. (Ritchie, 1959) and 100% sea water for *Pencilium notatum* Westling (Byrne, 1971) *B. bassiana* isolated and used in the present study showed optimum salinity requirement of 75% aged sea water similar to those species mentioned above.

The formulation of balanced fermentation media is critical to good growth and enzyme yields. The use of chemically defined media tends to be expensive and usually leads to slow growth and low yields. However, designing of an ideal medium for growth and enzyme production is inevitable while developing an ideal bioprocess. Hence, the nutritional factors were optimized Initial concentration of colloidal chitin, requirements of additional carbon and nitrogen sources were optimized.

Colloidal chitin, was required in large concentration for maximal biomass accumulation. In spite of growth at significant levels, at concentration varying from 1 to 10%, maximal growth was recorded with 8% colloidal chitin indicating strong chitinolytic nature of the fungus besides demonstrating the ability of the fungus to grow over chitinous wastes abundantly.

Among the various carbon sources tested glucose was found to support maximal growth. Most interestingly, the fungus could grow to a maximum with a very high concentration of glucose (12%) although 3-10% of glucose could promote high rate of growth. Glucose concentrations were not found to repress the growth of this marine fungus and instead boosted the growth. Results indicate the saccharophilic nature of the marine fungus.

According to Jone and Byrne (1976) there is an enormous variation in the range of carbohydrates utilized by the higher marine fungi. Ahearn *et al.* (1962) examined the assimilation of carbon and nitrogen by marine and terrestrial strains of *Rhodotorula* and found no differences ascribable to environmental conditions. According to Van Uden and Fell (1968) marine forms, on average, utilized 19.2 compounds. While non-marine forms assimilated on average 12.8 compounds. They attributed to the greater variability of the marine forms to their need to utilize any nutrients available in an environment where nutrient supplies are often critical.

In the present study, marine *B. bassiana* showed preference to utilize wide range of carbon sources tested. It could grow significantly, using, besides glucose, mannose, maltose, arabinose, sucrose, galactose and glycerol While lactose led to a poor growth, ribose and xylose, inhibited

growth. The inorganic carbon source trisodiumcitrate also led to poor growth. These observations could be attributed to the ability of *B. bassiana* to utilize any nutrients in its natural marine sedimemt environment, where it comes into contact wide range of substrates during the mineralizaton process.

Among the nitrogen sources tested, yeast extract supported maximal growth compared to beef extract and peptone, and malt extract inhibited growth. Yeast extract is an excellent stimulator of bacterial growth and frequently used in culture media in place of meat extract. It is a rich source of the `B vitamins and is used to supply these factors in culture media. For this reason it is superior to meet extract in most culture media (Salle, 1993) Thus yeast extract might have boosted the growth of the fungus in the culture medium compared to other nitrogen sources by satisfying the requirements of the fungus. Among the amino acids tried glycine supported maximal growth, indicating selective preference of this marine fungus for growing maximum.

*B. bassiana* was found to be fast growing marine fungus since its generation time was recorded as 7.5 h and it could record maximal growth by 54 h. It has been reported that the average mass doubling time of common fungi in submerged fermentation is 4-8 h. (Wang *et al.*, 1979)

According to Jennings (1986) for a fungus, sea water poses three problems. First, it is a medium of relatively low water potential. Second, it contains relatively high concentration of ions being potentially capable of exerting toxic effects on cell processes and that it has an alkaline pH. In batch culture, a fungus is able often to nullify relatively easily these properties of sea water which under natural conditions would pose problems for growth. Thus in culture the low water potential presents less of a problem, since the fungus is added in the generation of the turgor necessary for growth by the relatively high concentration of the carbon source, from which organic solutes can be synthesized within the cytoplasm and these can make a significant contribution to the solute potential and therefore turgor potential. Thus the need to absorb ions to generate turgor is kept to a minimum. Thus in the present study, the observations made with respect to signififant growth by *B. bassiana* in batch culture using aged sea water, NaCl and high concentration of carbon source, such as collodial chitin and glucose are in agreement with the hypothesis proposed by Jennings.

Submerged fermentation production of chitinase by *B. bassiana* was studied using a mineral salts colloidal chitin medium. Initially the optimal levels of the different environmental variables namely temperature, pH, NaCl,

concentration, substrate concentration, additional nitrogen sources, inoculum concentration, requirement for phosphate and incubation period were determined for attaining maximal enzyme production by the selected strain.

Maximal enzyme production was observed when the optimal conditions were incubation temperature 27°C, pH 6.0-9.0, NaCl concentration 9%, yeast extract 9%, colloidal chitin 8% 12 mg dry weight equivalent of vegetative inoculum per 100 ml and 120 h of incubation. $MgSO_4$ 0.25% and a combination of $K_2HPO_4$ and $KH_2PO_4$ at a ratio of 2 0.5% (w/v) enhanced chitinase production under submerged fermentation. Results indicated that in general those environmental conditions which promoted maximal growth favoured maximal enzyme production too. It was further noted that the enzyme synthesis was not growth associated since significant yield of enzyme was recorded during late stationary phase of growth.

Similar observations were made with *Benecka neptuna* which recorded maximal chitinase at 25-27°C after 4-5 days, and at pH 6.0 and 7.0 (Hood and Mayer, 1977a) *Serratia marcescens* elaborated higher quantities of chitinase with shrimp chitin (Boxby and Gray, 1968). However, the shrimp chitin used was a highly purified one compared to that used in the present study. The reduction of substrate particle size resulted in increased chitinase activity and maximal yields of

enzyme occured on 1.5% -2.0% chitin (Boxby and Gray, 1968 ). However, *Chytriomyces* required only 0.2% substrate for maximal chitinase production (Reisert and Fuller, 1962). Whereas in the present study B. bassiana was found to require a high concentration of colloidal chitin for maximal enzyme production. Similarly the requirement for NaCl and yeast extract were also high for *B. bassiana*. During the fermentation production of chitinase, lowering of pH in the media after bacterial growth and accumulation of a considerable amount of ammonia was recorded by (Seki and Toga, 1963) In a similar manner changes in the pH of the fermented medium was observed with *B. bassiana*, as presented in the previous chapter.

Maximal chitinase yields were obtained after 4-5 days for *Serratia marcescens* (Monreal and Reese, 1969) and for *B. bassiana* (Leopold and Saminkova, 1970) Very similarly in the present study also *B. bassiana* was found to require 5 days of incubation for maximal production of chitinase under shake flask culture.

*B. bassiana* was able to utilize all the three chitinous substrates, prawn waste, colloidal chitin and purified chitin for the growth and synthesis of chitinase. It was also noted that this marine fungus was capable of producing chitinase without any chitinous substrate present in the medium suggesting a constitutive nature to the chitinase

produced by this fungus, which was originally isolated from marine sediment might have became adapted to chitin rich environment in its natural marine environment and consequently has became natural for the strain to secrete chitinase for derivation of its carbon and nitrogen requirements. These observations indicate further, a marine nativity to the organism by virtue of its ability to utilize chitin through synthesizing chitinase as constitutive enzyme.

Chitinases are produced constitutively (Clarke and Tracey, 1956; Claus, 1961; Leopold and Saminkova, 1970) However, the addition of chitin to culture media greatly enhanced enzyme production (Reynolds, 1954; Otakara, 1964) In the case of *B. bassiana* Smith and Grula (1983) observed that chitinases are inducible and the sterilization of chitin by autoclaving or boiling causes the release of D-glucosamine and N-acetyl-D-glucosamine which function as inducers. In the present study a similar observation was made with respect to enhanced enzyme yield in the presence of chitin compared to absence of chitin. However no attempt was made to analyse the release of D-glucosamine and N-acetyl-D-glucosamine after sterilisation by boiling /autoclaving. However a process as suggested by Smith and Grula (1983) could have contributed to induction of chitinase. Monreal and Reese (1969) have also suggested that the probable inducer of chitinase system are short chitin units (three or more N-acetyl glucosamine units)

It was also observed that chitinase synthesis by *B. bassiana* is subject to feed back inhibition. N-acety-D-glucosamine, an end product of chitin degradation by chitinase was observed to repress chitinase synthesis under submerged fermentation. Monreal and Reese (1969) observed very little chitinase activity with glucosamine, chitosan, cellulose, cellobiose, lactose and glucose. Chitinase production was found to be repressed when *T. harziannum* mycelia was provided with a easily metabolized carbon source such as glucose or N-acetyl glucosamine (Ulhoa, *et al.*, 1993).

A comparative evaluation of chitinase production under shake flask, static and in a bubble column bio reactor culture conditions of submerged fermentation revealed an interesting information that *B. bassiana* respond positively to shake flask culture conditions by recording maximal enzyme production in 5 days compared to 9 days required under static culture and in bubble column bio reactor. The enzyme yield was almost double fold when the inoculated flasks were subjected to shaking Probably the process of shaking facilitated better contact of the fungal mycelia with the nutrients besides mild aeration and better nutrient uptake by the fungus which consequently could have accelerated growth and enzyme production. In most of the submerged fermentations, irrespective of the microorganism, whether it is a bacteria, yeast or fungi, agitation of the reactor vessel normally enhance rate of mass

transfer and accelerate growth of the microorganism in the fermentor. *B. bassiana* could have also responded positively to this process under shake flask culture unlike that in static condition and bubble column reactor.

The presence of the product in dilute form in submerged fermentation was recognized as a major obstacle in economic manufacture of the product mainly due to the consequent higher cost on down stream processing and the disposal of larger volume of waste water (Hahn, 1986) More over, the cost of seperation of the microbial cells from fermentation broth using centrifugation or microfiltration is reported to involve between 48 and 76% of total production cost of microbial metabolitite by submerged fermentation (Dator, 1986). Hence more interest in solid substrate fermentation (SSF) has been generated in recent years throughout the world (Steinkraus, 1984), as it not only give higher product concentration (Arima, 1964; Ghildyal *et al.*, 1985; Kumar and Lonsane, 1987) but also offers many other economic and practical advantages, mainly less cost of medium, lower capital investment and lower plant operating costs (Forge and Righelato, 1974; Hesseltine, 1977; Lonsane *et al.*, 1985).

Solid substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitrogen,

minerals and as well as growth factors. The bacterial and yeast culture grow by adhering to the surface of solid substrate particles (Lansane and Ramesh, 1990); while filamentous fungi are able to penetrate deep into the solid substrate particle (Lonsane *et al.*, 1985) for nutrient uptake. A commonly used solid substrate was wheat bran which contained 2.33% total nitrogen, 14.1% starch, 7.6% sugar, and 35.2% cellulose (Ramesh, 1989).

Solid substrate fermentation technique was employed for the production of extra cellular enzyme by filamentus fungi. Except for the report on the extra and intra cellular chitinase by *Aspegillus oryzae* from SSF (Ohtakara, 1961 a, b, 1962, 1963, 1964; Sumi, *et al.*, 1988), no detailed investigations have been conducted on chitinase production by SSF. Further there is an absolute lack of knowledge on the possibility of employing solid substrate fermentation techniques for the large scale production of chitinase by marine filamentous fungi. Hence an attempt was made to produce chitinase through SSF. The results strongly indicate that SSF method would be advantageous for chitinase production.

Production of *B. bassiana*, under solid substrate fermentation using wheat bran or a mixture of rice powder compost, humus and ground cornstalks, in China, is well known

(Mussey and Tinsley, 1981). *B. bassiana* conidia, which was used as a coarse dust often, after the bran medium has been diluted 1:10 with fine sand is a practice for controlling corn borers and pine beetles (Mussey and Tinsley, 1981). *B. bassiana* thus, have already became a potential candidate for use through solid substrate fermentation. In the present study, attempt was made to produce chitinase using this species under solid substrate fermentation using wheat bran as solid substrate. Results obtained clearly evidence the suitablility of this marine fungus for the SSF production of chitinase. Since filamentous fungi can grow on a solid substrate in the absence of free water surface adhesion plays a very important role in fungal growth and mycelial spread. *B. bassiana* has ideally demonstrated its ability to adsorb on to wheat bran, grow and produce chitinase during growth.

In the present study the optimal levels of different bioprocess parameters namely, ratio of moistening medium to solid substrate that impart initial moisture content in the SSF medium, particle size of solid substrate, incubation temperature, concentration of collioidal chitin, pre treatment of solid substrate, NaCl concentration, phosphate concentration inoculum concentration, type of inoculum, additional nitrogen source were optimised for maximal chitinase production by *B. bassiana* under SSF with wheat bran. From the results it is seen that *B. bassiana* could produce

maximal chitinase under solid substrate fermentation with wheat bran as solid substrate, when the following conditions were optimal. An initial moisture content obtained with 3:5 (v:w) ratio of moistening medium to wheat bran, 27°C incubation, 2 N NaOH for adjusting the pH of the substrate, 7.5% and 2% of colloidal chitin when moistened with sea water and with 2 N NaOH respectively, 2.5 % NaCl with sea water and 10% NaCl with NaOH, 1 1 and 2.5 1.0 ratio of $K_2HPO_4$ and $KH_2PO_4$, use of vegetative inoculum (952 mg dry weight of mycelia equivalent), < 425 micron of particle size of wheat barn and 60 minutes of autoclaving of solid substrate. Yeast extract addition and 75% aged sea water used for humidifying the wheat bran supported maximal enzyme yield.

The critical importance of moisture content of the medium and its control during fermentation are extensively documented for solid state fermentation processed involving fungal cultiners (Lonsane *et al.*, 1985). In the present study, *B. bassiana* required a minimal initial moisture content (about 40-50%) obtained with 3:5 ratio of moistening medium suggest that the water activity requirements of this fungus on wheat bran is in general similar to that of any filamentous fungi that normally grow with minimal moisture content.

Optimal incubation temperature, 27°C, is very much the same that observed for maximal growth and chitinase

production under submerged fermentation condition. The results indicate that temperature requirement of *B. bassiana* is independent of its culture conditions, submerged or solid substrate and temperature influences the rate of chitinase production by the fungus irrespective of other environmental parameters.

An interesting observation made during the present study is the impact of pretreatment of wheat bran with sea water and 2 N NaOH on the optimal requirement of the concentration of colloidal chitin. It was observed that the concentration of colloidal chitin required for maximal chitinase production was just 2% in the case of NaOH moistening of what bran compared to high requirement of 7.5% of colloidal chitin when sea water was used. Similarly NaCl requirement also changed according to the nature of moistening medium. While high NaCl concentrations (10%) were required when NaOH was used, only 2.5% NaCl was adequate in the case of sea water. It seems the moistening medium, NaOH, an alkali, and sea water drastically affected the nature of wheat bran and its constituents during pretreatment which consequently influenced the rate of chitinase production by *B. bassiana*. It may be noted that, in SSF, pretreatment of the solid substrate by mechanical or chemical means is also employed in order to improve its amenability to microbial modification by forming smaller permeable molecules, to provide sites for easy

microbial penetration to impart more fibrous structure or to bring about gelatinization and swelling. The pre treatment may be either mild or drastic and include soaking, ball milling, sieving and treatment with alkali or sodium chloride (Lonsane *et al.*, 1985). Hence in the present study, the pretreatment with alkali and sea water (rich in sodium chloride) could have improved the wheat bran condition better amenable for chitinase production by *B. bassiana*.

From the results it was also noted that requirement for $K_2HPO_4$ and $KH_2PO_4$ varied with reference to fermentation condition. It was observed that requirement for $KH_2PO_4$ was lesser in SmF compared to that in SSF unlike $K_2HPO_4$ which was required lesser in SSF than under SmF.

According to Mitchell (1992) in protease production by fungi, either spores or mycela can be used as inoculum. Spores are preferable because of the convenience of preparation, their suitability during storage and tolerance of mistreatment during harvesting (Mitchell, 1992). However for small scale fermentations, suspensions prepared from agar slants remain the most convenient (Hesseltine *et al.*, 1976).

In the present study, with *B. bassiana* vegetative inoculum was found to promote maximal chitinase

compared to spore inoculum. Perhaps the picture would be different in large scale fermentations.

Volume of culture vessel in which solid substrate fermentation was conducted, directly influenced the enzyme yield by the marine fungus. While in petriplates lesser quantity of wheat bran spread out uniformly promoted maximal enzyme yield compared to larger quantity, in conical flasks, larger volume of the flask with fixed quantity of wheat bran promoted maximal enzyme yield. The results of the present study indicate requirement for lesser bed height of the bran medium which facilitate better aeration that consequently accelerate growth and enzyme production compared to larger bed heights. *B. bassiana* required adequate aeration and minimal bed height of wheat bran for maximal enzyme production under SSF.

Shrimps take a major share in the fish processing industry and earns a significant amount of foreign exchange through export. Processing of penaeid prawns results in more than 50% of the raw material as waste (Prawn head and shall). Although these wastes are rich in protein, minerals and the natural carbohydrate polymer-chitin, they have not received proper attention. Except for the extraction of chitin, the wastes are not processed further for the preparation of edible products but used as manure, in poultry feed or discarded.

These solid wastes could be explored as probable solid substrates for fermentative production of enzymes and other desirable products using marine fungi and bacteria (Chandrasekaran, 1994).

Hence, suitability of prawn wastes as chitinous solid substrates for chitinase production by *B. bassiana* under SSF was standardized. Results indicated scope for utilization of prawn waste as solid substrate for production of chitinase. Optimal conditions of initial moisture content imparted by humidification with 1:5 ratio of moistening medium to solid substrate; initial pH of 5 and 11.0, 1% NaCl concentration 2.5% of $KH_2PO_4$ and 1:2.5 ratio of combination of $K_2HPO_4$ and $KH_2PO_4$; 425-600 micron particle size of the substrate, use of spore inocula and 5 days of incubation supported maximal enzyme yield.

Studies of Ashford and coworkers (1977) demonstrated that chitin represents 14-27% of the dry weight of shrimp. According to Carroad and Tom (1978) chitin waste is used to induce selected microorganism to secrete into solution an extracellular chitinase system in submerged culture of *Serratia marcescens*. The enzyme is harvested by filtration and combined with the bulk of the chitin waste for chitin hydrolysis. Hence, the chitin in the prawn waste, could have acted as the carbon source and as an inducer for the chitinase

production besides serving as the solid support for the adhesion and growth of *B. bassiana*.

It was observed that the requirement of the initial moisture content in terms of the ratios of moistening medium was found to be very different with prawn wastes compared to wheat bran. In the case of prawn waste very less moisture content (1:5 ratio) was sufficient for maximal enzyme production compared to 3:5 ratio in the case of wheat bran. NaCl concentration (1%) requirement was minimal with prawn waste compared to the high requirement in the case of wheat bran. Similarly the requirement of phosphate, $KH_2PO_4$ was higher in the case of prawn waste compared to wheat bran. Another notable feature observed with prawn waste was the preference for spore inoculum of maximal enzyme production unlike that of wheat bran, where vegetative inoculum led to maximal enzyme production.

Smaller particle size provide large surface area for heat and mass transfer. This results in higher surface nutrient concentration and shorter pathways for diffusion of nutrients. In the present study, *B. bassiana* showed varied preference for maximal chitinase production, with reference to different particle size of the different substrate. While it preferred very small particle size ( $< 425\ \mu$) of wheat bran,

it required 425-600 $\mu$ particle size of prawn shell waste for maximal enzyme production.

Thus altogether *B. bassiana* showed variation in its optimal requirements for maximal enzyme production under solid substrate fermentation when conducted with wheat bran, a plant source solid substrate and with prawn shell waste an animal source. Results indicate that nature of solid substrate directly influence the optimal conditions of fermentation. Interestingly, inspite of variations in optimal conditions for maximal enzyme production under SSF, the overall enzyme yield was almost similar, as 241.5 U/gIDS and 248.0 U/gIDS were recorded respectively with wheat bran and prawn waste. Further about 20 fold increase in enzyme yield was noted with SSF compared to SmF. Another interesting point is that, the period of incubation for attaining maximal enzyme under SSF and SmF were identical unlike in other cases reported (Renu, 1991; Mohanan, 1995).

The results obtained for the enzyme profile for *B.bassiana* indicate that these fungus is versatile in its mode of nutrition, since it has the ability to produce the wide range of hydrolytic enzymes. This rare ability of this fungus could be attributed to its nativity to marine sediments where normally it comes to contact with wide range of substrates.

Purification of mold chitinase from wheat bran culture under solid substrate fermentation have remained unstudied except for few reports (Ohtakara, 1961, 1963). Chitinase from *Aspergillus niger* was purified 40 fold with a yield of 11% (Ohtakara, 1961). whereas in the present investigation, for *B. bassiana* only upto 24.2 fold purification with 22.3% yield was achieved.

The purified enzyme was checked for homogeneity on native polyacrylamide gel electrophoresis, where the enzyme gave rise to a single band only. The purified chitinase also showed single band of protein on SDS-PAGE, indicating thereby that it does not contain any subunits. This result is in contrast to the observation of more than one protein band in SDS gel electrophoresis in the case of other fungal chitinases, *Neurospora crassa* (Angel, 1988) *Verticillium alb-atrum* (Pegg, 1988).

The molecular weight of pure chitinase from *B. bassiana* was 23,000 $\pm$ 500; 65,000 $\pm$ 2,000 by SDS PAGE and gel filtration respectively. The difference in molecular weight of the enzyme when determined by gel filtration and SDS-PAGE methods were also reported Chiang *et al.* (1979) and attributed to the interactions of enzyme protein molecules with dextran in gel filtration (Morgan and Priest, 1981; Chiang *et al.*, 1979). It was also related to the presence of subunits

(Morgan and Priest, 1981), but it is assumed that it may be due to the lower molecular size of the enzyme. Whereas the molecular weight of other fungal chitinase were 45,000 on SDS PAGE for *Aeromonium abclavatum* (Gunaratna and Balasubramanian, 1994), 54,900 on SDS PAGE and 21500 on gel filtration for *Pencillium oxalicum* (Rodrigues *et al.*, 1995), 38,000 on gel filtration for *Pycanoporns cinabarinus* (Ohtakara, 1988), 64,000 and 58,000 for *Verticillium albo -atrum* (Pegg, 1988). 3,3000 for *Metarhizium anisophiac* (St. Leger *et al.*, 1991), 1,18,000 on gel filtration and 64,000 on SDS PAGE for *Trichoderma harzaianum* (Ulhoa and Preberdy, 1991).

The optimal activity for the enzyme was recorded at pH 9.0. Chitinase from fungi have a pH optimum in the acidic range (Ohtakara, 1988; Reyes *et al.*, 1989). In general fungal chitinases are stable over a wide pH range (Srivasthava *et al.*, 1985; Reyes *et al.*, 1988).

The optimal temperature for maximal activity of chitinase varied among the microorganisms widely. Thus chitinase of *Aeromonium abclavatum* (Gunaratna and Balasubamanian, 1993) and *Trichoderma hrzianum* (Ulhoa and Preberdy, 1991) were maximally active at 50$^{o}$C while chitinase of *Penicillum oxalicum* (Rodrigues *et al.*, 1995). *Piromyces communis* (Sakurado *et al.*, 1975) were active at 35$^{o}$C and 40$^{o}$C respectively. Chitinase of *Verticillium albo-atrum* was

maximally active at 51°C (Pegg, 1988). In the present study chitinase of *B.bassiana* was maximally active at 32°C.

The purified chitinase of *B. bassiana* was partially inhibited by $Ca^{2+}$ $Ni^{2+}$ $Cd^{2+}$ and $Co^{2+}$ and not inhibited by $Na^{2+}$ $Hg^{2+}$ $Ag^{+}$ and $Mg^{2+}$ In *Mucor mucedo* an increase of the activity with magnesium has been described (Humphreys and Goody, 1984). For *Pencillium oxalicum* $Hg^{2+}$ and $Ag^{+}$ were chitinase inhibitors (Rodrigues *et al.*, 1995). It should be pointed out that the inhibitory action of metal ions on chitinase differs with the sources of the enzyme (Vyas and Deshpande, 1993). $HgCl_2$ and $MnCl_2$ inhibited the enzyme activity almost completely in *Aeremonium abclavatum* (Gunaratna and Balasubramanian, 1994). Kimura (1960) reported that calcium, lead and mercury ions inhibited chitinase activity whilst manganese, iron, tin and zinc enhanced the chitinase activity.

Substrate specificity of the purified chitinase from *B. bassiana* was tested against various substances. The activity was higher in the crude substrate than with purified chitin and colloidal chitin. The chitinase of *B. bassiana* showed a wide range of substrate specificity. It could hydrolyze non-chitinous substrate such as laminarin and CM cellulose. It also showed bacterial cell wall lytic activity. It hydrolysed native chitin most rapidly among the substrates

examined and also hydrolyzed chitosan at higher rate. The action of the chitinase towards chitin related compound and non-chitinous compounds varied. The enzyme also showed high rate of action towards chitotriose and no action against chitobiose.

The ability of this enzyme to hydrolyse native chitin at higher rate shows that the substrate specificity of this chitinase is different from that of the chitinases which were reported earlier.

The chitinase of *B. bassiana* isolated from marine sediments, produced under solid substrate fermentation was tested for their fungal and yeast lytic activity in order to assess its suitability as a biocide against microbial pathogens. The lytic activity of the crude enzyme preparation was examined toward phytopathogenic and non-phytopathogenic filamentous fungi and yeast. The enzyme preparation liberated reducing sugars from the mycelial preparation of both fungal and yeast. However, the liberation of reducing sugar was in higher rate in non-phytopathogenic fungus than in phytopathogenic fungus. The liberation of reducing sugar from non-phytopathogenic fungus, *Aspergillus niger* and *Mucor* sp. was almost same.

Ramirez'- Leon' *et al.* (1972) and Monaghan *et al.* (1973) reported that only the chitosanase was able to degrade the cell wall of fungi belonging to mucorales but chitinase did not participate in the lysis of them. However, in the present study chitinase from *B. bassiana* showed lysis of *Mucor* cell wall and liberated reducing sugars. These facts indicate that the substrate specificity of the chitinase produced by *B. bassiana* is different from that of the chitinases which were reported earlier.

Earlier reports have pointed out that the cell walls of *Aspergillus* and *Penicillium* which belong to Euascomycetes consists of same kinds of glucon and chitin (Applegarth, 1976), and there fore the carbohydrolases participating are the same kinds of glucanase and chitinase (Horikoshi and Ida, 1959; Skujins *et al.*, 1965). It has been known, however, that the cell wall of *Rhizopus* and *Mucor*, which belong to Zygomycetes, are distinguishable from those of Euascomycetes in that they contain chitosan as a main component besides glucons and chitin (Bartnicki-Garcia and Nickerson, 1962). Almost all lytic enzymes reported so far, are not able to degrade these cell walls (Muzzarelli, 1977). Whereas in the present study we observed that the chitinase preparation of *B. bassiana* could hydrolyse cell walls of both group. The chitinase preparation were also active against yeast.

The present study highlight several interesting informations which include availability of potential chitinase producing fungi from marine environments; *Beauvaria bassiana* as the ideal marine fungi for large scale production of chitinase, employing both submerged and solid substratefermentation; potential of using chitinous prawn wastes as solid substrates for SSF production of chitinase and possible uses of this fungal chitinase as biocide and in single cell protein production from chitinous waste.

# CHAPTER - FIVE

# SUMMARY AND CONCLUSIONS

Chitinase producing fungi were isolated from marine environments of Kochi on colloidal chitin agar medium prepared in 50% aged sea water.

Chitinase producing fungi were present in higher levels in water and sediment samples of marine and environments of Kochi.

Chitinase producing fungi, isolated during the study, included only two genera, *Beauveria* and *Mucor*. Of the two *Beauveria* sp. were dominant in all the samples.

Among the isolates obtained one strain identified as *Beauveria bassiana* BTMF S 10 was selected for further studies based on the efficiency to produce higher levels of chitinase under solid substrate fermentation using wheat bran.

The selected strain was initially characterized for the optimal growth conditions, such as incubation temperature, initial pH of the medium, NaCl requirement, colloidal chitin utilization, carbon and nitrogen sources, concentration, incubation time and salinity for maximal growth.

*B. bassiana* could grow maximal over a wide range of temperature 22-32°C. However, the maximal growth was recorded at 27°C.

*B. bassiana* could grow maximal over a wide range of pH,(3-11.0) with maximal at pH 9.0 and with two peaks of optima, one large at pH 9.0 and a small at pH 6.0.

*B. bassiana* could grow maximal over a wide range of NaCl, concentration 0-12%, with optimal at 3.5% NaCl concentration in the medium.

Among the various organic carbon sources tested glucose enhanced significant level of growth, while xylose, and ribose inhibited growth. Inorganic carbon source, trisodiumcitrate led to a minimal growth.

Among the various organic nitrogen sources tested yeast extract supported maximal growth, while malt extract inhibited the growth completely. Among the various inorganic nitrogen sources tested $NH_4Cl$ supported maximal growth. Of the different amino acids tested glycine promoted maximal growth. However all the amino acids, except tryptone could support significant growth.

Growth medium, (GPYM broth), prepared with 75% aged sea water promoted maximal growth followed by 100%, 50%, and 25% aged sea water, distilled water plus 5% NaCl, and distilled water both in liquid and solid media.

Growth curve of the fungus indicated that the strain entered into the logarithmic phase after 24 h and continued till 54 h. The generation time was recorded as 7.5 h.

Chitinase production by *B.bassiana* in mineral salt colloidal chitin (MSCC) medium, under Submerged Fermentation (SmF) conditions were studied.

Chitinase production by *B. bassiana* under SmF-shake flask culture showed that addition of glucose to MSCC medium supported maximal chitinase yield followed by dextrin, mannose, and sorbitol, while soluble starch, sucrose, maltose and mannitol decreased the enzyme synthesis. Inorganic carbon source, trisodiumcitrate inhibited the enzyme synthesis.

Chitinase production by *B. bassiana* under SmF with a various chitinase substrate showed that all chitinous substrate, excluding glucosamine, promoted similar level of chitinase production although a maximum was noticed with prawn waste followed by colloidal chitin, purified prawn shell chitin powder and chitosan.

*B. bassiana* produced chitinase under SmF in the absence of chitinous substrate indicating that the chitinase of *B. bassiana* was a constitutive enzyme.

Chitinase production by *B. bassiana* under SmF was maximal with 10% of prawn waste (w/v) after 10 days of fermentation.

Optimal conditions for maximal chitinase production by *B. bassiana*, under SmF shake flask culture, were recorded as 27°C, pH 9.0, 8% colloidal chitin as substrate and 9% sodium chloride.

Yeast extract among the various organic nitrogen sources and $NaNO_3$ among the inorganic nitrogen sources promoted maximal chitinase under SmF by *B. bassiana*.

Maximal chitinase production by *B. bassiana* under SmF shake flask culture was recorded with 9% yeast extract (v/w).

Chitinase production under SmF by *B. bassiana* was maximum with the inoculum concentration of 120 mg dry weight equivalent of mycelia per 100 ml of medium.

Maximal chitinase production by *B. bassiana* under SmF at optimized condition was recorded after 5 days of fermentation.

Chitinase production by *B. bassiana* under SmF was optimal in the presence of 0.25% $MgSO_4$ (w/v) and with 2 0.5% (w w) ratio of $K_2HPO_4$ and $KH_2PO_4$.

Chitinase production by *B. bassiana* under SmF - static culture was maximal on the 9th day of incubation. Enzyme production was not growth associated.

Addition of N-acetyl-D-glucosamine in the chitinase production medium of *B. bassiana*, under SmF shake flask culture, showed that chitinase synthesis by *B. bassiana* was catobolically reperessed at 4% (w/w) level.

Chitinase production under SmF by *B. bassiana* in a Bubble Column Reactor was maximal on 9th day of fermentation, and growth associated.

Chitinase production by *B. bassiana* was studied under solid substrate fermentation (SSF) using commercial wheat bran.

Optimal initial moisture content required for the enhanced chitinase production by *B. bassiana* under SSF using wheat bran (WB) was 3 5 (v w) ratio of moistening medium to WB and 3 days of incubation.

Optimum incubation temperature and initial pH of the substrate on chitinase production by *B. bassiana* under SSF using wheat bran was 27°C and pH 10.0.

Treatment of wheat bran with NaOH solution greatly influenced chitinase production by *B. bassiana* under SSF, and enhanced level of chitinase production was observed with the treatment of wheat bran with 2 N NaOH.

Optimum concentration of colloidal chitin required for maximal chitinase production by *B. bassiana* under SSF using wheat bran was 7.5% (w/w) and 3% (w/w), respectively in the SSF medium moistened with sea water and 2 N NaOH.

Chitinase production by *B. bassiana* was increased by the addition of NaCl in the wheat bran medium. However, the optimal concentration required for maximal yield varied with the choice of the solvent used for humidifying the wheat bran medium.

The maximal enzyme yield was observed with 2.5% (w/w) and 10% (w/w) NaCl, respectively with sea water and 2 N NaOH as moistening medium of WB.

Chitinase production by *B. bassiana* under SSF with wheat bran medium was influenced by the addition of phosphate ($K_2HPO_4$ and

$KH_2PO_4$). Maximal chitinase production was observed with addition of $K_2HPO_4$ independently at 5% (w/w) level and with a combination of $KH_2PO_4$ at a ratio of 2.5 1, compared to other ratios.

Chitinase production was high throughout the course of fermentation with spore inocula (32 x $10^6$ per 5 g of wheat bran) and a maximum was reached at 62 h of fermentation. The enzyme yield with vegetative inoculum was maximum, with 952 mg dry weight equivalent of mycelia per 100 g of wheat bran, after 42 h. Among the spore and vegetative inoculum, the latter was preferable for obtaining maximal yield of enzyme, since almost 2.3 fold yield could be obtained after 42 h as compared to the maximal obtained with spore inoculum at 72 h.

Maximal chitinase production by *B. bassiana* was promoted by the wheat bran medium prepared with < 425 micron particle size of the substrate, irrespective of the type of inoculum used. However, maximal yields were observed after 72 h and 48 h of incubation respectively with spore and vegetative inocula.

The enzyme yield decreased along with increase in particle size of the substrate, irrespective of the type of inoculum used.

Chitinase production increased linearly along with the increase in the autoclaving time and a maximal yield was observed with the wheat bran medium autoclaved for 60 minutes.

Addition of yeast extract in the wheat bran medium, at 1% (w/w) level, enhanced chitinase production by *B. bassiana*. However, concentration above 1% level affected adversely and resulted in a decrease in chitinase yield.

Addition of $NH_4Cl$ in the wheat bran medium adversely affected the chitinase synthesis by *B. bassiana*, resulting in a sharp decrease in enzyme yield.

Chitinase production by *B. bassiana* under SSF using wheat bran medium was influenced by the volume of culture vessel. Maximal level of chitinase synthesis was recorded with 5 g of wheat bran medium in 500 ml conical flask.

Chitinase production by *B. bassiana* was maximal in wheat bran medium humidified with 75% aged sea water, followed by 100% and 50% aged sea water. The chitinase synthesis was lesser in wheat bran medium moistened with tap water and distilled water compared to that observed with the various dilutions of aged sea water.

Chitinase production by *B. bassiana* was maximal in the wheat bran medium inoculated with vegetative inoculum after 48 h and spore inoculum after 72 h of fermentation. The rate of enzyme synthesis was rapid with vegetative inoculum, compared to spore inoculum.

Chitinase production by *B. bassiana* under SSF using prawn waste (chitinous) as solid substrate was studied.

Optimal initial moisture content for maximal chitinase production by *B. bassiana* under SSF using prawn waste (PW) medium was 1:5 (v:w) ratio of moistening medium to PW and 4 days of incubation.

Maximal chitinase production by *B. bassiana* was observed with the prawn waste medium humidified with aged sea water, with an initial pH 10.0, However, two peaks of optima, a small peak at pH 5.0 and a large peak at pH 10.0 were recorded.

Maximal yield of chitinase by *B. bassiana* could be observed when NaCl was added to the prawn waste medium at 1% (w/w) level.

Addition of phosphate as $KH_2PO_4$ individually or in combination with $K_2HPO_4$; at higher proportion led to enhanced enzyme yield compared to $K_2HPO_4$.

Particle size of 425-600 micron supported maximal enzyme yield by *B. bassiana*, with the spore inoculum throughout the period of incubation after 5 days. Whereas in the case of vegetative inoculum, maximal enzyme yield was recorded with a maximal particle size of > 1400 micron after 5 days.
Addition of yeast extract to the prawn waste medium led to reduction in chitinase synthesis by *B. bassiana* significantly unlike in the wheat bran.

Chitinase from *B. bassiana* produced under SSF using wheat bran medium was purified by $(NH_4)_2SO_4$ fractionation followed by gel filtration on Sephadex $G_{100}$ and Sephadex $G_{200}$ A 24.2 fold purification was achieved.

Polyacrylamide gel electrophoretic analysis of the purified enzyme preparation gave rise to a single band only.

Molecular weight of chitinase of *B. bassiana* as estimated by calibrated gel filtration on Sephadex $G_{100}$ was 66,000 $\pm$ 2,000.

Sub unit molecular weight of chitinase of *B. bassiana* as estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis was 22,000 $\pm$ 500 with a single band.

Purified chitinase was maximally active at 32$^{o}$C and pH 9.0.

Two peaks of optimal activity one small peak at pH 6.0 and a large peak pH 9.0 was recorded for this enzyme.

Enzyme was stable upto a temperature $50^{o}C$ and over a pH 5.6-9.6.

Metal ions viz $Ni^{2+}$ $Cd^{2+}$ $Co^{2+}$ and $Ca^{2+}$ reduced the chitinase activity $Sn^{2+}$, $Hg^{2+}$ $Ag^{2+}$and $Mg^{2+}$ did not effect chitinase activity.

Chitinase was active against a wide range of substrate, viz highly purified crab shell chitin to very crude prawn shell powder. The enzyme showed maximum activity against prawn shell powder followed by colloidal chitin, purified crab shell, chitin powder and purified prawn shell chitin powder. The enzyme showed considerable activity on chitotriose and no activity on chitobiose. The chitinase also hydrolysed chitosan powder, CM cellulose, laminarin and *Micococcus lysodeikticus* cells.

Crude chitinase hydrolysed whole fungal cells. The hydrolysis of yeast cells was significantly higher compared to filamentous fungi such as *Rhizoctonia solani*, *Aspergillus niger* and *Mucor* sp. The chitinase was more active on the non-phytopathogenic fungi such as *A. niger*, and *Mucor* sp. than on the phytopathogenic fungus *R. solani*.

Present study indicate scope for utilisation of *B. bassiana* for industrial production of chitinase using prawn waste as solid substrate employing solid substrate fermentation.

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