L-GLUTAMINASE PRODUCTION BY MARINE *VIBRIO COSTICOLA* UNDER SOLID STATE FERMENTATION

Thesis submitted
under the Faculty of Science
Cochin University of Science and Technology
for the degree of
DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

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JUNE, 1996

Dedicated to

My Beloved Parents

and

Mr. M. R. Mohammed Riaz
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CERTIFICATE

This is to certify that the work presented in the thesis entitled L-GLUTAMINASE PRODUCTION BY MARINE *VIBRIO COSTICOLA* UNDER SOLID STATE FERMENTATION is based on the original research done by Mr.G. Nagendra Prabhu, under my guidance and supervision, at the Department of Biotechnology and no part of the work has been included in any other thesis for the award of any degree.

M. CHANDRASEKARAN

DECLARATION

I hereby declare that the work presented in the thesis entitled "L-GLUTAMINASE PRODUCTION BY MARINE *VIBRIO COSTICOLA* UNDER SOLID STATE FERMENTATION" is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Dr. M. Chandrasekaran, Reader in Microbiology and UGC Career Awardee, and no part thereof has been presented for the award of any other degree.

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COCHIN - 22

4 -07-1996

ACKNOWLEDGEMENTS

I have genuine feeling of gratitude, indebtedness and gratefulness to all those who were instrumental in the successful completion of this work. I know that all those who made this thesis work possible know me and my feelings of gratitude for them, though I may not be able to express them here because of my limited vocabulary and unbound emotions.

I have great pleasure in expressing my sincere gratitude and obligation to Dr. M. Chandrasekaran, Reader in Microbiology and U.G.C. Career Awardee, Department of Biotechnology, Cochin University of Science and Technology, for introducing me to the exciting world of Microbiology. He has been a constant source of inspiration and guidance throughout the course of my work and I bow my head before his vision of treating his research students as his own "Academic Children".

I take this opportunity to thank Dr. G.S. Selvam, Dr. C.S. Paulose and Dr. Padma Nambisan, of the Department of Biotechnology for their kind co-operation and encouragement. I am indebted to Dr. P. Madhavan Pillai, Head, Dr. K.L. Sebastian, S.S. Bhatnagar Awardee, and all other faculty members of the Department of Applied Chemistry for allowing me to use the excellent facilities of the Department, without which this work could not have been completed.

I wish to acknowledge the kind help of Dr. Ashok Pandey, Scientist, Regional Research Laboratory (CSIR), Trivandrum, India in the scanning electron microscopic studies and also for making available valuable reprints. M/s. Madhavan Nampoothiri, K. Balakrishnan, and P. Selvakumar of the Biotechnology Unit, RRL, also deserve my special thanks for their help during my stay there.

I am greatly indebted to Dr. Kenji Soda, Professor of Microbial Biochemistry, Institute of Chemical Research, Kyoto University, Japan, for his valuable suggestions and encouragement during the 16th IUBMB Congress at New Delhi, September, 1994, and also for sending highly useful study materials. Thanks are also due to Dr. P. Ajitkumar, I.I.Sc, Bangalore, Dr. Rakesh Sehgal, PGIMER, Chandigarh, Dr. R. Harikrishnan, Indian Immunologicals Ltd, Hyderabad, and Mr. Smarajit Pal, Chittaranjan National Cancer Institute, Calcutta for sending me several useful reprints in my research area which have helped me to complete the work successfully. The authorities of RRL, and Regional Cancer Centre, Trivandrum; All India Institute of Medical Sciences and National Medical Library, New Delhi; Central Food Technological Research Institute, Mysore; Anna University and Madras University, Madras are gratefully acknowledged for permitting me to utilize the library facilities.

The support, encouragement and help which I received from my lab-mates cannot be expressed in words. The stimulating discussions and suggestions of M/s. Mohanan, P.V, Suresh, P.V, Jayachandran, K, Abi. N. Eldo, Williams, C, Ms. Krishna, C, Ms. Keerthi, T.R. and Mrs. Shylaraj, K.S. have helped me a lot in completing my work successfully. M/s. Sabu, A, Unnikrishna Pillai, P.R, Pius. S. Padayatti, Ms. Sudha. B. and all other colleagues in the Department are gratefully remembered for their help and encouragement.

Thanks are also due to my seniors Dr. M.P. Nandakumar, CFTRI, Mysore; Dr. S. Renu, CSRI, Mysore, and Dr. C. Mohandass, NIO, Goa, for their help and suggestions. Dr. (Mrs) Manpal Sridhar, CMFRI, Cochin; Dr. K.G. Raghu CDRI, Lucknow, and Mr. E.V. Dasan, Medical College, Trivandrum, also deserves special mention for their interest and help in my work.

Mr. Harilal, S.S. and Mr. Riju C. Isac, International School of Photonics, CUSAT and Mr. N. Saravanan, Department of Applied Chemistry have helped me in the preparation of the manuscript and figures. They have spent many sleepless nights and sacrificed lot of time and energy for me. I take this opportunity to convey my heartfelt gratitude to them.

My friends M/s. Ajit Sacheendran, Jyothish, Bimal Lal, Gopakumar, Anzar, Sreekumar and Subramanian have constantly encouraged and supported me. I thank them all for what they have done to me. I humbly remember all my teachers at the P.G. Department of Zoology, S.D. College, Alleppey, for motivating and encouraging me to pursue my Ph.D. research.

The administrative staff of the Department of Biotechnology, CUSAT, Mrs. Lalithambal, Mrs. Sumathi, Mrs. Girija Devi and Mrs. Thilakamani are also acknowledged for their support and cooperation. I also thank the authorities of CUSAT for providing necessary facilities for carrying out the work. Financial support from the Council of Scientific and Industrial Research, Govt. of India, New Delhi in the form of a research fellowship is gratefully acknowledged.

The support I received from my parents, brothers and other members of my family has enabled me to spend most of my time in the laboratory and complete the work successfully. I also thank Ms. Latha for silently and patiently waiting for the completion of my research work. Finally, I would like to dedicate my work to my beloved parents and Mr. M.R. Mohammed Riaz (1969-1994) who was like a brother to me and was always ready to sacrifice anything for our friendship. Remembering him now is the least I can do in his everlasting memory.

G. NAGENDRA PRABHU

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INTRODUCTION

1.1. PREFACE

World trade in enzymes is about 500 million US dollars and the total market for food enzymes alone is presently estimated to be about Rs. 300 crores, with India contributing a mere 0.5 %. Food industry is identified as the largest consumer for commercial enzymes (Lorrane & Ramakrishna, 1989; Bhotmange & Shastri, 1994). The enzymatic processing in our country has not received the attention it demands. The total market for enzymes in India today is limited around 20 crores with a predicted growth rate of 20–30 % annually (Nair, 1994). Except papain which is produced in plenty, we depend on imports for majority of enzymes used in the food industry. Therefore, there exists a potential not only for the production of industrially important enzymes, but also for their economical utilization (Nair, 1994). Microbial enzymes are preferred over plant or animal sources due to their economic production, consistency, ease of process modification and optimization. Thus, about 80 % of the commercial enzymes are produced from microorganisms, owing to the wide spectrum of enzymes elaborated by them which offer an opportunity to select specific enzymes for specific purposes (Bhotmange & Shastri, 1994)

Microbial enzymes are in great demand because of their usefulness in several industries such as brewing, baking, leather, dairy, textile, starch processing, pharmaceuticals etc. Inspite of their utilization in industry for many centuries, only recently a detailed knowledge relating to their nature, properties and functions has become more evident. In fact, the commercial success of alkaline proteases 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 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 & Kelly, 1990).

Interest in amidohydrolases such as L-asparaginase and L-glutaminase (L-glutamine amidohydrolase E.C. 3.5.1.2) started with the discovery of their antitumour properties (Broome, 1961; Roberts *et al*, 1970; Bauer *et al* 1971; Abell & Uren, 1981; Raha *et al*, 1990; Pal & Maity, 1992 a). Apart from its therapeutical value, L-glutaminase is also useful in the food industry as it increases the glutamic acid content of fermented foods thereby imparting a unique flavour (Yokotsuka, 1985). Since the sources for L-glutaminase are limited,

the search for potential microbial strains that hyper produce the enzyme with novel properties and economically viable bioprocesses for their industrial production is being pursued all over the world.

Marine microorganisms remain as untapped sources of many metabolites with novel properties and several marine metabolites are closer in physiological functions and compatible to terrestrial animal systems compared to that from terrestrial sources (Faulkner, 1986). The marine bacteria and their enzyme systems are useful in many industries because of their high salt tolerance and consequently they are being intensively screened in order to obtain products of economic importance (Austin, 1988; Moriguchi *et al*, 1994).

The oceans are the richest known source of many bioactive compounds of which only a small percentage have been studied (Halevy, 1990). Marine environment is being appreciated as a potential source of novel pharmaceutical compounds, enzymes and fine chemicals. There is an urgent need to develop selective isolation methods for commercially useful organisms and screening programmes for potentially interesting compounds. Despite the fact that the terrestrial environment has contributed to the overwhelming majority of commercially useful strains of microorganisms, there are reports on marine microorganisms which are potent producers of DNases, lipases, alginases, proteases chitinases and glutaminase (Austin, 1988; Venu and Chandrasekaran, 1992 a; Moriguchi *et al*, 1994).

Apart from the ability of marine bacteria to prc luce extracellular enzymes, they also posses the ability to colonize be ·ren surfaces (Austin, 1988). This adsorption or attachment property has been well documented in the literature (Zobell & Allen, 935; Fletcher, 1980; Hermanson & Marshall, 1985). The unique proper' / of marine bacteria to adsorb on to solid particles is a highly desi able feature for their use in the solid state fermentation proc sses (Chandrasekaran, 1994, 1996). Further, they are salt tolerant anc are capable of surviving in a wide range of pH and temperature (Mori; ıchi *et al*, 1994). Salt tolerant microbes and their products are extr nely important in industries which require high salt concentrations suc ı as the production of soy sauce (Japan and other Oriental count ies) where the final salt concentration is as high as 20–25 %. Hence t ıere is an increasing interest in the salt tolerant marine microorga sms for their use in such industries (Moriguchi *et al*, 1994).

Traditionally, large scale production of useful metabolites rom microorganisms is carried out by submerged fermentation (SmF). olid state fermentation (SSF) is the culturing of microorganisms on oist solid substrates in the absence or near absence of free water (C ınel & Moo Young, 1980). It is also described as any fermentation pr cess that takes place on solid or semi solid substrate or that occurs n a nutritionally inert solid support, which provides some advantag ı to the microorganisms with respect to access to nutrients (Aidoo e *al*, 1982). Recently there is renewed interest all over the world on SSF,

in spite of the fact that this technique is being practiced for centuries. Currently, SSF is being used for the production of traditional fermented foods; mushroom cultivation; protein enrichment of animal feed; single cell protein; fuel generation; production of ethanol; organic acids; antibiotics; alkaloids; food flavours; enzymes such as amylase, glucoamylase, cellulase, protease etc., and in the disposal of solid wastes (Lonsane, 1994).

Although most commercial solid state fermentations basically lack the sophisticated control systems normally achieved in SmF, there is an increasing interest and awareness of the value of SSF and the expanding role they could play in developing biotechnology (Moo Young *et al*, 1983, Rolz, 1984). The major advantages of SSF processes over SmF include (1) higher product titres in concentrated form, (2) lower capital & recurring expenses, (3) lower waste water output, (4) reduced energy requirement, (5) absence of foam formation, (6) simplicity of operation, (7) high reproducibility, (8) simpler fermentation medium, (9) lesser fermentation space, (10) absence of rigorous control of parameters, (11) less complex plant & machinery, (12) easier aeration, (13) use of less water, (14) easier control of contamination, (15) storage of product for longer periods, and (16) lower cost of downstream processing (Lonsane, 1994).

Solid state fermentation does have some disadvantages w ich include (1) the types of substrates and microorganisms that can be used in SSF are limited, (2) in large scale operations the remova of heat generated by the microorganisms creates lot of problems, (3) r ass transfer and oxygen transfer is difficult to control du ng fermentation, (4) measurement of moisture levels, pH, O_2 & CO_2 le els and product yield is difficult, (5) biomass estimation is hard to perform, (6) scale up strategies are more complex, and (7) very l tle engineering data on the design of reactors is available (Cannel nd Moo Young, 1980). Further, extensive pretreatment of the substrat is sometimes necessary (Aidoo *et al*, 1982).

The selection of a raw material for bioprocessing is dictated by price, availability, composition and the oxidation state of the car on source (Hacking, 1986). The most widely exploited substrates for SF are mainly materials of plant origin and includes food crops (gra ns, roots, tubers & legumes), agricultural and plant residues nd lignocellulosic materials (wood, straw, hay & grasses; Smith & Ai oo, 1988). An essential prerequisite of all potential substrates is at the microbial colonizer must be able to derive energy and cell lar constituents from these compounds by oxidative metabolism. Sev ral natural substrates are usually water insoluble and form a multi fa ed complex surface on which the microorganisms grow and the rate nd direction of growth will be dependent on the nutrient availability nd geometric configuration of the solid matrix (Moo Young *et al*, 1983).

It is usual for the crude raw material to contain most, if not all, of the necessary nutrients for growth. Some degree of pretreatment is normally necessary for successful colonization by the microorganis ıs. Pretreatment methods can be physical, chemical or biological. In m ıst cases, some degree of particle size reduction will be necessary to ensure rapid fermentation (Smith & Aidoo, 1988).

The success of SSF largely depends on the type of the substr .te and the nature of the microorganism used. SSF processes will uti ze a wide range of solid organic materials such as wood, straw etc. S: ɔh compounds are invariably polymeric molecules, insoluble or sparin ly soluble in water. However, they are cheap, readily available ı ıd provide a concentrated source of nutrients (Smith and Aidoo, 19(3). Although fungi are traditionally used for SSF processes, bacte ia, yeasts and actinomycetes are also being used now (Ramesh & Lonsa ıe, 1989; Satyanarayana, 1994; Lonsane, 1994). Majority of he microorganisms used in SSF processes are native of terrest ial environments and reports on the use of marine microorganis ıs, especially bacteria are not available. Whereas the uni(ue characteristics of marine bacteria to survive in extreme conditions of pH, temperature and salinity coupled with their ability to adsorb ɔn to solid particles, in fact, makes them ideal for their use in S SF (Chandrasekaran, 1994; 1996).

Use of inert supports have been recommended for SSF in orc ɔr to overcome its inherent problems and efforts are being made to s arch for newer and better materials to act as inert solid supports lidoo *et al*, 1982; Zhu *et al*, 1994).

In the present study an attempt was made to pr duce L-glutaminase, which is industrially and therapeutically impc tant, from marine bacteria under solid state fermentation using nt ural, inert and mixed substrates with a view to develop an ideal biop ɔcess for its large scale production.

1.2. REVIEW OF LITERATURE

1.2.1. Sources of microbial L-glutaminase

L-Glutaminase is widely distributed in plants, animal and microorganisms. In animals they are mainly present in the kidn y and liver and are called the Kidney type and Liver type glut inase respectively. It plays a central role in cellular n rogen metabolism. Glutaminase from mammalian tissues are phc phate dependent and is distinctly different from the microbial glutar inases (Tanaka *et al*, 1988 Pal & Maity, 1992 b). Only the lit ature available on microorganisms is reviewed here since any atte pt on other sources is out of scope of the present study.

Several species of bacteria, yeasts and fungi are repor ed to produce L-glutaminase. The details are summarized in Table 1.

Table - 1. Sources of microbial L-glutaminase

Organism	Source	Reference
BACTERIA		
Achromobacter	Terrestrial	Roberts *et al* (1972)
Acinetobacter sp.	Terrestrial	Holcenberg *et al* (197)
A. glutaminasificans	Terrestrial	Schmidt & Roberts (1 74)
Aerobacter aerogenes	Terrestrial	Imada *et al* (1973)
Azotobacter	Terrestrial	Ehrenfeld *et al* (1963
Bacillus subtilis	Terrestrial	Harayama & Yusuhar (1991)
B. licheniformis & *B. circulans*	Terrestrial	Cook *et al* (1981)
Clostridium welchii	Terrestrial	Hughes & Williamson 1952)
E. coli	Terrestrial	Peterson *et al* (1974)
Enterobacteriaceae	Terrestrial	Roberts (1976)
Erwinia carotovora	Terrestrial	Wade *et al* (1971)
Micrococcus luteus	Marine	Moriguchi *et al* (199
Proteus vulgaris	Terrestrial	Wade *et al* (1971)
Pseudomonas sp.	Terrestrial	Arima *et al* (1972)
Pseudomonas 7 A	Terrestrial	Oki *et al* (1973)
P. aeruginosa	Terrestrial	Schmidt & Roberts (374)
P. aureofaciens	Terrestrial	Meister (1955)
P. acidovorans	Terrestrial	Davidson *et al* (1977
P. fluorescence	Marine	Renu & Chandrasek an (1992 a)
P. putrefaciens	Terrestrial	Holcenberg *et al* (19 3)
Serratia marcescens	Terrestrial	Wade *et al* (1971)
Vibrio costicola	Marine	Renu & Chandrasek an (1992 a) Nagendra Prabhu & Chandrasekaran (1995;1996 b)
V. cholera	Marine	Renu & Chandrasek an (1992 a)

FUNGI

Actinomucor elegans	Terrestrial	Chou *et al* (1993)
Aspergillus oryzae	Terrestrial	Furuya *et al* (1985) Yano *et al* (1988) Chou *et al* (1993)
A. sojae	Terrestrial	Kuroshima *et al* (196)
A. taiwanensis	Terrestrial	Chou *et al* (1993)
Tilachlidium humicola	Terrestrial	Imada *et al* (1973)

YEASTS

Candida utilis	Terrestrial	Kakinuma *et al* (1987
Cryptococcus sp.	Terrestrial	Furuya *et al* (1985)
Cryptococcus laurentii	Terrestrial	Kakinuma *et al* (1987
C. albidus	Terrestrial	Iwasa *et al* (1987); Yokotsuka (1987); Fukushima & Motai (990)
Hansenula	Terrestrial	Furuya *et al* (1985)
Torulopsis candida	Terrestrial	Kakinuma *et al* (1987

1.2.2. METHODS OF L-GLUTAMINASE PRODUCTION

1.2.2.1. Submerged Fermentation

Majority of reports on bacterial glutaminase production deal with submerged fermentation. Summary of reports on productio of L-glutaminase under submerged fermentation by bacteria and fun i is presented in Table 2.

Table - 2. L-Glutaminase production by microorganisms under submerged fermentation

Microorganism	Intra/Extra cellular	Referenc
Achromobacteraceae	Intracellular	Roberts *et al* (1972)
Acinetobacter	Intracellular	Holcenberg *et al* (1972)
Aspergillus oryzae	Intracellular	Furuya *et al* (1985)
A. oryzae	Intra & Extra	Yano *et al* (1988)
Bacillus subtilis	Intracellular	Mardashev *et al* (1970)
Bacillus licheniformis	Intracellular	Cook *et al* (1981)
Clostridium welchii	Intracellular	Hughes & Williamson (1 52)
E. coli	Intracellular	Prusiner *et al* (1976)
Erwinia carotovora	Intracellular	Mardashev *et al* (1970)
Micrococcus luteus	Intracellular	Moriguchi *et al* (1994)
Pseudomonas sp.	Intracellular	Ramadan *et al* (1964)
P. acidovorans	Intracellular	Davidson *et al* (1977)
P. aeruginosa	Intracellular	Soda *et al* (1972)
P. fluorescence	Intra & Extra	Renu & Chandrasekaran
Proteus vulgaris	Intracellular	Mardashev *et al* (1970)
Serratia marcescens	Intracellular	Ramadan *et al* (1964)
Vibrio cholera	Intra & Extra	Renu & Chandrasekar n (1992 a)
V. costicola	Intra & Extra	Renu & Chandrasekar n (1992 a)

1.2.2.2. Solid State Fermentation

Reports on the use of solid state fermentation for extracel ılar glutaminase production are limited and mainly deal with fungi. The production of glutaminase by *Aspergillus oryzae* using wheat ran (Tomita *et al*, 1988; Yano *et al*, 1988), *Pseudomonas fluoresc*ıce, *Vibrio costicola* and *V. cholera* using wheat bran (Renu, 1991; Renu ınd Chandrasekaran 1992 b); *Aspergillus oryzae, Actinomucor elega*ı s & *A. taiwanensis* using a mixed substrate system (Chou *et al*, 1993) are known. Nagendra Prabhu and Chandrasekaran (1995, 1996 a, b) repo ted that marine *Vibrio costicola* produced extracellular L-glutami ase under SSF using different natural substrates and polystyrene. In fact, except for the report of Renu and Chandrasekaran (1992 b) ınd Nagendra Prabhu and Chandrasekaran (1995, 1996 a, b), no other rep rts are available on the use of bacteria for L-glutaminase produc ion under SSF.

1.2.2.3. Use of nutritionally inert supports in SSF

SSF processes using conventional, nutritionally rich substr tes such as wheat bran have certain inherent problems such as the lacı of methods for accurate monitoring of process parameters, scaling up strategies and various engineering aspects. Biomass estimation is almost impossible and unreliable with nutritionally rich substr :es which also contributes to undesirable product formation besi .es

creating problem of solid waste disposal (Mitchell, 1992; Zhu *et al*, 1994). Use of nutritionally inert supports have been recommended for SSF in order to overcome most of the inherent problems of SSF, since these materials when impregnated with a suitable media provide a homogeneous aerobic condition throughout the fermenter and contribute no impurities to the fermentation product (Aidoo *et al*, 1982). They also offer several other advantages such as yield of product with high specific activity and free from the presence of undesired proteins, maximum recovery of the leachate with reduced viscosity, easy optimization of the media which gives maximum product yield, feasibility of biomass estimation, lower chances of contamination etc., which are all highly desirable features in the fermentation industry (Zhu *et al*, 1994; Nagendra Prabhu & Chandrasekaran, 1995, 1996 a, b).

It was Meyrath (1966) who first used a synthetic inert solid material, vermiculite, for the production of amylase by *Aspergillus oryzae*. He found that the rate of enzyme production on vermiculite impregnated with 4 % starch solution was as high as on wheat bran and the yield was almost double than that obtained with wheat bran. Polyurethane foam was used as inert support for enzyme production by fungi (Fujishima *et al*, 1972) and for higher yields of citric acid by *Aspergillus niger* as compared to submerged or surface culture methods (Aidoo *et al*, 1982).

More recently, materials such as computer cards for β-glucos ıase production by *Aspergillus niger* (Madamwar *et al*, 1989); ion-exch ınge resin, Amberlite IRA 900 for the growth studies of *Aspergillus iger* (Auria *et al*, 1990); polyurethane foam for glucoamylase productio by *Aspergillus oryzae* (Kobayashi *et al*, 1991) and for the productio ı of nuclease P 1 from *Penicillium citrinum* (Zhu *et al*, 1994), and polystyrene, for producing L-glutaminase from marine *Vibrio cos icola* (Nagendra Prabhu & Chandrasekaran, 1995, 1996 b) have been tri l as inert supports for SSF. Except for the reports of Nagendra Prabhı and Chandrasekaran, all other reports are based on fungal systems.

1.2.2.4. Use of Mixed Substrates in SSF

Use of mixed substrates for the production of enzymes, or anic acids, mushroom cultivation etc., have been reported earlier (Ro ssos *et al*, 1991 a; Pandey & Radhakrishnan, 1993; Jwanny *et al*, 1995 A mixture of sugar cane bagasse and wheat bran at a ratio of 80 2(was used for the production of cellulases by *Trichoderma harz. ınum* (Roussos *et al*, 1991 a). Maximum glucoamylase production by *A. ıiger* was obtained when wheat bran was mixed with corn flour at 9 1 v/w) ratio, but mixtures of wheat bran with other substrates such as rice flour, rice husk, rice bran, wheat flour, corn starch and bengal gram flour showed varying trend of enzyme production (Pande & Radhakrishnan, 1993). Decrease in protease yield by *Rhi opus oligosporus* was observed when a mixed substrate system of wheat ıran,

wheat flour, soy flour, soy oil, soy bean, rice flour, rice, rice ran and cassava starch were used in varying combination (Ikasa & Mitchell, 1994). The technical feasibility of using mango and late industry wastes for mushroom cultivation was evaluated and it was observed that the highest yield of fruiting bodies was obtained u ing a mixture of date waste and rice straw at 1 1 ratio, followed by 3 1 and 2 1 ratios respectively (Jwanny *et al*, 1995). They also reported that mango waste supplemented with rice straw could be sed for mushroom cultivation. Whereas no reports are available on the use of a mixed substrate system involving both nutritionally rich and inert substrates.

1.2.3. Impact of mutation on L-glutaminase production

Literature on the impact of mutation on L-glutaminase produ ion is limited to very few. Induced mutation increased protease and glutaminase production by koji moulds such as *Aspergillus oryzae* and *A. sojae* (Nasuno & Ohara, 1972; Yamamoto, 1974; Furuya *et al*, 983, 1985). NTG treatment of *Torulopsis famata* resulted in a 3 fold increase in glutaminase yield (Kakinuma *et al*, 1987). Spontaneous and induced mutation of streptomycin sensitive strains of *E. coli* led o a 60 % decrease in L-glutaminase and L-asparaginase activ ties (Mugnetsyan & Stepanayen, 1987).

1.2.4. Purification of microbial L-glutaminase

Earliest reports on the purification and characterizatio of glutaminase dates back to the 1950's. Glutaminase have been iso ated and purified from several bacteria including *Clostridium w chii* (Hughes & Williamson, 1952); *E. coli* (Rudman & Meister 1953; Mei ter, 1955; Hartman, 1968; Prusiner *et al* 1976; Yokotsuka, 1 87); *Cryptococcus albidus* (Iwasa *et al*, 1987; Yokotsuka, 1987); *Ba illus subtilis* GT (Shimazhu *et al* 1991); *Pseudomonas fluores nce* (Yokotsuka, 1987; Renu, 1991) and *Micrococcus luteus* K 3 (Moriguc i *et al*, 1994). Glutaminase from *Achromobacteraceae* was purified wit an overall yield of 40–60 %, crystallized and characterized for its properties (Roberts *et al* 1972). L-Glutaminase from *Ser atia marcescens* was purified using DEAE-Cellulose chromatography (Nov k & Phillips, 1974).

Glutaminase - glutamyltransferase was purified from *Azotob cter agilis* (Ehrenfeld *et al*, 1963). L-glutaminase - asparaginase rom *Pseudomonas acidovorans* was purified to homogeneity by Davidson t *al* (1977), while glutaminase isozymes from *P. aeruginosa* were also purified and characterized (Soda *et al*, 1972; Oshima *et al*, 1 76). L-Glutaminase with asparaginase activity from *Pseudomonas* was pur fied by butanol treatment, ammonium sulphate fractionation and one electrophoresis (Ramadan *et al*, 1964). The preliminary cry al structure of *Acinetobacter glutaminasificans* glutaminase–asparag ase

was reported by Ammon *et al*, (1988). Similarly, glutaminase - asparaginase from *Pseudomonas* 7 A was purified and structurally characterized (Lubkowski *et al*, 1994).

Glutaminases from the fungus, *Aspergillus oryzae* was purified employing different methods including ammonium sulphate fractionation and ion exchange chromatography (Shikata *et al*, 1978; Teramoto *et al*, 1985). Intra and extracellular glutaminases from *A. oryzae* were purified with a 730 fold purification with 6.2 % recovery for the intracellular fraction and a 1100 fold purification with 3.1 % recovery for the extracellular fraction (Yano *et al*, 1988; Tomita *et al*, 1988).

1.2.5. Properties of L-glutaminase

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

The pH and temperature tolerance of glutaminases from various microorganisms differed greatly. While optimal activities of glutaminase A and B of *P. aeruginosa* were at alkaline pH of 7.5 9.0 and 8.5 respectively (Soda *et al*, 1972), glutaminase from *Pseudomonas* was reported to be active over a broad range of pH (5-9) with an optimum near pH 7.0 (Roberts, 1976). Glutaminase of *Pseudomonas acidovorans* showed optimum activity at pH 9.5 and retained 7 % activity at pH 7.4 (Davidson *et al*, 1977). An intracellular glutaminase from *Cryptococcus albidus* preferred an optimal pH of 5 - 8.5 (Yokotsuka *et al*, 1987). Whereas glutaminase I and II isolated from marine *Micrococcus luteus* were active at alkaline pH values of 8.0 and 8.5 respectively (Moriguchi *et al*, 1994). Glutaminases from *A. oryzae* and *A. sojae* recorded pH optima of 9.0 and 8.0 respectively (Shikata *et al*, 1978). The intra and extracellular glutaminases from *A. oryzae* were most active and stable at pH 9.0 (Yano *et al*, 1988).

The temperature stability of glutaminases also showed wide variation. Glutaminase from *Pseudomonas* showed maximum activity at 37^{o}C and were unstable at high temperatures (Ramadan *et al*, 1984), whereas the enzyme from *Clostridium welchii* retained activity upto 60^{o}C (Kozlov *et al*, 1972). Glutaminase from *Cryptococcus albidus* retained 77 % of its activity at 70^{o}C even after 30 minutes of incubation (Yokotsuka *et al*, 1987). Glutaminase I & II from *Micrococcus luteus* had a temperature optima of 50^{o}C and the presence of NaCl (10 %) increased the thermostability (Moriguchi *et al*, 1991).

The optimum temperature for activity of both intra and extrace ular glutaminases from *A. oryzae* was 45°C while they became inacti e at 55°C (Yano *et al*, 1988).

Glutaminases also differed in their affinity towards L-gluta ine. While the enzyme from *Acinetobacter sp.* recorded a Km of 5.8 ± .5 X 10^{-6} M, those from *C. welchii* had a Km of 10^{-3} M (Kozlov *et al*, 972). The enzyme from *Achromobacteraceae* had a Km of 4.8 ± 1.4 X 1($^{-6}$ M (Roberts *et al*, 1972). The average Km values for glutami ase-asparaginase from *Pseudomonas* 7A was 4.6 ± 0.4 X 10^{-6} M (Rol rts, 1976) whereas that from *P. acidovorans* had 2.2 X 10^{-5} M (Davids n *et al*, 1977). The glutaminase I and II from marine *Micrococcus l teus* had a Km of 4.4 mM and 6.5 mM respectively (Moriguchi *et al*, 199

The isoelectric point of glutaminase varied for diff ent organisms. Thus it was 5.5 for *Clostridium welchii* (Kozlov e *al*, 1972); 5.4 for *E. coli* (Prusiner *et al*, 1976); 8.43 for *Acinetob cter glutaminasificans* (Roberts *et al*, 1972); 5.8 for *Pseudo nas* (Holcenberg *et al*, 1976); 7.6 for another species of *Pseudo nas* (Katsumata *et al*, 1972); 3.94 - 4.09 for *Cryptococcus ali idus* (Yokotsuka, 1987) *and* 7.2 for *Pseudomonas acidovorans* (Davidson al, 1977).

Glutaminase activity was found to be inhibited by va ious substances and heavy metals. Cetavlon, while accelerating glutam ase of *Clostridium welchii, E. coli* and *Proteus morganii* in crude ext acts and intact cells, inhibited the purified enzyme (Hughes & Willia son, 1952). Glutaminase of *E. coli* was found to be sensitive to h avy metals (Hartman, 1968) and *Acinetobacter* glutaminase-asparaginase was inactivated by glutamine analogue 6-diazo 5-oxo L-norleucine eve at very low concentration while unaffected by EDTA, NH_3, L-glutama or L-aspartate (Roberts *et al*, 1972). Various investigations have s own that glutaminase from *Pseudomonas* was activated by certain div lent anions and cations while inhibited by monovalent anions and by ce tain competitive inhibitors like NH_3, D and L - glutamic acid and 6- iazo 5-oxo L-norleucine (Ramadan *et al*, 1964; Soda *et al*, 1972; Rob rts, 1976). In the case of fungi, both intra and extracellular glutam ase from *Aspergillus oryzae* were inhibted by Hg, Cr and Fe but were not affected by sulphydroxyl reagents (Yano *et al*, 1988). EDTA, Na_2SO_4, and *p*-chloromercuribenzoate strongly inhibited the *Micrococcus l eus* glutaminase I while glutaminase II was inhibited by EDTA, $HgCl_2$, Na_2SO_4, $CuCl_2$ and $FeCl_3$ (Moriguchi *et al*, 1994).

The bacterial amidohydrolases are reported to be homotetrame s of identical subunits and the individual subunits are not catalyti ally active. The molecular weight ranges from 120,000 - 147,000 da ons (Ammon *et al*, 1988). The enzyme from *Achromobacteraceae* show d a molecular weight of 138,000 daltons with a subunit molecular weig of

35,000, whereas that from *P. acidovorans* had a larger molecular w ight of approximately 156,000 and subunit weight of 39,000 da tons (Davidson *et al*, 1977). The glutaminase-asparaginase from *Er inia chrysanthemi* had a subunit molecular weight of 35,100 and approximately 140,000 for the native protein (Tanaka *et al*, 1988).

Enzyme with smaller molecular weight has also been rep rted (Prusiner *et al*, 1976; Moriguchi *et al*, 1994). Glutaminase B fr m *E. coli* had a molecular weight of 90,000 daltons when estimated b gel filtration on Sephadex G-200 and 100,000 daltons under electroph esis (Prusiner *et al*, 1976). Glutaminase I and II from *Micrococcus* h d a molecular weight of 86,000 daltons when measured by gel filtratic n on Superose 12 column. Glutaminase I also showed a subunit mole ular weight of 43,000 daltons upon SDS-PAGE (Moriguchi *et al*, 1994).

1.2.6. APPLICATIONS OF L-GLUTAMINASE

1.2.6.1. Glutaminase in flavour industry

Flavour industry is a rapidly developing industry and microorganisms and their products play an important role in fla our production in various food stuffs (Delest, 1995). In additio to being used for the production of flavour chemicals, ome microorganisms and their enzymes can be used for the improveme of food flavour. For example, glucose oxidase from *Penicillium sp.* was used to inhibit endogenous oxidoreductases in tomato fruit to imp ove its sensory and nutritional qualities (Montedoro 1995).

L-Glutaminase enhances the flavour of fermented foods by increasing their glutamic acid content thereby imparting a palat ble taste (Yokotsuka, 1985, 1986). It is widely used in countries suc as Japan where fermented foods such as soy sauce is a highly valu ble commodity. Of the many Oriental fermented products, soy sauce is the one most widely consumed in China, Japan, Korea and other As tic countries as a condiment and colouring agent in preparation of f ds and for table use (Luh, 1995).

In soy sauce fermentation, it is important to increase the amc nt of glutamic acid for a delicious taste. Glutaminase is gener lly regarded as a key enzyme that controls the quality and taste of oy

sauce and other fermented foods (Yamamoto & Hirooka, 1974; Tomi a *et al*, 1988; Yano *et al*, 1988). Salt tolerant glutaminase rom *Cryptococcus albidus* was used to increase the glutamic acid conte t of soy sauce (Nakadai and Nasuno, 1989).

Yokotsuka *et al* (1987) selected 3 strains *E. coli, Pseudo* nas *fluorescence* 3021 and *Cryptococcus albidus* IAM 4830 from 292 st ains of bacteria and 450 strains of yeasts which produced heat and salt tolerant glutaminase. A glutaminase with γ - glutamyl transpept lase activity was also isolated from *A. oryzae* with a view to improv the glutamic acid content of fermented foods (Tomita *et al*, 1 88). Protoplast fusion among the species of *A. sojae* was employe by Ushijima and Nakadai (1984) to induce protease and glutam ase production. *Cryptococcus albidus* producing salt tolerant glutam ase was immobilized on silica gel and alginate - silica gel comple for obtaining a continuous production of glutamic acid from glut ine (Fukushima and Motai, 1990).

Glutaminase from *Aspergillus oryzae* is traditionally used for soy sauce fermentation in many countries. However the enzym from *A. oryzae* has been shown to be markedly inhibited by the high salt concentration in the fermentation process (Yano *et al*, 1988). U of salt tolerant glutaminase from marine bacteria provides an intere ting alternative in the soy sauce fermentation industry (Moriguchi *et al*, 1994).

1.2.6.2. L-Glutaminase in cancer treatment

L-Glutaminase is a potential candidate for enzyme therapy of cancer. It is found that administration of L-glutaminase will deplete L-glutamine which is required for asparagine synthesis in the cells thereby inhibiting asparagine dependent protein synthesis and eventually the synthesis of DNA and RNA. Certain tumour cells utilize L-glutamine as the major carbon and energy source even in the presence of glucose (Reitzer *et al*, 1979). At the same time they are deficient in glutamine synthetase enzyme which is needed for the synthesis of glutamine. Thus, by depleting the amount of circulating L-glutamine in the blood, it is possible to selectively starve the tumour cells without causing any harm to the normal cells who have normal glutamine synthetase enzyme (Tanaka *et al*, 1988).

A number of glutaminases with antitumour activity have been isolated from *Acinetobacter glutaminasificans, Pseudomonas aureofaciens, P. aeruginosa, Pseudomonas* 7 A & *Achromobacter* (Roberts, 1976; Spiers *et al*, 1976). Several of these enzymes reduced both asparagine and glutamine concentration in tissues and their therapeutic effect may depend on the combined depletion of both these amino acids. *E. coli* glutaminase was effective in lowering the tumour burden, in Ehrlich ascites tumour bearing mice and led to increase in the life span of the host (Pal & Maity, 1992 a). It was found that glutaminase-asparaginase from *Pseudomonas aureofaciens* was inhibitory

to 9 out of 16 tumours in mice and rats, one of which was an asparaginase resistant mouse adenosarcoma (Bauer *et al*, 1971).

Kien *et al* (1985) observed that glutaminase - asparaginase from *Acinetobacter sp.* markedly reduced the tumour burden in mice bearing Ehrlich ascites tumour. When purified glutaminase from *E. coli* was used in combination with copper-ATP for the treatment of mice bearing Ehrlich ascites carcinoma, it was found that it was more effective in inhibiting tumour growth and in increasing the life span of hosts, compared with the individual efficacies of these two agents (Pal & Maity, 1994). O'Dwyer *et al* (1984) used a glutamine antagonist, acivicine, in clinical trials. Crystalline bacterial glutaminase - asparaginase in combination with acivicin and 6-diazo-5-oxo-norleucine enhanced therapeutic effectiveness of glutamine antimetabolites against human and murine solid tumours *in vivo* (McGregor & Roberts, 1989). They reported that the antitumour effectiveness of glutamine antimetabolites is enhanced when the available pool of glutamine is depleted by glutaminase. Roberts and McGregor (1989) also reported that glutaminase had potent antiretroviral activity *in vivo*. They found that murine leukaemia virus required glutamine for replication and glutaminase mediated depletion of glutamine in animals resulted in potent inhibition of retrovirus replication, thereby increasing the median survival time of the animals.

1.3. OBJECTIVES OF THE PRESENT STUDY

Considering the growing importance of enzymes from marine bacteria and need for developing ideal SSF processes, studies were undertaken to produce L-glutaminase by a marine bacterium employing SSF using different natural substrates and an inert support. Marine *Vibrio costicola*, isolated during an earlier investigation in our laboratory, that produced extra cellular L-glutaminase under both SmF and SSF using wheat bran, was used in the present study. The natural solid substrates used for SSF included Wheat bran, Rice husk, Copra cake powder, Groundnut cake powder and Saw dust, which are easily available, cheap materials procured from the local market. Polystyrene [Poly (1-phenylethylene)], a commercially available insulating and packaging material was tried as a model synthetic inert support for L-glutaminase production by marine *V. costicola* under SSF.

The specific objectives include

1) Production of L-glutaminase by marine *Vibrio costicola* under SSF using natural substrates.

2) Use of Polystyrene as a model inert support for L-glutaminase production by the bacteria.

3) Use of a mixed substrate system (Natural + Inert) for L-glutaminase production.

4) To study the impact of mutation on the rate of glutaminase production by the bacterium under SSF using Polystyrene.

5) Purification and characterization of the enzyme.

MATERIALS & METHODS

2.1. MICROORGANISM

Vibrio costicola (ACMR 267) was used throughout the study.

2.1.1. Source

The bacteria was isolated from the marine environments of Cochin, and available as stock culture in the culture collection of the Department of Biotechnology, Cochin University of Science and Technology, India (Renu, 1991).

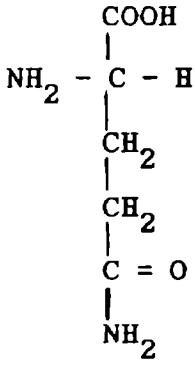
2.1.2. Maintenance

The culture was maintained on ZoBell's marine agar slants and subcultured once in a month. One set was maintained as stock culture, layered over with sterile mineral oil. Another set was used as the working culture for routine experiments.

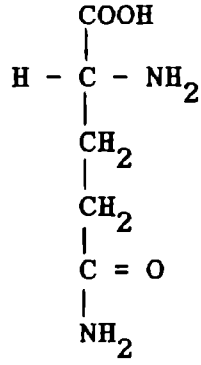
2.2. SUBSTRATE

L-Glutamine (SRL, India) was used as the substrate for growth and enzyme production by *Vibrio costicola*. Chemical names of glutamine are 2-amino 4-carbomylbutanoic acid and α-amino γ-carbamidobutyric acid. Empirical formula for glutamine is $C_5H_{10}O_3N_2$ (C - 41.09 % H - 6.90 % O - 32.84 % and N - 19.17 %), having a molecular weight of 146.15. Glutamine is found to occur as optical isomers ie., L-glutamine and D-glutamine (Greenstein & Winitz, 1961).

L-Glutamine



D-Glutamine



Wheat glutan hydrolyzate and beet sugar molasses are two natural sources of glutamine.

2.3. L-GLUTAMINASE PRODUCTION UNDER SOLID STATE FERMENTATION USING NATURAL SUBSTRATES

Production of extracellular L-glutaminase by *V. costicola* under SSF was studied using five nutritionally rich solid substrates which included Wheat Bran (WB), Rice Husk (RH), Copra Cake Powder (CCP), Groundnut Cake Powder (GCP) and Saw Dust (SD). Wheat bran and Rice Husk are widely accepted solid substrates for exoenzyme production under SSF. They have high nutrient contents, large surface area and support the growth of a wide variety of microorganisms (Ghildyal, 1991). Saw dust forms the cellulose rich waste from the saw mills and commonly used as fuel and packing material. Copra cake

powder and Groundnut cake powder are byproducts obtained after oil extraction from Copra and Groundnut respectively and are used as poultry and cattle feed (Wealth of India, 1950). These solid substrates were selected for fermentation studies based on their easy availability, water holding capacity and non-requirement of extensive pretreatment procedures.

2.3.1. Preparation of solid substrates

All the substrates were purchased in bulk quantities from the local market as a single lot. They were powdered and stored in air tight containers after drying in a hot air oven overnight at $60^{o}C$. All experiments were carried out using 10 g of the solid substrate taken in 250 ml Erlenmeyer flasks. The substrates were moistened with phosphate buffer (0.1 M pH 7.0) containing L-glutamine at 1 % (w/w) level, autoclaved for 1 h at $121^{o}C$ and cooled to room temperature ($28\pm2^{o}C$) before inoculation (unless otherwise stated).

2.3.2. Medium

Mineral salts glutamine medium (MSG medium) with the following composition was used for the preparation of the inoculum throughout the course of the study (Renu, 1991).

KH_2PO_4	- 1.0 g
$MgSO_4.7H_2O$	- 0.5 g
$CaCl_2$	- 0.1 g
$NaNO_3$	- 0.1 g
Trisodium citrate	- 0.1 g
Glucose	- 5.0 g
L-Glutamine	- 10.0 g
Distilled Water	- 1 L
pH	- 7.0 $\pm$ 0.2

The prepared medium was autoclaved before use.

2.3.3. Preparation of Inoculum

1) A loopful of 18-24 h old slant culture was transferred aseptically to 10 ml of the prepared MSG medium taken in a test tube and incubated for 24 h on a rotary shaker at 150 rpm, at room temperature.

2) Using the test tube grown culture, 100 ml of sterile MSG medium taken in 250 ml flask was inoculated and incubated for 24 h at room temperature, on the rotary shaker at 150 rpm.

3) After incubation, the cells were centrifuged, washed twice with sterile saline, resuspended in the same and transferred aseptically to 1000 ml of sterile MSG medium taken in a 3 L flask. The inoculated medium was incubated on the rotary shaker (150 rpm) for 24 h at room temperature.

4) The cultured cells were harvested by centrifugation under sterile conditions in a high speed refrigerated centrifuge (Kubota, Japan), washed twice with sterile saline and resuspended in the same and used as the inoculum. The final cell suspension had a cell concentration of 10^8 cells/ml (or 0.1 mg dry wt/ml).

2.3.4. Inoculation and Incubation

The sterilized solid substrate media was inoculated with the prepared inoculum containing 1.0 mg dry weight cells/10 gram dry solids (arbitrarily selected before optimization of inoculum concentration). The contents were mixed thoroughly and incubated in a slanting position at $30^{o}C$ for 24 h (unless otherwise mentioned) under 75-80 % relative humidity (Renu, 1991).

2.3.5. Enzyme Recovery

Enzyme from the fermented solid substrates was extracted using phosphate buffer (0.1 M, pH 7.0) by simple contact method (Renu, 1991), as described below.

Phosphate buffer was added to the fermented medium at 1:5 (w/v) ratio, mixed thoroughly and subjected to simple contact for 30 min on a rotary shaker (150 rpm). Later the contents were pressed in dampened cheese cloth to recover the leachate. The process was repeated twice, the extracts were pooled and centrifuged at 10,000 rpm for 15 min at $4^{o}C$ in a refrigerated centrifuge. The supernatant was used as the crude enzyme.

2.3.6. ASSAYS

2.3.6.1. L-Glutaminase

L-Glutaminase was assayed by following the methodology of Imada *et al* (1973) with slight modifications, as given below.

1) An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of 0.04 M L-glutamine solution in the presence of 0.5 ml of distilled water and 0.5 ml of phosphate buffer (0.1 M, pH 8.0).

2) The mixture was incubated at $40^{o}C$ for 15 min. and the reaction was arrested by the addition of 0.5 ml of 1.5 M trichloro acetic acid.

3) To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added.

4) The absorbance was measured at 450 nm using a UV-Visible spectrophotometer (Shimadzu 160 A, Japan).

5) A standard graph was plotted using ammonium chloride as the standard for computation of concentration of ammonia.

6) One international unit of glutaminase was defined as the amount of enzyme that liberates one μ mol of ammonia under optimum conditions. The enzyme yield was expressed as Units/gram dry substrate (U/gds).

7) Appropriate controls were also maintained.

2.3.6.2. Protein

Protein content was estimated using Lowry's method with bovine serum albumin as the standard (Lowry *et al,* 1951). The values were expressed as mg/ml.

2.3.6.3 Viscosity

Viscosity of the extracts was measured using Ostwald viscometer and values are expressed as Newtons/m^2 (Ns/m^2 Ramesh & Lonsane, 1989).

2.3.6.4. Other Enzymes

Presence of L-asparaginase (Imada *et al,* 1973), amylase (Medda & Chandra, 1980), cellulase (Mandels *et al,* 1981) and protease (Kunitz, 1946) in the leachate were also tested.

2.3.7. Impact of process parameters on L-glutaminase production during solid state fermentation using natural substrates

The impact of various process parameters influencing L-glutaminase synthesis by *V. costicola* under SSF was studied as detailed below. The parameters studied included, initial moisture content of the medium, initial pH, particle size of the substrates, incubation temperature, L-glutamine concentration, additional carbon and nitrogen sources, NaCl concentration, additional mineral salts, inoculum concentration and incubation time. Preparation of solid

substrates and inoculum, inoculation and incubation, extraction and assay of enzymes were carried out as described in earlier sections 2.3.1., 2.3.3., 2.3.4., 2.3.5. and 2.3.6. respectively.

The protocol adopted for optimization of various process parameters influencing glutaminase yield was to evaluate the effect of an individual parameter and to incorporate it at the standardized level before optimizing the next parameter (Sandhya Xavier & Lonsane, 1994). All the experiments were carried out in triplicate and mean values are reported.

2.3.7.1. Initial moisture content

The effect of initial moisture content of the solid medium on enzyme production was determined by preparing the solid substrates with varying initial moisture contents in the range of 30–80 %. This was achieved by altering the amount of buffer used to moisten the substrates.

2.3.7.2. Initial pH of the medium

The impact of initial pH of the medium on enzyme yield by bacteria during SSF was studied by adjusting the pH of the solid substrate medium with various buffers in the range of pH 5-9. The buffers used to obtain the desired pH included acetate buffer (pH 5), phosphate buffer (pH 6-8) and glycine–NaOH buffer (pH 9).

2.3.7.3. Particle size of the substrate

Impact of particle size of the solid substrates on glutaminase production by the bacteria was evaluated using substrates of different particle size. The solid substrates purchased from the local market contained particles of different sizes and hence they were sieved using standard sieves (Filterwel, India) of known mesh sizes ranging from 425 μ to 2000 μ to obtain uniform particles of desired size.

2.3.7.4. Incubation temperature

The effect of incubation temperature on enzyme production by bacteria during SSF was determined by incubating the inoculated flasks at different temperatures (25-50^{o}C) under 75-80 % relative humidity.

2.3.7.5. L-Glutamine concentration

The influence of L-glutamine concentration on glutaminase yield by bacteria during SSF was assessed by incorporating L-glutamine at different levels (0.5 - 5.0 % w/w) in the medium.

2.3.7.6. Inoculum concentration

The impact of inoculum concentration on enzyme yield by bacteria during SSF was determined by using inoculum of increasing size in the range 0.125 - 1.250 mg dry wt/10 gds.

2.3.7.7. Incubation time

The effect of incubation time on enzyme production by bacteria during SSF was studied by incubating the inoculated flasks for a total period of 72 h and estimating the enzyme production at regular intervals of 12 h.

2.3.7.8. Additional carbon sources

The effect of additional carbon sources on enzyme synthesis by bacteria during SSF was evaluated by incorporating them at 1 % (w/w) level in the medium. The carbon sources analyzed included Sucrose, Maltose, Glucose, Lactose, Galactose and Trisodium citrate.

2.3.7.9. Additional nitrogen sources

The impact of additional nitrogen sources on glutaminase production by the bacteria during SSF was evaluated by incorporating them at 1 % (w/w) level in the SSF medium. The nitrogen sources tested include Casein, Ammonium sulphate, Ammonium nitrate, Sodium nitrate, Yeast extract, Peptone and Urea.

2.3.7.10. Amino Acids

The impact of amino acids as additional nitrogen sources on glutaminase yield by bacteria under SSF was assessed by incorporating them at 1 % (w/w) level in the SSF medium. The amino acids tested were L-Asparagine, L-Glutamic acid, Tyrosine, Alanine, Arginine, Methionine and Cysteine.

2.3.7.11. Mineral salts

The effect of various mineral salts on glutaminase production by bacteria during SSF was also tested by incorporating them at 1 % (w/w) level in the medium. The salts tested included KH_2PO_4, NaH_2PO_4, KCl, $CaCl_2$, $MgSO_4.7\ H_2O$, and $MnCl_2$.

2.3.7.12. NaCl concentration

The effect of NaCl concentration on glutaminase production by bacteria under SSF was determined by adding NaCl at different levels (0–10 % w/w) in the medium.

2.4. L-GLUTAMINASE PRODUCTION UNDER SOLID STATE FERMENTATION USING POLYSTYRENE AS INERT SUPPORT

Expanded polystyrene [Poly (1-phenylethylene)], is a commercially available insulating and packaging material. It is odourless, non-toxic, tasteless, low in weight, less brittle and non-biodegradable. Its maximum water absorbency is 2.0 g/100 cm^3 (Brydson, 1982). Although it is nutritionally inert, it could act as a support for the attachment of microorganisms during fermentation. Hence, in the present investigation, polystyrene was used as a model inert support for glutaminase production by bacteria under SSF.

2.4.1. Selection of suitable medium for L-glutaminase production under SSF using polystyrene

Suitable media that can support enhanced production of glutaminase by *Vibrio* using polystyrene as inert support was determined by using different media, which included

1) Aged sea water (25-30 ‰ salinity) containing L-glutamine 1 % (w/w) (SWG Medium).
2) Mineral salts glutamine (MSG) media described in section 2.3.2
3) Phosphate buffer (0.1 M pH 7) with 1 % (w/w) L-glutamine (PBG Medium)
4) Physiological saline (0.85 % NaCl) containing 1 % (w/w) L-glutamine (PSG Medium).
5) Distilled water containing 1 % (w/w) L-glutamine (DWG Medium).

They were used at a solid substrate to media ratio of 1 1 (w/v). The media that supported maximal glutaminase yield was used in the subsequent optimization studies.

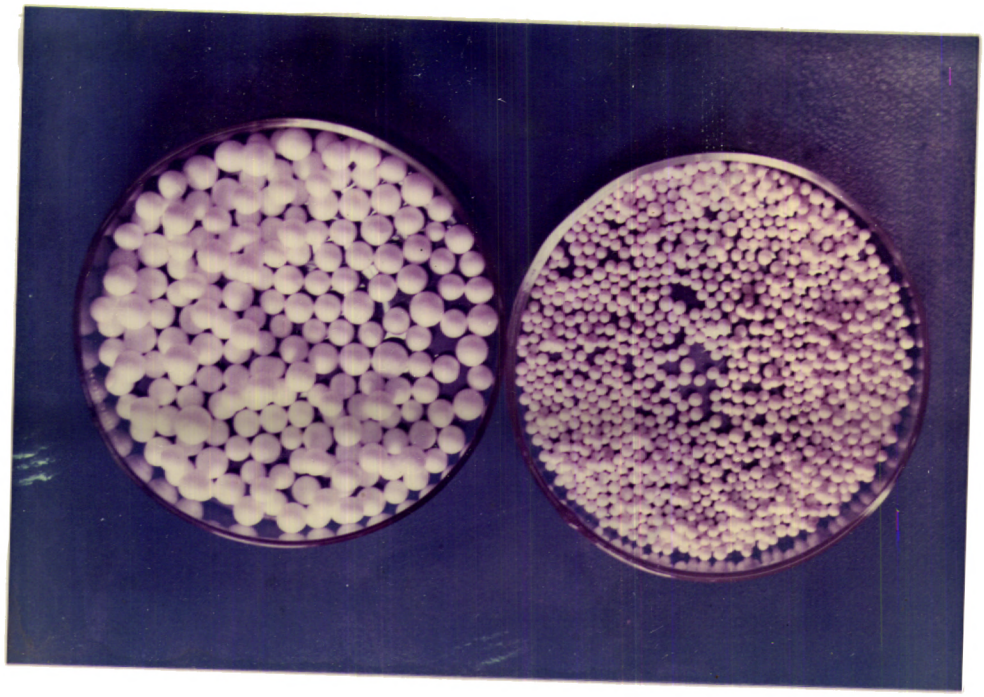
2.4.2. Preparation of solid substrate

Polystyrene beads of 2-3 mm diameter were pretreated by autoclaving at 121^{o}C for 15 min. during which the beads collapsed and reduced to about one third of their original size (Brydson, 1982). The reduced beads of uniform size (1 - 1.5 mm) were selected for fermentation studies (Plate - I).

PLATE - I

PHOTOGRAPH OF POLYSTYRENE BEADS

(A) Before pretreatment (B) After pretreatment



A B

5 g of pretreated polystyrene beads were taken in 250 ml Erlenmeyer flasks, moistened with the medium, autoclaved for 1 h and cooled to room temperature before inoculation.

2.4.3. Preparation of Inoculum

Inoculum was prepared by growing the cells in MSG medium as described earlier under section 2.3.3.

2.4.4. Inoculation and incubation

The sterilized media was inoculated with the prepared inoculum containing 1.0 mg dry weight cells/10 gds (arbitrarily selected before optimization of inoculum concentration). Care was taken such that no free water was present after inoculation, The contents in the flasks were mixed thoroughly and were incubated in a slanting position for 24 h at $30^{o}C$ (unless otherwise stated) under 75-80 % relative humidity.

2.4.5. Enzyme recovery and assay

Enzyme was recovered from the polystyrene medium after incubation, using phosphate buffer (0.1 M, pH 7) as described earlier (section 2.3.5). Dampened cheese cloth was used to filter the extractant which was centrifuged at 10000 rpm at $4^{o}C$ for 15 min. The supernatant was used for the various assays as described earlier under section 2.3.6.

2.4.6. Impact of process parameters on L-glutaminase production during SSF using Polystyrene

The impact of various process parameters influencing L-glutaminase production by *V. costicola* under SSF on polystyrene was studied as detailed below. The parameters optimized included, initial pH, solid substrate media ratio, incubation temperature, L-glutamine concentration, inoculum concentration, incubation time, additional carbon and nitrogen sources, amino acids and mineral salts. Preparation of the solid substrate and inoculum, inoculation and incubation, extraction and assay of enzymes were carried out as described earlier under sections 2.4.2., 2.4.3., 2.4.4., 2.4.5. and 2.3.6. respectively.

The protocol adopted for optimization of various process parameters, influencing glutaminase production by the bacteria, was similar to that used for optimization studies using natural substrates (section 2.3.7). Determination of the optimum incubation temperature, L-glutamine concentration, additional carbon and nitrogen sources, amino acids, mineral salts, inoculum concentration and incubation time required for maximal glutaminase production by bacteria under SSF using polystyrene system were carried out as described earlier (sections 2.3.7.4., 2.3.7.5., 2.3.7.6., 2.3.7.7., 2.3.7.8., 2.3.7.9., 2.3.7.11. and 2.3.7.12. respectively). Other parameters which influenced glutaminase yield by the bacteria under SSF using polystyrene system were optimized as described below.

2.4.6.1. Substrate Media ratio

The effect of solid substrate media ratio on glutaminase production by bacteria under SSF was tested by altering the amount of the medium used to moisten the beads to different ratios in the range of 1 0.2 to 1 1.5 (w/v).

2.4.6.2. Initial pH of the medium

The impact of initial pH of the medium used to moisten the beads on glutaminase production by bacteria under SSF was studied by using media with different pH in the range of 5-10. Either 1 N HCl or 1 N NaOH was used to attain the desired pH of the medium.

2.4.6.3. Impact of Solid Extractant Ratio for L-glutaminase recovery from the polystyrene SSF system

The impact of the solid substrate extractant ratio on L-glutaminase recovery from the polystyrene SSF system was studied by using phosphate buffer (0.1 M pH 7.0) at different ratios (% w/v) ranging from 1 2 to 1 10. Extraction was carried out as described earlier (section 2.4.5.) except that the volume of the buffer was varied. The methodology adopted for the study is described below.

Phosphate buffer (0.1 M; pH 7.0) was added to the polystyrene beads at ratios of 1 2, 1 3, 1 4, 1 5 and 1 10 (% w/v) and allowed to remain in contact for 30 min. on a rotary

shaker (150 rpm) at room temperature. Later the leachate was recovered as described earlier (section 2.4.5).

2.4.7. Scanning Electron Micrography (SEM)

SEM studies were performed with polystyrene beads to confirm the bacterial adsorption to the beads during SSF. The protocol followed was that of Pandey & Radhakrishnan (1993).

SSF using polystyrene beads were conducted as described earlier (sections 2.4.2. to 2.4.4.). Sample beads, uninoculated and 24 h after inoculation, were fixed with UV light and were mounted on brass stubs. They were then coated with a thin layer of gold (100 A°) and were viewed with a JEOL 35 scanning electron microscope (JEOL, Japan) at an accelerating voltage of 15 kV.

2.5. ESTIMATION OF BIOMASS DURING SSF USING POLYSTYRENE

Direct estimation of biomass is not feasible in SSF using natural substrates while it is necessary to correlate the progress of the fermentation to product formation over the duration of the fermentation period. So various indirect methods have been devised for the estimation of biomass in SSF which are difficult to perform and are of questionable reliability (Mitchell, 1992).

Since the use of inert supports such as polystyrene enables the separation of bacteria from the solid media, biomass can be directly estimated. In the present study, biomass was estimated in terms of cell protein (Herbert *et al*, 1971) and dry weight of cells obtained after enzyme extraction.

For biomass estimation, SSF was carried out using 5 g of polystyrene beads taken in 250 ml Erlenmeyer flasks, under optimized conditions. Preparation of the substrate and inoculum, inoculation and incubation were carried out as described earlier (sections 2.4.2., 2.4.3. and 2.4.4.). The inoculated flasks were incubated for a total period of 72 h at 35^{o}C and samples, as whole flasks, were withdrawn at regular intervals of 12 h. Enzyme extraction was performed as described under section 2.4.5.

The cell pellet obtained after enzyme extraction from the polystyrene system was washed twice with sterile distilled water and resuspended in a known volume of fresh sterile distilled water. 1 ml of this cell suspension was mixed with 1 ml of 3 N NaOH, heated in a boiling water bath for 5 min. and cooled to room temperature. It was then centrifuged at 10000 rpm for 10 min. and the supernatant was assayed for protein by modified Lowry's method (Herbert *et al*, 1971).

Dry weight of the cells was calculated after drying at 105^{o}C overnight.

2.6. L-GLUTAMINASE PRODUCTION UNDER SSF USING MIXED SUBSTRATES

Enzyme production by bacteria using a mixed substrate system (Natural + Inert) was evaluated under fermentation conditions optimized with polystyrene since it helped in the accurate optimization of process parameters. Wheat bran and Rice husk, which supported maximum glutaminase yield among the natural substrates tested, were used in combination with polystyrene. They were used at different (w/w) ratios.

2.6.1. Medium

Sea water medium containing L-glutamine (2 % w/w), maltose and KH_2PO_4 (1 % w/w of each), obtained after optimization studies using the polystyrene system, was used as the nutrient medium used to moisten the mixed substrates. It was added at 1 1 (w/v) ratio.

2.6.2. Preparation of solid substrates

Wheat bran / Rice husk were mixed with polystyrene in different (w/w) ratios. Wheat bran, rice husk and polystyrene were also used individually as controls. 10 g of the substrates (mixed / individual) in each ratio were taken in 250 ml Erlenmeyer flasks and moistened with the sea water glutamine media (section 2.6.1) at 1:1 (w/v) ratio, autoclaved for 1 h at 121^{o}C and cooled to room temperature before inoculation.

2.6.3. Preparation of Inoculum

Inoculum was prepared by growing the cells in MSG medium as described earlier under section 2.3.3.

2.6.4. Inoculation and incubation

The sterilized media was inoculated with the prepared inoculum containing 0.750 mg dry weight cells/10 gds. The contents in the flasks were mixed thoroughly and were incubated in a slanting position for 24 h at 35^{O}C under 75-80 % relative humidity.

2.4.5. Enzyme recovery and assay

Enzyme was recovered from the mixed substrate medium after incubation, using phosphate buffer (0.1 M, pH 7) at 1 5 (w/v ratio) as described earlier (section 2.3.5). Dampened cheese cloth was used to filter the extractant which was centrifuged at 10000 rpm at 4^{O}C for 15 min. The supernatant was used for the various assays as described earlier under section 2.3.6.

2.7. MUTATION STUDIES

The effect of mutation by UV irradiation and chemical mutagenesis on *V. costicola* with respect to L-glutaminase production was studied by following the methodology of Jayaraman and Jayaraman (1979). N-Methyl-N'-nitro-N -nitrosoguanidine (NTG Sigma, USA) was used as the chemical mutagen.

2.7.1. Preparation of cell suspension

ZoBell's broth was used for cultivation of cells. The log phase culture grown in ZoBell's broth (16 h) was centrifuged at 10000 rpm for 15 min, washed in sterile saline (0.85 % NaCl) repeatedly, and suspended in 25 ml of sterile phosphate buffer (0.1 M, pH 7.0). The cell density in the suspension was adjusted to 10^8 cells/ml with sterile buffer.

2.7.2. Mutagenesis with Ultra Violet irradiation

1) One ml of the cell suspension prepared as mentioned above was serially diluted up to 10^{-8} and plated on ZoBell's agar. The plates were incubated overnight at room temperature ($28 \pm 2^{o}C$) and total viable counts were made. The data obtained was used as control.

2) 15 ml of the cell suspension was taken in a sterile petridish with the lid open and was exposed to UV irradiation from a 20 W UV lamp (Phillips, Holland) kept at about a distance of 15 cm. Care was taken to switch-on the UV lamp at least 15 min. before the commencement of the experiment in order to stabilize the emission.

3) 1 ml aliquot of samples, in duplicate, were drawn at regular intervals of 0, 20, 40, 60, 80, 100, and 120 seconds after exposure to irradiation into sterile test tubes.

4) One set of samples were wrapped in dark paper and stored in the refrigerator overnight.

5) The other set of irradiated cells were serially diluted, 10^{-4} to 10^{-6}dilutions for early samples upto 60 seconds, and 10^{-1} to 10^{-4} dilutions for the late samples.

6) Diluted samples were plated on ZoBell's agar and incubated overnight at room temperature ($28\pm2^{O}C$).

7) Total viable count in each plate was made and the survivors per ml for each sample were calculated.

8) Using a semi log graph, a graph was plotted with time of exposure to UV *vs* survivors. Optimal time for mutagenesis was determined based on 99 % lethality.

2.7.3. Mutagenesis with NTG

1) Aqueous solution of NTG was prepared using distilled water and filter sterilized. The final concentration was 1 mg/ml.

2) This NTG solution was dispensed in duplicate into 1.5 ml eppendorf tubes with concentrations varying from 10 μg to 100 μg/ml. 1 ml of the prepared cell suspension was added to the NTG solution in eppendorf tubes and incubated for 30 min at $30^{O}C$ with shaking.

3) Cells were then separated by centrifugation at 10000 rpm for 20 min, washed repeatedly with sterile physiological saline and resuspended in 50 ml of fresh sterile saline.

4) 0.1 ml of this cell suspension was transferred to ZoBell's broth, and rest of the solution was stored under refrigeration.

5) The inoculated ZoBell's broth was incubated overnight on a rotary shaker at 150 rpm, at room temperature.

6) Cells were harvested by centrifugation at 10000 rpm for 15 min, washed repeatedly with sterile physiological saline and used for L-glutaminase production studies.

2.7.4. L-Glutaminase production by UV irradiated and NTG treated cells.

Cells obtained after mutagenesis by UV irradiation and NTG treatment were checked for L-glutaminase production using SSF technique using polystyrene under optimized conditions, following the procedures described earlier under sections 2.4.1. to 2.4.5. The stability of the treated cells, with respect to glutaminase production, was confirmed by subculturing the cells for 5 generations and using the cells of each generation for enzyme production studies.

2.8. ENZYME PURIFICATION

L-Glutaminase was purified by following the enzyme purification methodologies available in the literature (Davidson *et al*, 1977; Moriguchi *et al*, 1994). The strategy adopted included fractionation by ammonium sulphate followed by dialysis and gel-filtration chromatography. Purity was checked by using polyacrylamide gel electrophoresis (native as well as denaturing).

2.8.1. Ammonium sulphate fractionation

Finely powdered ammonium sulphate (enzyme grade, SRL, India) was added slowly into the crude enzyme preparation so as to reach 40 % saturation. A magnetic stirrer was used for continuous stirring and the procedure was carried out at $4^{O}C$ in an ice bath. The precipitated protein was removed by centrifugation at 10000 rpm for 20 min. at $4^{O}C$. Fresh ammonium sulphate was added to the supernatant to increase the concentration upto 80 % and the precipitate obtained was resuspended in a minimum volume of 0.01 M phosphate buffer (pH 8.0). The protein content of the fraction was determined by Lowry's method.

2.8.2. Dialysis

The precipitate obtained after ammonium sulphate fractionation was dialysed against 0.01 M phosphate buffer (pH 8) for 24 h at $4^{O}C$ with continuous stirring and occasional changes of the buffer. Cellulose membrane dialysis tubes (Sigma, USA) were used for

dialysis. The glutaminase activity and protein content of the dialysate was determined as described earlier (sections 2.3.6.1. & 2.3.6.2 respectively).

2.8.3. Gel-filtration chromatography

The dialysate was concentrated by lyophilization (Yamoto-Neocool, Japan) and was applied on top of a gel-filtration column. Sephadex G 100 (Pharmacia) was used for gel-filtration experiments, which was carried out at $4^{o}C$ and included the following steps.

1) Two g of Sephadex G 100 was suspended in phosphate buffer (0.1 M, pH 8) containing 0.1 M NaCl and 0.001 M EDTA and placed on boiling water for 5 h. The solution was stirred occasionally during the pretreatment procedure.

2) The swollen gel was then poured into a chromatography column (1.5 x 50 cm) and allowed to settle under gravity while maintaining a slow flow rate through the column. Care was taken to avoid trapping of air bubbles in the column. The column was stabilized and equilibrated by passing about three bed volumes of buffer.

3) The lyophilized dialysate was applied to the top of the column and eluted with buffer. The flow rate was adjusted to about 50 ml/h and fractions of 2.5 ml were collected using a fraction collector (Redifrac, Pharmacia).

4) Each fraction was analyzed for protein by measuring absorbance at 280 nm and glutaminase was measured by the method described earlier (section 2.3.6.1.).

5) The active fractions were pooled and concentrated by lyophilization.

2.8.4. Electrophoresis

The purified enzyme was subjected to electrophoretic studies to confirm purity and to determine the molecular weight. Electrophoresis was performed as suggested by Hames (1990) using polyacrylamide (PAGE). Both native PAGE and denaturing PAGE (SDS-PAGE) were conducted using a slab gel apparatus (modified Studier model) with a notched glass plate system. Gels of 1.5 mm thickness were prepared by using perspex spacers of the same size.

2.8.4.1. Stock solutions

1) Acrylamide-bisacrylamide (30:0.8) was prepared by dissolving 30 g of acrylamide and 0.8 g of bisacrylamide in a total volume of 100 ml of distilled water. The solution was filtered through Whatman No.1 filter paper and stored at $4^{o}C$ in a dark bottle.

2) TEMED (N,N,N',N'-tetramethylethylenediamine) was used as supplied. It was stored in a dark bottle at $4^{o}C$.

3) Ammonium persulphate (1.5 % w/v) - 0.15 g of ammonium persulphate was dissolved in 10 ml of distilled water. It was prepared fresh just before use.

4) Sodium dodecyl sulphate (SDS - 10 % w/v). It was prepared by dissolving 10 g of SDS in 100 ml distilled water and stored at room temperature.

5) Buffers for SDS-PAGE

a) Stacking gel buffer stock (0.5 M Tris-HCl, pH 6.8) 6.0 g of Tris was dissolved in 40 ml of distilled water, titrated to pH 6.8 with 1 M HCl and brought to 100 ml final volume with distilled water. The solution was filtered through Whatman No. 1 and stored at $4^{o}C$.

b) Resolving gel buffer stock (3.0 M Tris-HCl, pH 8.8) 36.3 g of Tris and 48 ml of 1 M HCl were mixed and brought to 100 ml final volume with distilled water. This was filtered through Whatman No.1 and stored at $4^{o}C$.

c) Reservoir buffer stock (0.25 M Tris, 1.92 M glycine, 1 % SDS, pH 8.3) 30.3 g of Tris, 144.0 g of glycine and 10.0 g of SDS were dissolved in and made upto 1 L with distilled water and stored at $4^{o}C$.

6) Buffers for native PAGE

Stacking and Resolving gel buffers were the same as that of SDS-PAGE. Reservoir buffer was prepared as described below

3.0 g of Tris and 14.4 g of glycine were dissolved in and made upto 1 L with distilled water. Stored at $4^{o}C$.

Slab gels of 1.5 mm thickness were prepared with either 7.5 % or 10 % resolving gel for SDS-PAGE and 5 % nd 7.5 % for native PAGE. Stacking gel of 2.5 % concentration was used throughout the experiments. SDS-PAGE was performed at room temperature while native PAGE was conducted at $4^{O}C$ to avoid denaturation of the enzyme.

2.8.4.2. Sample preparation

For SDS-PAGE, the sample was prepared as follows.

Enzyme solution	100 µl
Bromophenol blue (0.002 %)	30 µl
10 % SDS	40 µl
10 % Sucrose	20 µl
β-mercaptoethanol	10 µl

The contents were mixed well in a clean eppendorf tube and heated in a boiling water bath for 3 min. After cooling to room temperature, insoluble materials, if any were removed by centrifugation. For molecular weight determination of the subunits, marker proteins purchased from Sigma (wide range markers) were used. They included Myosin (205,000), β-Galactosidase (116,000), Phosphorylase b (97,000), Fructose-6-phosphate kinase (84,000), Bovine serum albumin (66,000), Glutamic dehydrogenase (55,000), Ovalbumin (45,000), Glyceraldehyde-3-phosphate dehydrogenase (36,000), Carbonic

anhydrase (29,000), Trypsinogen (24,000), Trypsin inhibitor (20,000), α-Lactalbumin (14,200) and Aprotinin (6,500).

For native PAGE, the sample included the following.

Enzyme solution	100 µl
Bromophenol blue (0.002 %)	30 µl
Sucrose (10 %)	20 µl

The samples were prepared in 1.5 ml eppendorf tubes at $4^{o}C$ and used immediately. To determine the molecular weight of the native protein, marker proteins (Sigma, USA) such as Albumin, chicken egg (45,000), Albumin, bovine serum (66,000, monomer 132,000, dimer and 272,000, trimer) were used. Glutaminase grade II from *E. coli* (Sigma, USA) was also used for comparative studies. Samples for electrophoresis were prepared as done with the purified enzyme sample.

Electrophoresis was performed using a constant voltage of 50 V till the samples entered the resolving gel and then the voltage was increased to 75-150 V depending on the resolving gel concentration.

2.8.4.3. Staining and destaining

Gels were stained using Coomassie Brilliant Blue R 250 (Sigma, USA). It was prepared in water methanol glacial acetic

acid (5:5:2 by volume) and filtered through Whatman No.1 filter paper to remove insoluble materials. After electrophoresis, gel was placed in a gel staining tray containing the stain solution and was kept for about 6 h at room temperature. After staining was complete, excess stain was removed by putting in a destaining solution (mixture of 30 % methanol & 10 % acetic acid). The destaining solution was renewed regularly as stain leached out of the gel. The destained gels were photographed and preserved in 7 % acetic acid.

2.8.5. CHARACTERIZATION OF THE PURIFIED ENZYME

The purified enzyme was characterized and its various properties were studied. The characters analyzed included effect of pH and temperature on enzyme activity and stability and effect of substrate concentration and NaCl concentration on enzyme activity. The effect of NaCl and substrate on temperature stability of the enzyme and the substrate specificity of the enzyme were also studied. The details of the procedures are given in the following sections.

2.8.5.1. Effect of pH on enzyme activity and stability

The effect of pH on the activity of the enzyme was studied by performing the enzyme assay at different pH using acetate buffer (pH 4-5), phosphate buffer (6-8) and glycine-NaOH buffer (9-10), as described in section 2.3.6.1. The pH stability of the enzyme was determined by incubating the enzyme with the buffers described above

for 1 h at 40^{O}C and then carrying out the enzyme assay as mentioned under section 2.3.6.1.

2.8.5.2. Effect of temperature on enzyme activity and stability

Optimum temperature needed for enzyme activity was estimated by incubating the reaction mixture described earlier under section 2.3.6.1. for 15 min at different temperatures (30–80^{O}C). The temperature stability of the enzyme was determined by incubating the enzyme solution at temperatures in the range 30–80^{O}C for 1 h and estimating the residual activity of the treated enzyme as described earlier (section 2.3.6.1.).

2.8.5.3. Effect of substrate concentration on enzyme activity

The effect of substrate (L-glutamine) concentration on the activity of the enzyme was determined by using different concentrations of L-glutamine (0.01 - 1.0 M), in the reaction mixture described in section 2.3.6.1. and estimating the enzyme activity. The Km value of the enzyme was also calculated from the data using Lineweaver-Burk plot.

2.8.5.4. Effect of NaCl concentration on enzyme activity

The effect of NaCl concentration on glutaminase activity was tested by incorporating different concentrations of NaCl ranging from 0–20% in the reaction mixture and estimating the enzyme activity as described in section 2.3.6.1.

2.8.5.5. Effect of substrate and NaCl on temperature stability of glutaminase

The effect of substrate and NaCl on temperature stability of glutaminase was studied by incubating the enzyme at different temperatures (30–80^{O}C) in the presence of 0.01 M L-glutamine / 10 % NaCl for 1 h. Then the residual enzyme activity was determined as described earlier (section 2.3.6.1.).

2.8.5.6. Substrate specificity

The substrate specificity of the purified enzyme was checked by using different substrates viz. L-glutamine, L-asparagine, L-glutamic acid, L-aspartic acid and a mixture of L-glutamine and L-asparagine (0.04 M of each) in the reaction mixture and estimating the activity as described in section 2.3.6.1.

RESULTS

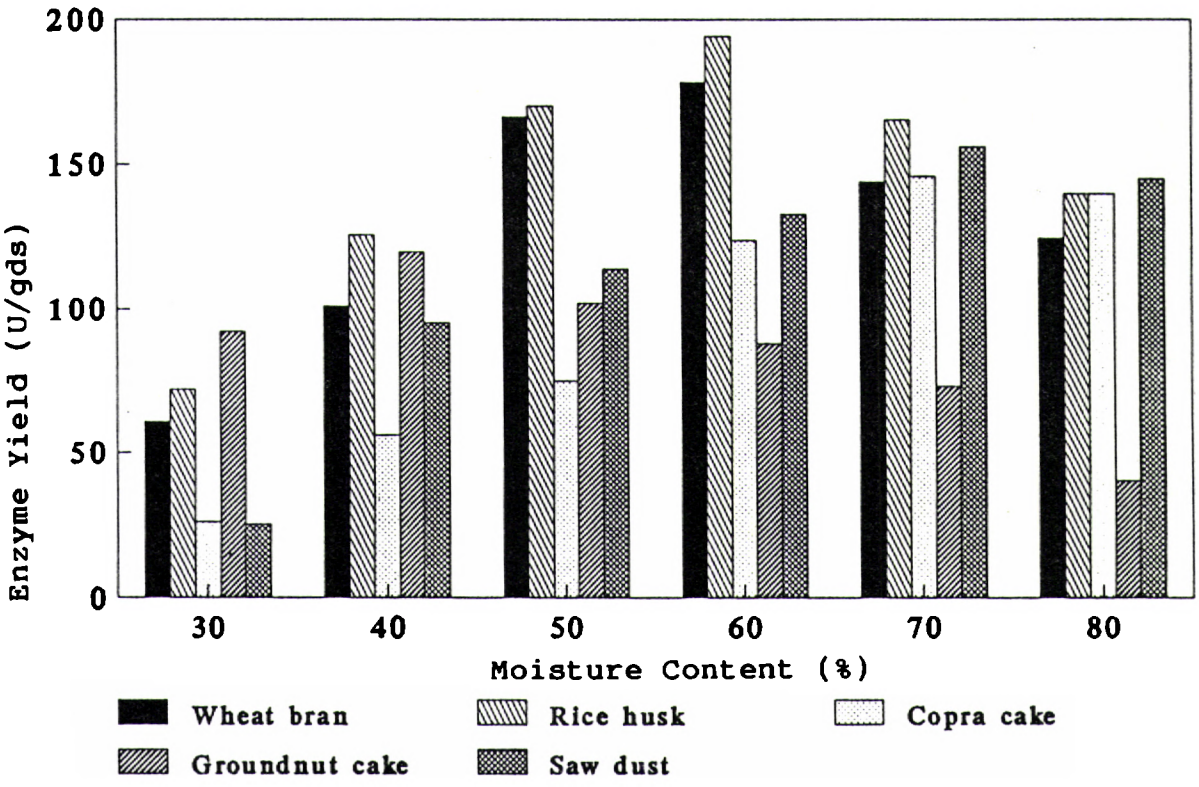
3.1. L-GLUTAMINASE PRODUCTION UNDER SSF USING NATURAL SUBSTRATES

Vibrio costicola, isolated from the marine environment, produced extracellular L-glutaminase under SSF using all the natural substrates tested. The effect of various process parameters are given in the following sections.

3.1.1. Effect of initial moisture content

The initial moisture content of the medium influenced glutaminase production by bacteria, irrespective of the nature of the substrates studied. Results given in Fig. 1 show that an initial moisture content of 60 % was optimum for maximal enzyme yield with RH and WB, compared to CCP and SD where the moisture requirement was 70%. In contrast, with GCP, 40 % moisture was found to be optimum and any further increase in the moisture content resulted in clumping of the solid particles and consequent reduction in enzyme yield. Among all the substrates tested, RH supported maximal yield of glutaminase at 60 % (194 U/gds) and 50 % (170 U/gds) moisture contents compared to other substrates. WB could also record maximal enzyme yield of 177 U/gds and 166 U/gds at 60 % and 50 % respectively. In general, RH supported enhanced yield of enzyme (126 - 194 U/gds) over a broad range of moisture contents (40 - 70 %). All the other substrates recorded lesser levels of glutaminase compared to RH and WB at their optimum moisture content.

FIGURE 1: EFFECT OF INITIAL MOISTURE CONTENT ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING NATURAL SUBSTRATES



3.1.2. Effect of initial pH of the medium

The results presented in Fig. 2 clearly indicate that the pH preference of the organism is independent and is not influenced by the nature of the substrates. Glutaminase yield was significant over a range of pH 6 - 8 with optimum at pH 7.0. Maximum yield of enzyme (259 U/gds) was obtained with WB at pH 7.0 followed by RH (212 U/gds), SD (132 U/gds), GCP (139 U/gds) and CCP (132 U/gds). Irrespective of the initial level of pH tested, WB promoted maximal enzyme yield (range 132 - 259 U/gds) followed by RH (range 110 - 212 U/gds). CCP supported lowest titres of enzyme among the substrates.

3.1.3. Effect of substrate particle size

The substrate particle size that supported maximal glutaminase yield varied according to the type of the substrate (Fig. 3). While small sized particles of WB and RH (0.6–1.0 mm size) supported maximum enzyme yield (167 & 164 U/gds respectively), with all the other substrates larger particles (1.0–1.4 mm size) were needed for obtaining maximal enzyme yield. In general, particle size below 0.6 mm did not promote significant enzyme yield compared to larger particle sizes. Further, it was observed that irrespective of the particle size, WB supported maximal glutaminase yield (96 - 167 U/gds).

FIGURE 2: EFFECT OF INITIAL pH ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING NATURAL SUBSTRATES

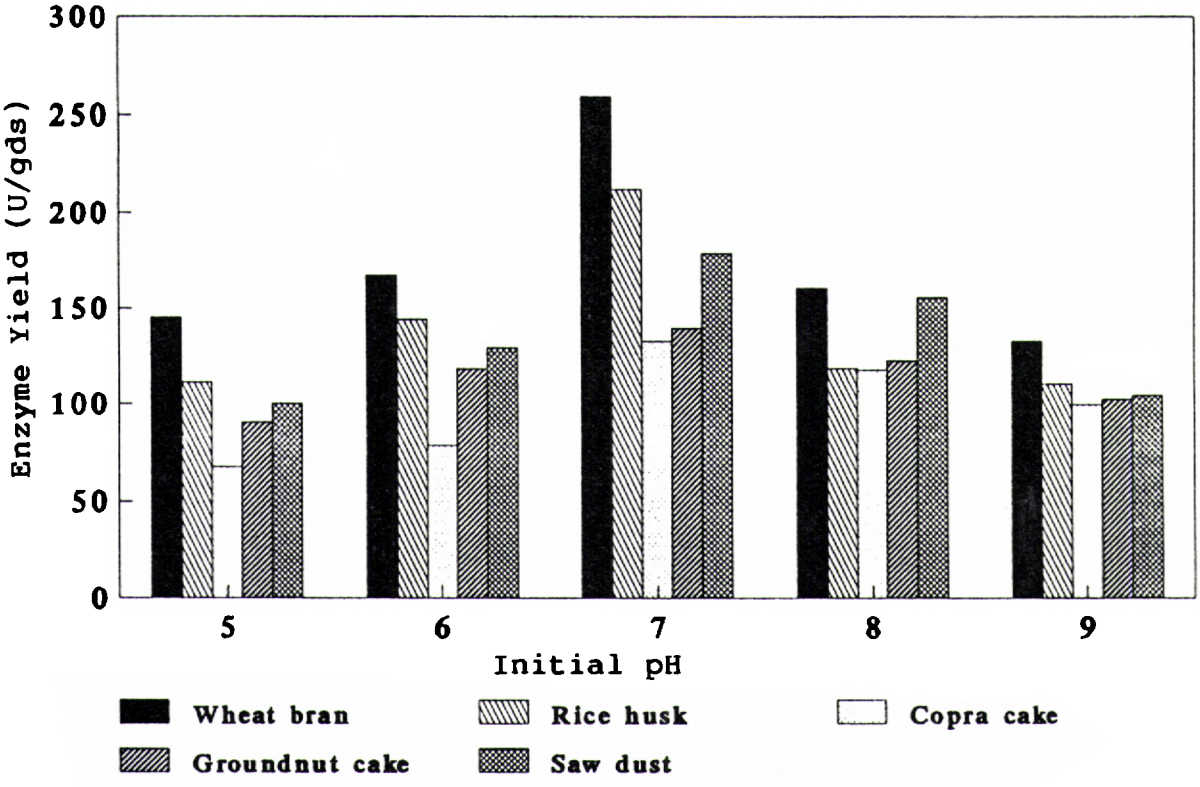
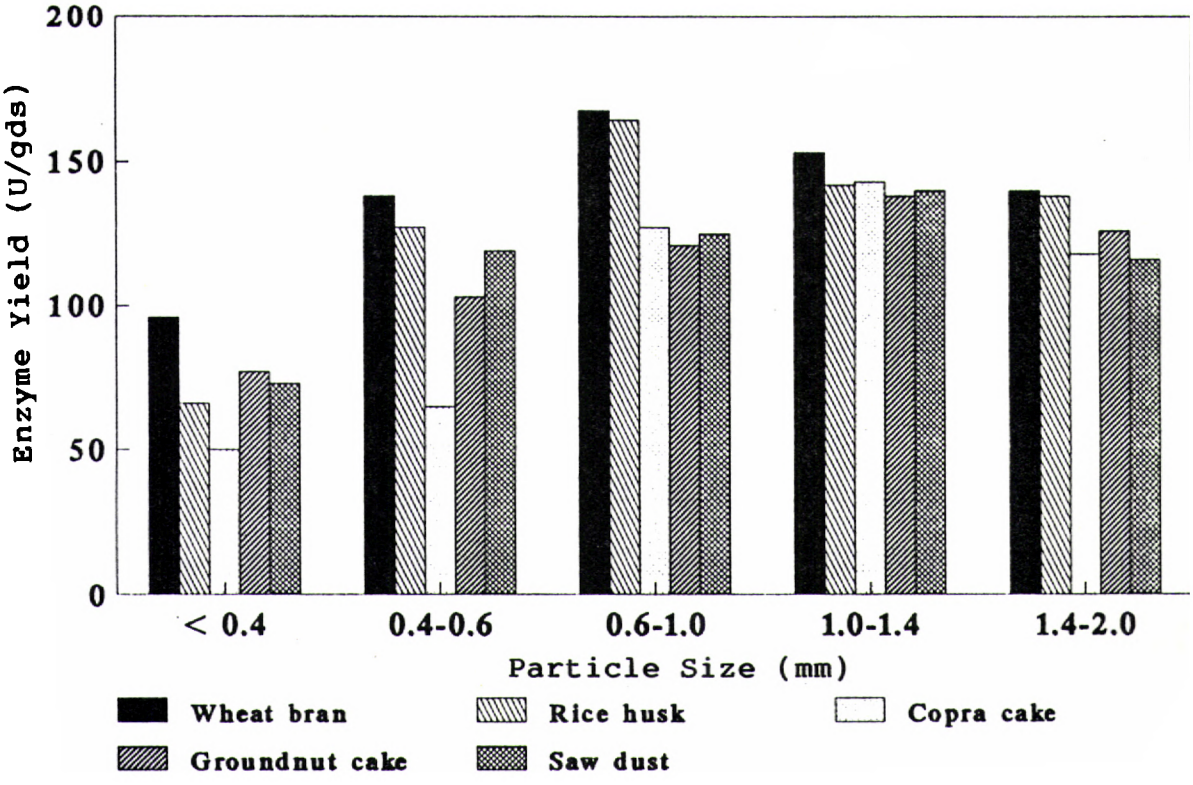


FIGURE 3: EFFECT OF SUBSTRATE PARTICLE SIZE ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING NATURAL SUBSTRATES



3.1.4. Effect of incubation temperature

Significant levels of enzyme could be produced by the bacteria with all the substrates over a range of temperatures 30–45^{o}C, with an optimum at 35^{o}C (135 - 186 U/gds; Fig. 4). It was noted that higher temperatures led to a decline in enzyme production. As experienced with pH, temperature also showed a similar pattern of recording the same temperature as optimum for maximal glutaminase yield irrespective of the substrates used. At higher temperatures (35 - 50^{o}C), RH promoted maximal enzyme yield (100 - 186 U/gds) followed by CCP, WB, SD and GCP. Whereas, at ambient temperatures (25 - 30^{o}C), WB supported maximal yield (97 - 157 U/gds) followed by SD, RH, GCP and CCP.

3.1.5. Effect of L-glutamine concentration.

L-Glutamine concentration needed for maximal glutaminase yield was similar irrespective of the nature of the substrates used. It is evident from the Fig. 5 that enzyme yield was significant over a wide range of substrate concentration (1-5 % w/w) with an optimum at 2 % level (range 120 - 239 U/gds). Glutaminase production showed a linear increase along with increase in L-glutamine concentration upto 2 % level. However, further increase in substrate concentration did not enhance glutaminase yield. WB followed by RH promoted maximal enzyme yield compared to other substrates irrespective of L-glutamine concentration.

FIGURE 4: EFFECT OF INCUBATION TEMPERATURE ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING NATURAL SUBSTRATES

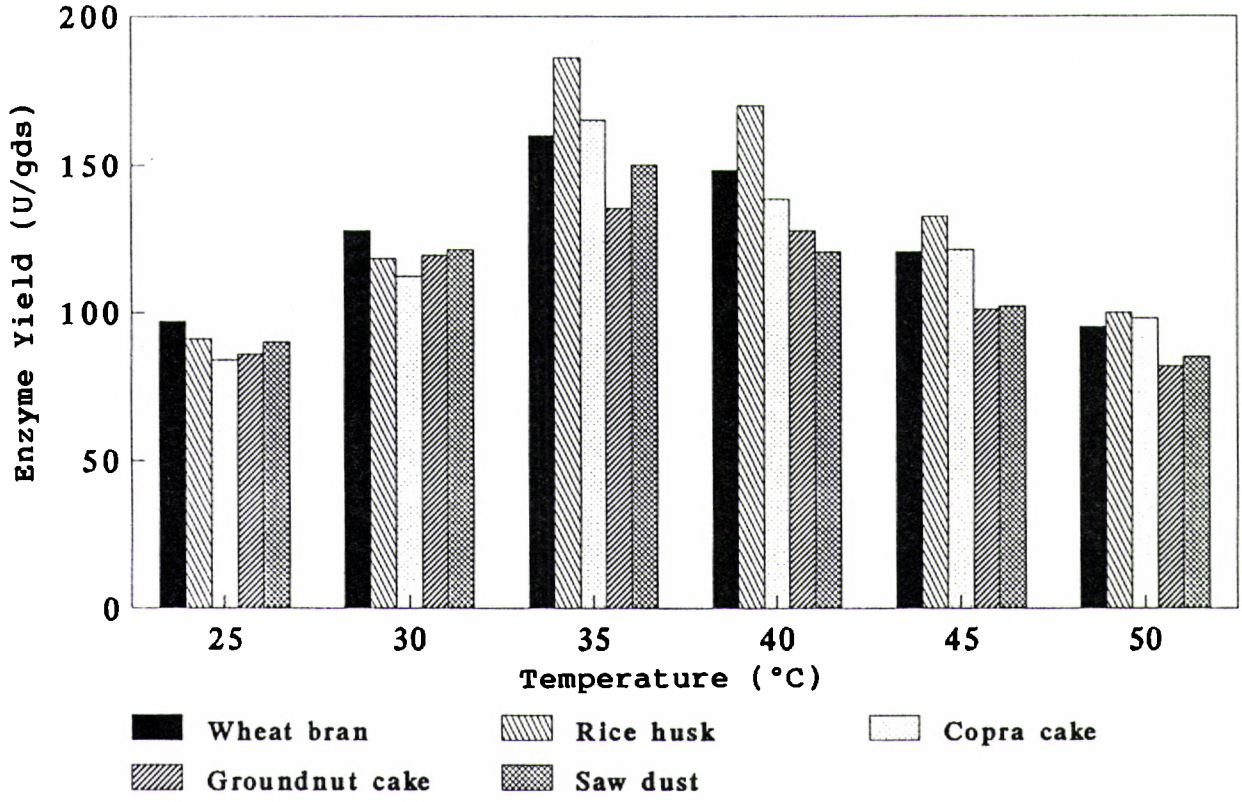
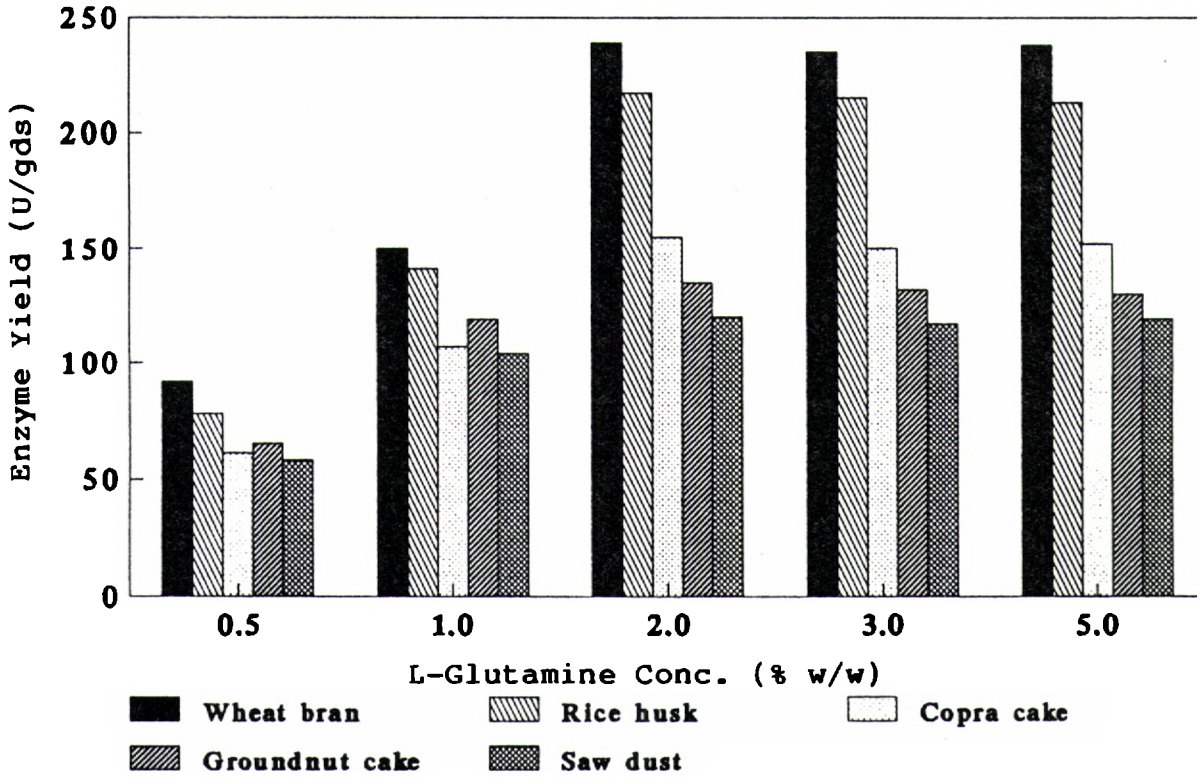


FIGURE 5: EFFECT OF L-GLUTAMINE CONCENTRATION ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING NATURAL SUBSTRATES



3.1.6. Effect of incubation time

Incubation time required for maximal glutaminase production varied according to the type of the solid substrate used (Fig. 6). While the fermentation period required for maximal enzyme yield was 24 h for WB (238 U/gds) and RH (229 U/gds), the optimum incubation period for the other substrates were longer (36 h). Further incubation beyond the optimal time led to a progressive decline in enzyme yield. WB followed by RH supported maximal enzyme yield at all periods of incubation compared to other substrates.

3.1.7. Effect of inoculum concentration

Enzyme production recorded a linear fashion of increasing trend along with increase in inoculum size with the yield reaching a maximum at 0.750 mg dry wt/10 gds of inoculum, irrespective of the substrates studied (Fig. 7). Further increase in inoculum concentration did not promote additional enzyme yield. At the optimal inoculum concentration, RH supported maximal glutaminase yield (143 U/gds) followed by WB, CCP, GCP and SD.

3.1.8. Effect of additional carbon sources

Incorporation of different carbon sources as additional nutrients did not show significant effect on the enzyme yield with the substrates except SD where a significant increase (8.9 - 26.4 %) was observed. Of the carbon sources tested, maltose glucose and sucrose could effect a marginal enhancement in the enzyme yield compared to

FIGURE 6: EFFECT OF INCUBATION TIME ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING NATURAL SUBSTRATES

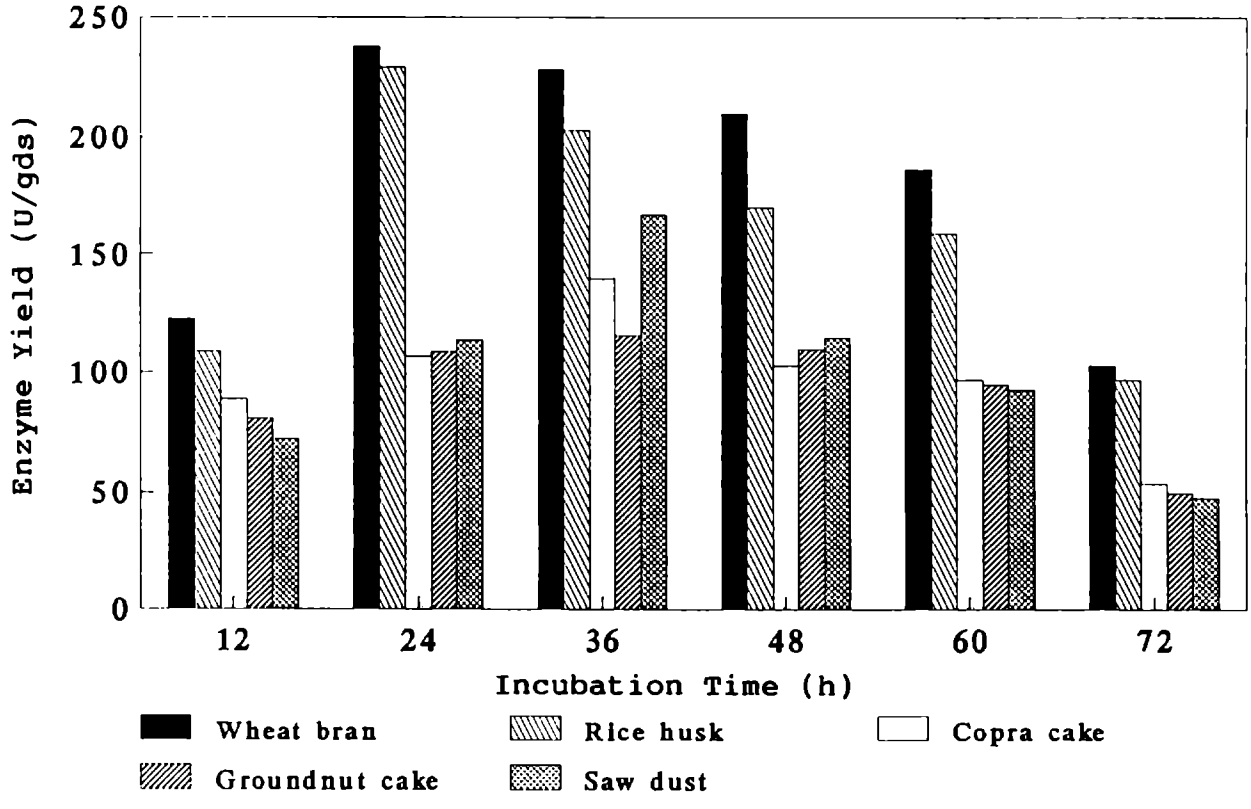
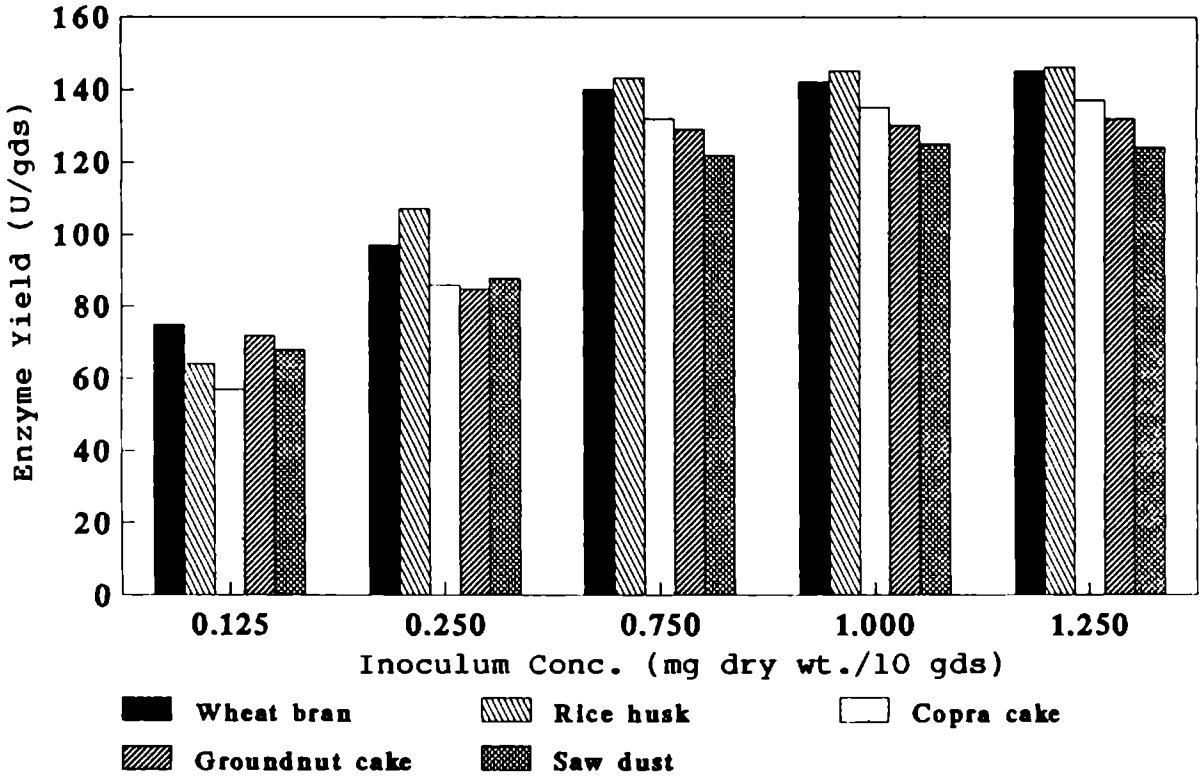


FIGURE 7: EFFECT OF INOCULUM CONCENTRATION ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING NATURAL SUBSTRATES



others (Table 3). Although a decline in enzyme yield was observed at a few instances with galactose, lactose and trisodium citrate with CCP, RH and GCP respectively, it was insignificant.

3.1.9. Effect of additional nitrogen sources

The addition of nitrogen sources at 1 % (w/w) to the SSF medium did not have any major impact on L-glutaminase yield with all the substrates studied (Table 4). However, peptone, yeast extract and ammonium sulphate led to a marginal decline in glutaminase yield compared to other nitrogen sources, with respect to all the substrates tested. A marginal increase in enzyme yield could be observed after the addition of casein to RH, GCP and SD. Similarly, ammonium nitrate enhanced glutaminase yield marginally with GCP and CCP. Sodium nitrate was also found to effect a marginal increase in glutaminase yield with SD.

3.1.10. Effect of amino acids

A similar trend was also noticed when amino acids were tried as additional nitrogen sources at 1 % w/w level (Table 5). In general, addition of amino acids did not enhance enzyme yield. Instead, it led to a significant decline in enzyme yield at most of the instances, except with cysteine, which led to a marginal increase in the case of GCP and SD. A decline in enzyme yield, greater than 15 %, was observed with asparagine (WB), glutamic acid (WB & CCP), lysine (RH), methionine (RH) and tyrosine (WB, RH & CCP). Similarly,

Table - 3

EFFECT OF ADDITIONAL CARBON SOURCES ON L-GLUTAMINASE PRODUCTION BY *V. COSTICOLA* UNDER SSF USING NATURAL SUBSTRATES

(Enzyme yield expressed as U/gds)

CARBON SOURCE (1 % w/w)	SOLID SUBSTRATE				
	Wheat bran	Rice bran	Copra cake powder	Groundnut powder	Saw dust
Control	225	230	165	150	146
Sucrose	230	235	168	152	164
Glucose	233	234	170	156	170
Maltose	234	236	172	160	185
Galactose	228	227	162	157	165
Lactose	229	228	167	154	159
Trisodium citrate	226	231	169	149	162

Table - 4

EFFECT OF ADDITIONAL NITROGEN SOURCES ON L-GLUTAMINASE PRODUCTION BY *V. COSTICOLA* UNDER SSF USING NATURAL SUBSTRATES

(Enzyme yield expressed as U/gds)

NITROGEN SOURCE (1 % w/w)	SOLID SUBSTRATE				
	Wheat bran	Rice bran	Copra cake powder	Groundnut powder	Saw dust
Control	220	226	170	165	150
Yeast extract	200	205	158	159	145
Peptone	215	225	166	160	140
Urea	218	220	171	162	144
Casein	222	230	167	169	152
Ammoniun sulphate	208	210	161	152	139
Ammonium nitrate	217	221	177	170	148
Sodium nitrate	212	215	170	161	153

Table - 5

EFFECT OF AMINO ACIDS ON L-GLUTAMINASE PRODUCTION BY *V. COSTICOLA* UNDER SSF USING NATURAL SUBSTRATES

(Enzyme yield expressed as U/gds)

AMINO ACIDS (1 % w/w)	SOLID SUBSTRATE				
	Wheat bran	Rice bran	Copra cake powder	Groundnut powder	Saw dust
Control	196	189	178	162	175
Alanine	181	162	154	155	172
Asparagine	167	170	159	156	175
Arginine	172	169	165	160	169
Cysteine	175	172	170	165	180
Glutamic acid	162	175	148	152	165
Lysine	190	160	169	157	171
Methionine	188	159	170	148	174
Tyrosine	163	158	149	147	170

10 - 15 % decline in enzyme yield was noticed when alanine (CCP), asparagine (RH & CCP), arginine (WB & RH) and cysteine (WB) were incorporated into the medium. In general, significant reduction in level of enzyme yield after incorporation of amino acids was recorded with RH, WB and CCP, compared to that obtained with GCP and SD which were less than 10 %. Interestingly, methionine did not have any influence on glutaminase yield with SD.

3.1.11. Effect of mineral salts

Supplementation of mineral salts in the SSF medium resulted in enhanced glutaminase yield with all the substrates, when compared to the control (Table 6). Among the mineral salts tested, KH_2PO_4, followed by NaH_2PO_4 effected maximal increase in the enzyme yield with all the five substrates. In the case of chlorides tested, KCl was observed to enhance enzyme yield with WB, RH and GCP compared to $MnCl_2$ and $CaCl_2$ which increased the enzyme yield with GCP and SD respectively. Whereas $MgSO_4.7H_2O$ promoted significant level of enzyme production with all the substrates studied.

3.1.12. Effect of NaCl concentration

Results presented in Table 7 clearly indicates that L-glutaminase production increased along with an increase in NaCl conc. upto 3 % (w/w) and conc above 3 % led to a linear decline in the enzyme yield. Results suggest further that differences in the nature of the substrate did not influence glutaminase yield.

Table - 6

EFFECT OF MINERAL SALTS ON L-GLUTAMINASE PRODUCTION BY *V. COSTICOLA* UNDER SSF USING NATURAL SUBSTRATES

(Enzyme yield expressed as U/gds)

MINERAL SALT (1 % w/w)	SOLID SUBSTRATE				
	Wheat bran	Rice bran	Copra cake powder	Groundnut powder	Saw dust
Control	196	189	178	162	165
KH_2PO_4	268	239	217	205	196
NaH_2PO_4	244	221	212	194	189
$MgSO_4$	220	210	208	190	180
$CaCl_2$	219	200	192	181	185
KCl	225	215	198	184	177
$MnCl_2$	212	206	195	187	171

Table - 7

EFFECT OF NaCl CONCENTRATION ON L-GLUTAMINASE PRODUCTION BY *V. COSTICOLA* UNDER SSF USING NATURAL SUBSTRATES

(Enzyme yield expressed as U/gds)

SUBSTRATE	NaCl Concentration (% w/w)										
	0	1	2	3	4	5	6	7	8	9	10
Wheat bran	210	217	231	260	258	253	208	200	188	180	172
Rice husk	201	218	232	240	237	235	202	189	178	170	156
Copra cake powder	189	204	210	218	215	211	199	183	171	165	148
Groundnut cake powder	176	180	200	216	212	208	190	178	160	149	133
Saw dust	153	169	187	194	191	190	177	162	148	132	126

Data documented in Table 8, on the comparison of suitability of different substrates for L-glutaminase production under optimized conditions, clearly evidences that WB followed by RH favoured maximal yield of glutaminase compared to CCP, GCP and SD. However, when viscosity of the leachate obtained from various substrates is considered, RH followed by SD, WB, GCP and CCP, showed an increase in viscosity.

3.2. L-GLUTAMINASE PRODUCTION UNDER SSF USING POLYSTYRENE

V. costicola could also grow well and produce L-glutaminase at significant levels, when SSF was carried out using the polystyrene system. The results of various optimization experiments are given in the following sections.

3.2.1. Nature of media used

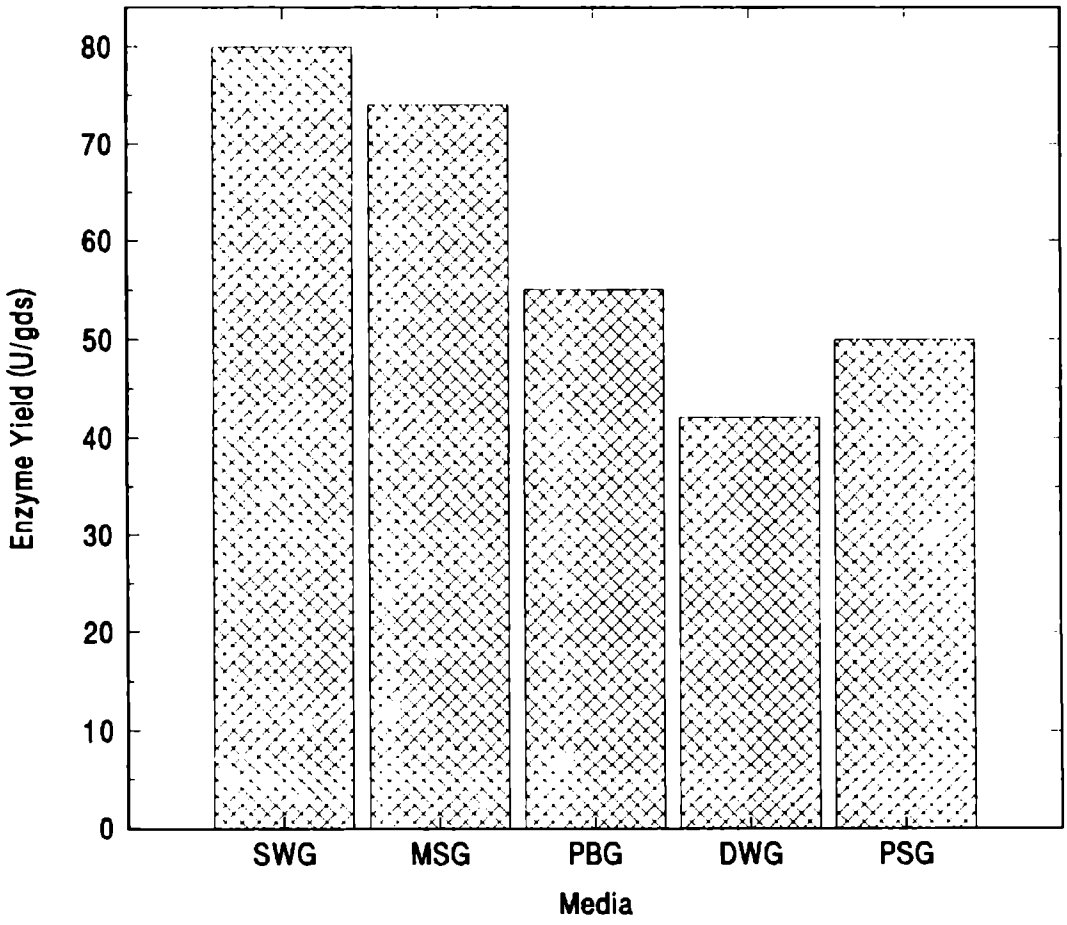
Results presented in Fig. 8 suggest that the nature of the media used to moisten the substrate had clear impact on the glutaminase yield. Of the five media tested, maximum enzyme yield was obtained using sea water containing 1 % (w/w) L-glutamine (80 U/gds) followed by MSG medium, PBG medium, PSG medium and DWG medium. Similarly, MSG medium could record higher enzyme yield (25 - 43 %) than PBG, PSG and DWG media. Based on these results, the sea water-glutamine (SWG) media was selected for use in the subsequent optimization experiments.

Table - 8

COMPARISON OF THE NATURAL SUBSTRATES FOR L-GLUTAMINASE PRODUCTION BY *V. COSTICOLA* UNDER SSF AT OPTIMIZED CONDITIONS

Substrate	Glutaminase Yield (U/gds)	Viscosity of Leachate (Ns/m^2)
Wheat bran	268	1.988
Rice husk	239	1.652
Copra cake powder	217	2.320
Groundnut cake powder	205	2.176
Saw dust	196	1.760

FIGURE 8: EFFECT OF THE TYPE OF MEDIA ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE



SWG - Sea water glutamine media
MSG - Mineral salts glutamine media
PBG - Phosphate buffer glutamine media
DWG - Distilled water glutamine media
PSG - Physiological saline glutamine media

3.2.2. Substrate media ratio

The enzyme yield increased along with increase in substrate to media ratio from 1 0.2 to 1 1 (w/v) reaching a maximum at 1 1 (83 U/gds; Fig. 9). Further increase in the proportion of media in the ratio resulted in the existence of free water and consequent reduction in enzyme yield (48.2 %).

3.2.3. Effect of initial pH of the medium

L-Glutaminase production was significant over a range of pH 6 - 8 with optimum at 7.0 (98 U/gds; Fig. 10). Enzyme yield was considerably lower at highly acidic (pH 5) and alkaline (pH 9 - 10) conditions. It was also observed that increase in pH above 8.0 led to a rapid decline in glutaminase yield.

3.2.4. Effect of incubation temperature

Maximal enzyme yield was obtained at a temperature of 35^{o}C (116 U/gds) as in the case of SSF with organic substrates (Fig. 11). It was observed that further increase in incubation temperature resulted in a decline in enzyme yield. However, significant levels of enzyme could be recovered over a range of 30 - 45^{o}C.

3.2.5. Effect of L-glutamine concentration

Results documented in Fig. 12 indicate that maximal glutaminase yield could be obtained with an optimum substrate concentration of 2 % (w/w) (129 U/gds) and further increase

FIGURE 9: EFFECT OF SUBSTRATE TO MEDIA RATIO ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE

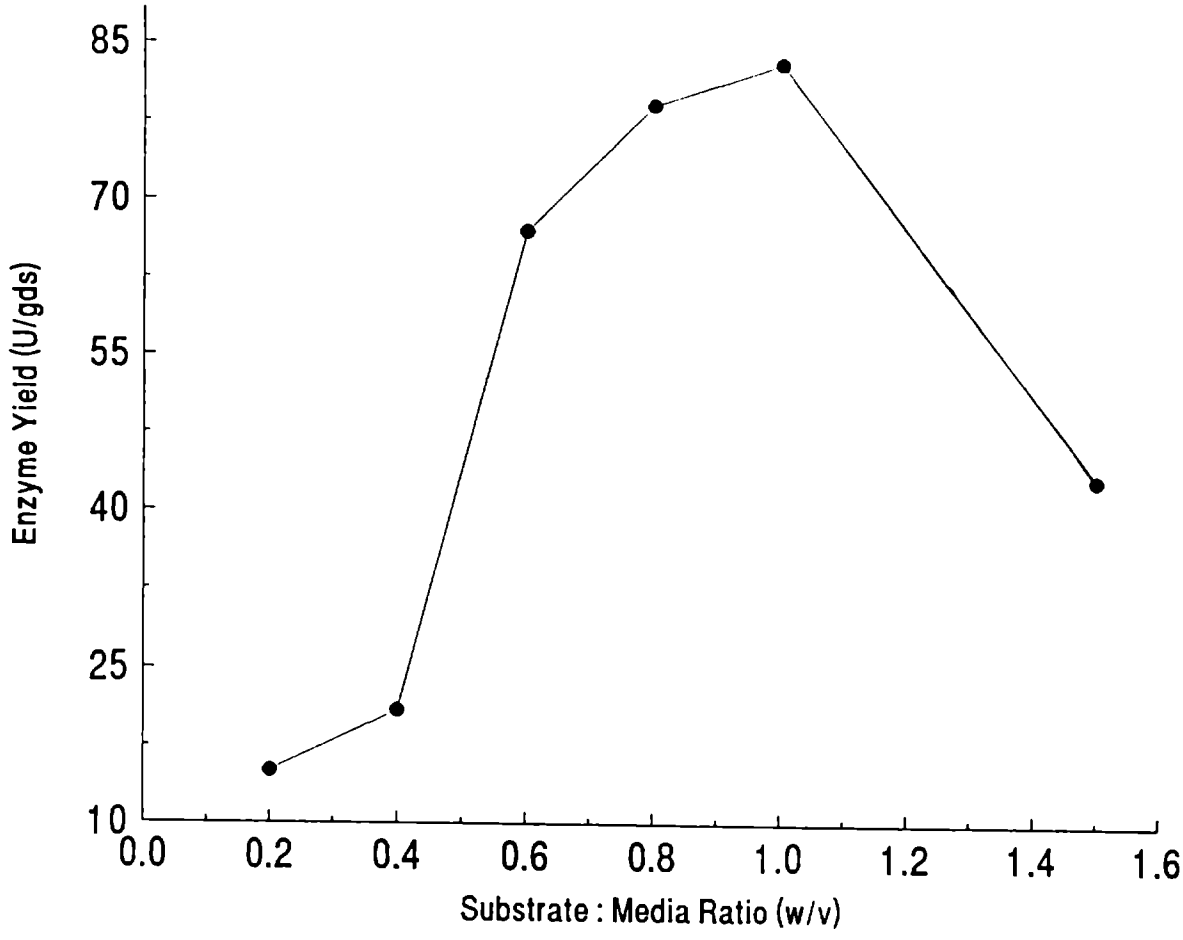


FIGURE 10: EFFECT OF INITIAL pH ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE

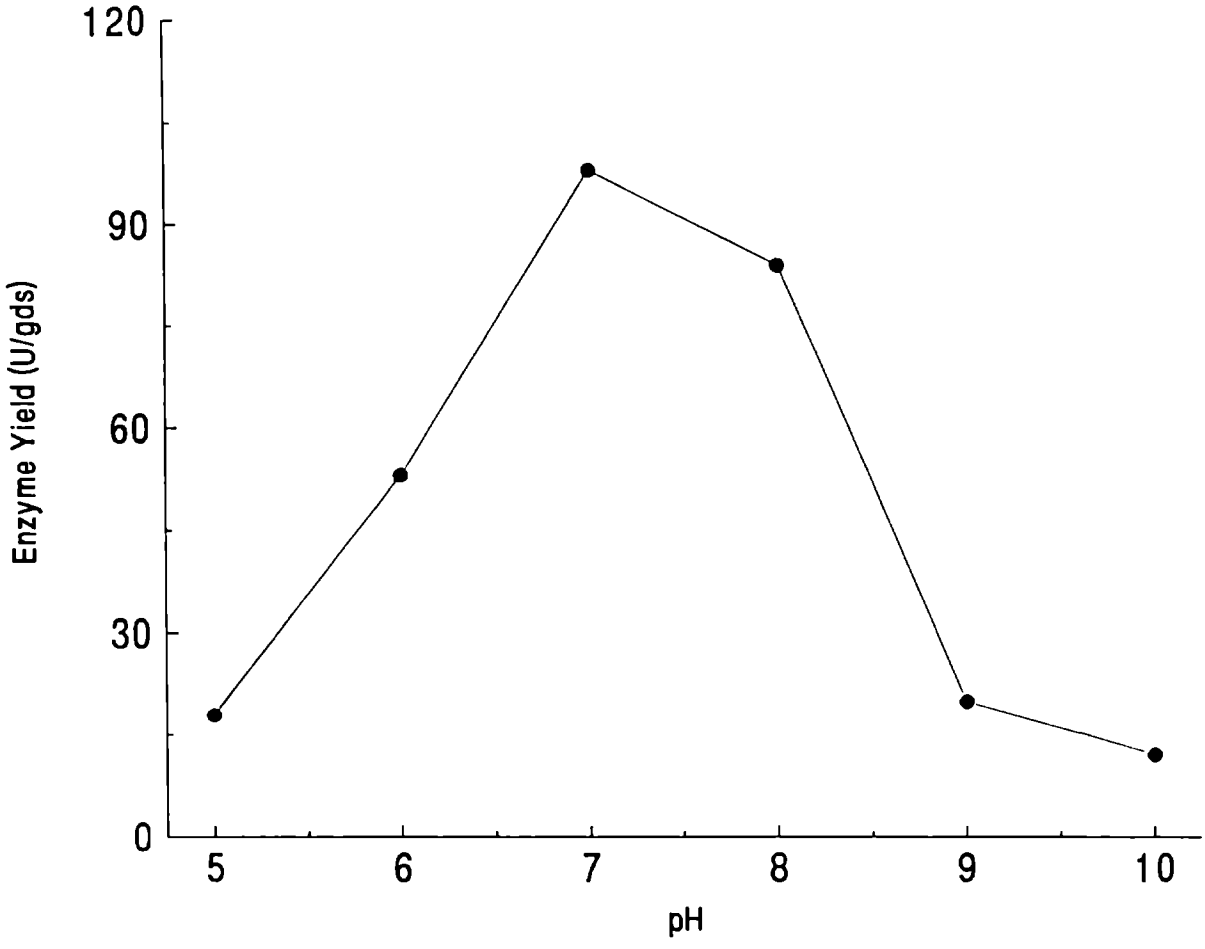


FIGURE 11: EFFECT OF INCUBATION TEMPERATURE ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE

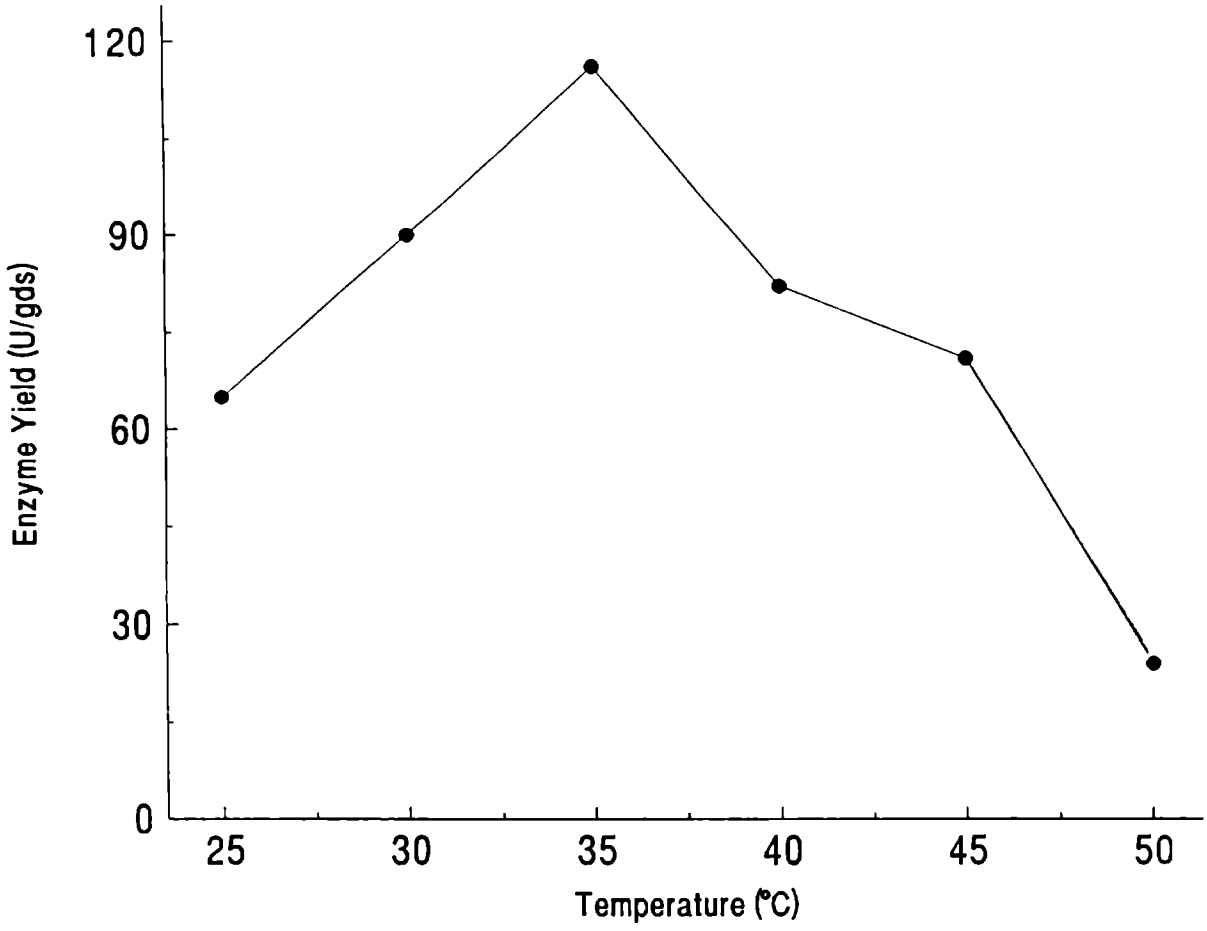
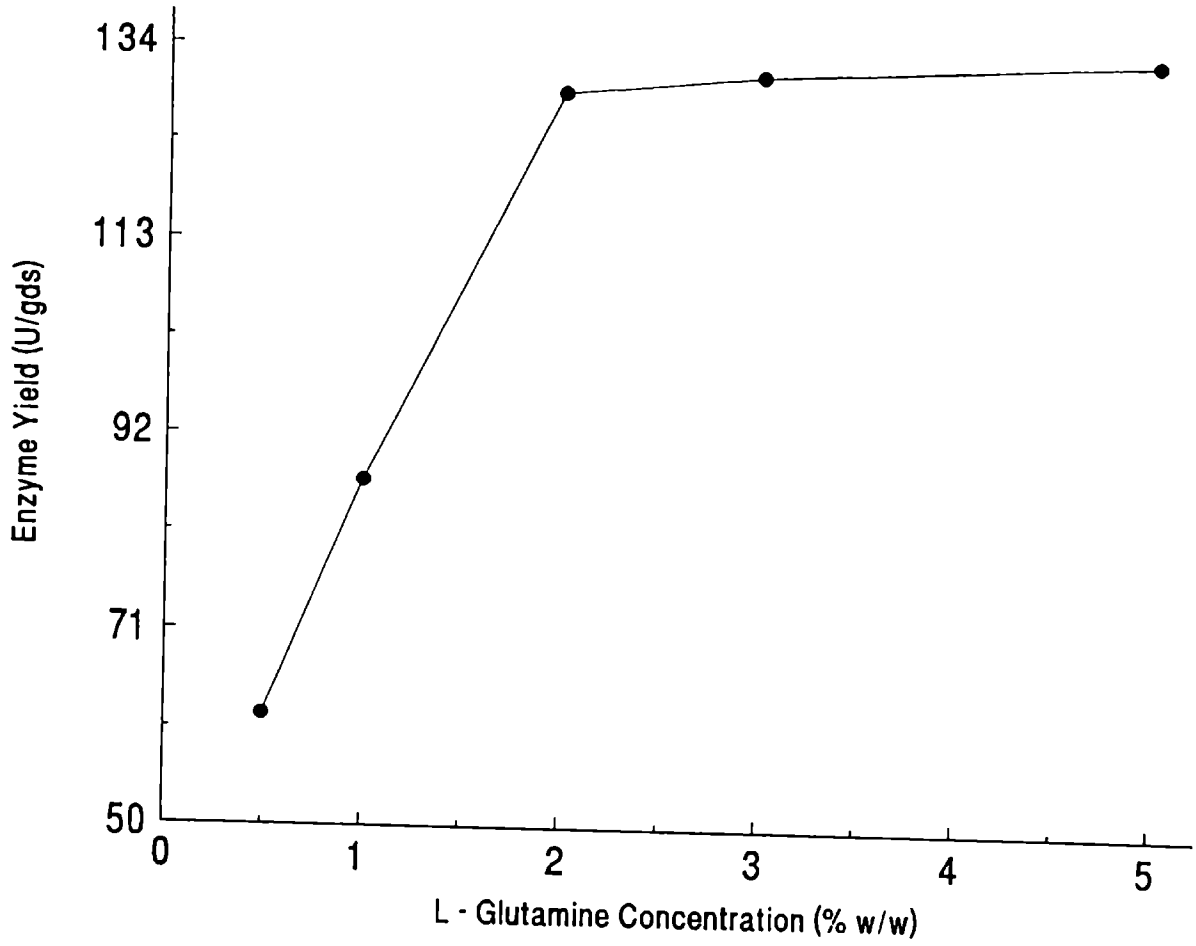


FIGURE 12: EFFECT OF L-GLUTAMINE CONCENTRATION ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE



in L-glutamine concentration did not enhance glutaminase production.

3.2.6. Effect of inoculum concentration

Glutaminase yield showed a linear increase along with increase in inoculum concentration (Fig. 13). Maximum enzyme yield was obtained with an inoculum containing 0.750 mg dry wt/10 gds (140 U/gds). Further increase in inoculum concentration did not effect additional enzyme yield.

3.2.7. Effect of incubation time

Results presented in Fig. 14 suggest that the optimum incubation time needed for maximum glutaminase yield was 24 h, when a total of 157 U/gds was obtained, in spite of recording a maximum of 160 U/gds at 36 h of incubation. The increase obtained during 24 - 36 h is insignificant compared to the increase observed during 12 to 24 h. It was also noted that fermentation beyond 48 h led to a decrease in enzyme yield

3.2.8. Effect of additional carbon sources

Incorporation of additional carbon sources to the SSF medium enhanced the enzyme yield significantly (Fig. 15). Among the carbon sources tested, maltose incorporated at 1 % (w/w) level in the sea water glutamine medium promoted maximal enzyme yield (22.5 % more) followed by glucose (16.9 %), galactose (10.6 %) and sucrose (7.5 %).

FIGURE 13: EFFECT OF INOCULUM CONCENTRATION ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE

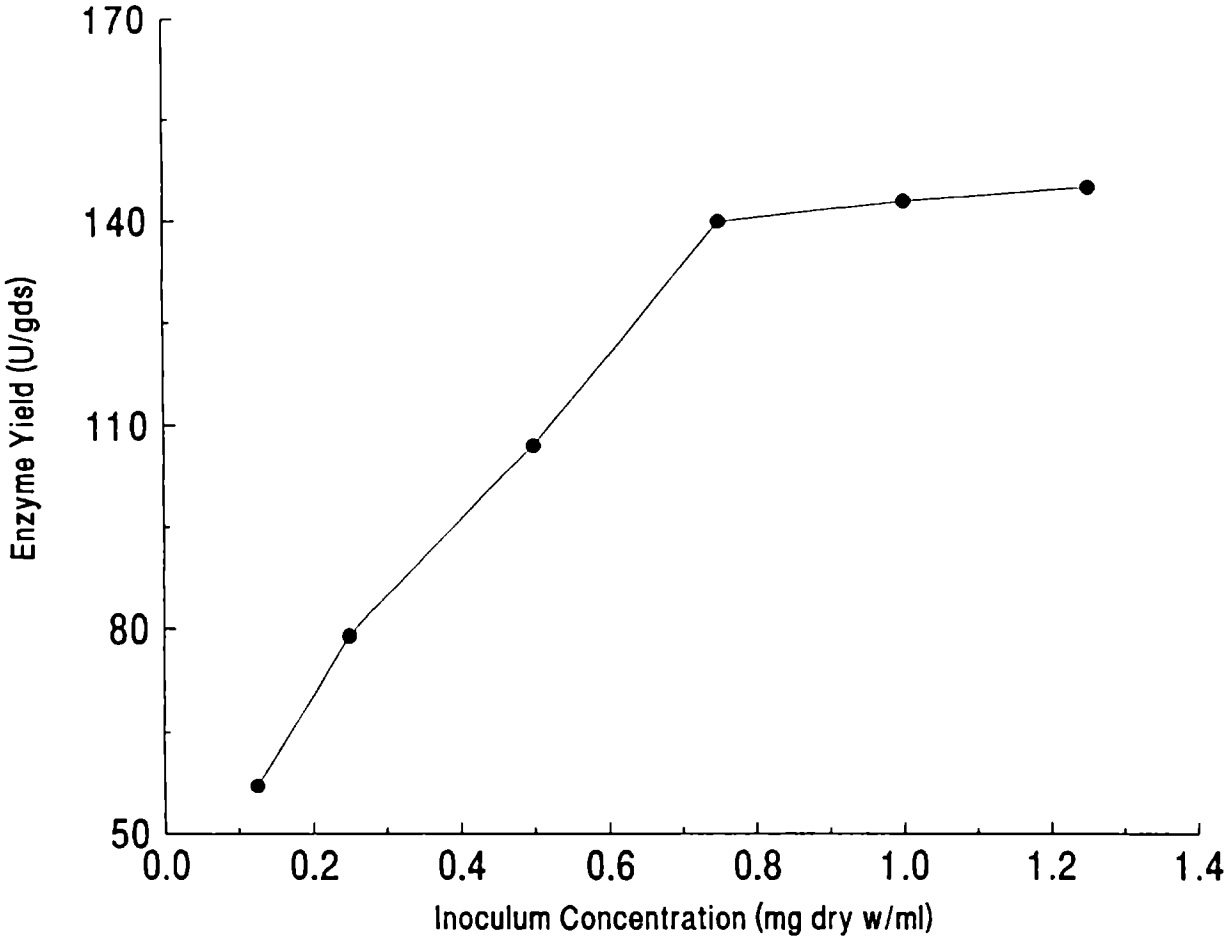


FIGURE 14: EFFECT OF INCUBATION TIME ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE

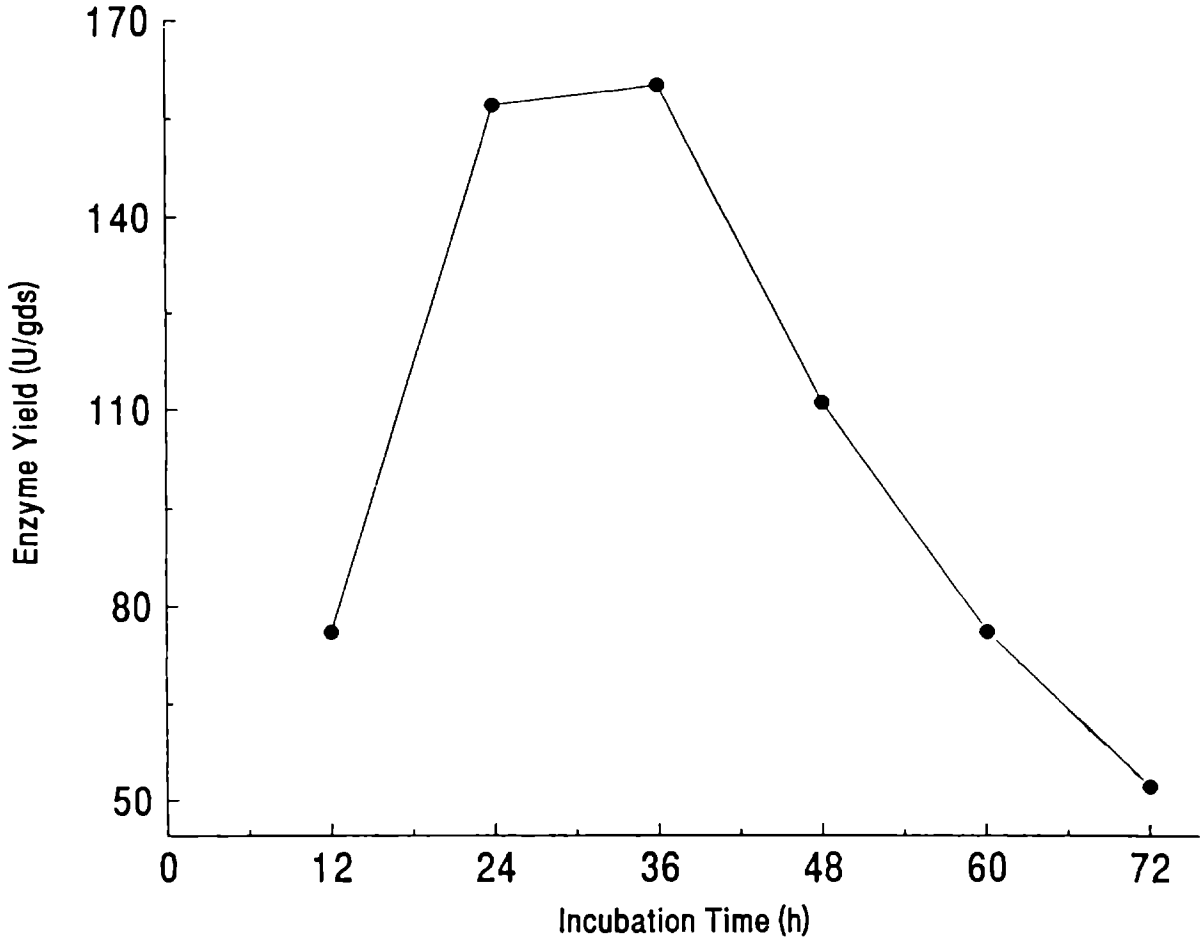
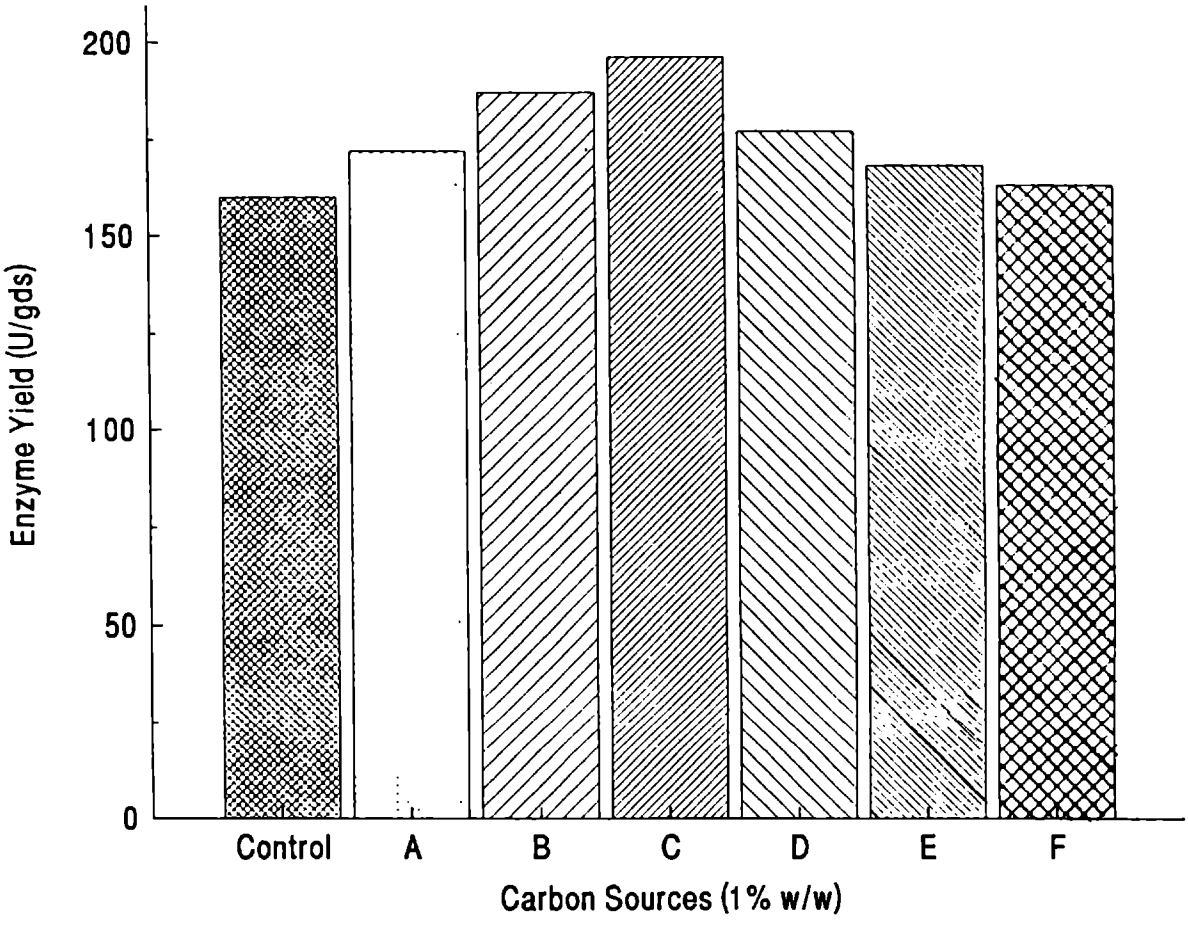


FIGURE 15: EFFECT OF ADDITIONAL CARBON SOURCES ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE



A - Sucrose
B - Glucose
C - Maltose
D - Galactose
E - Lactose
F - Trisodium Citrate

3.2.9. Effect of additional nitrogen sources

The incorporation of additional nitrogen sources at 1 % (w/w) had a negative impact on glutaminase yield (Fig. 16). The enzyme yield decreased from 196 U/gds, observed in the absence of various nitrogen sources, by 24.5 %, 24 %, and 22.4 % respectively with ammonium sulphate, urea and ammonium nitrate. Comparatively, organic nitrogen sources exerted a lesser impact on enzyme production than the inorganic nitrogen sources, except sodium nitrate.

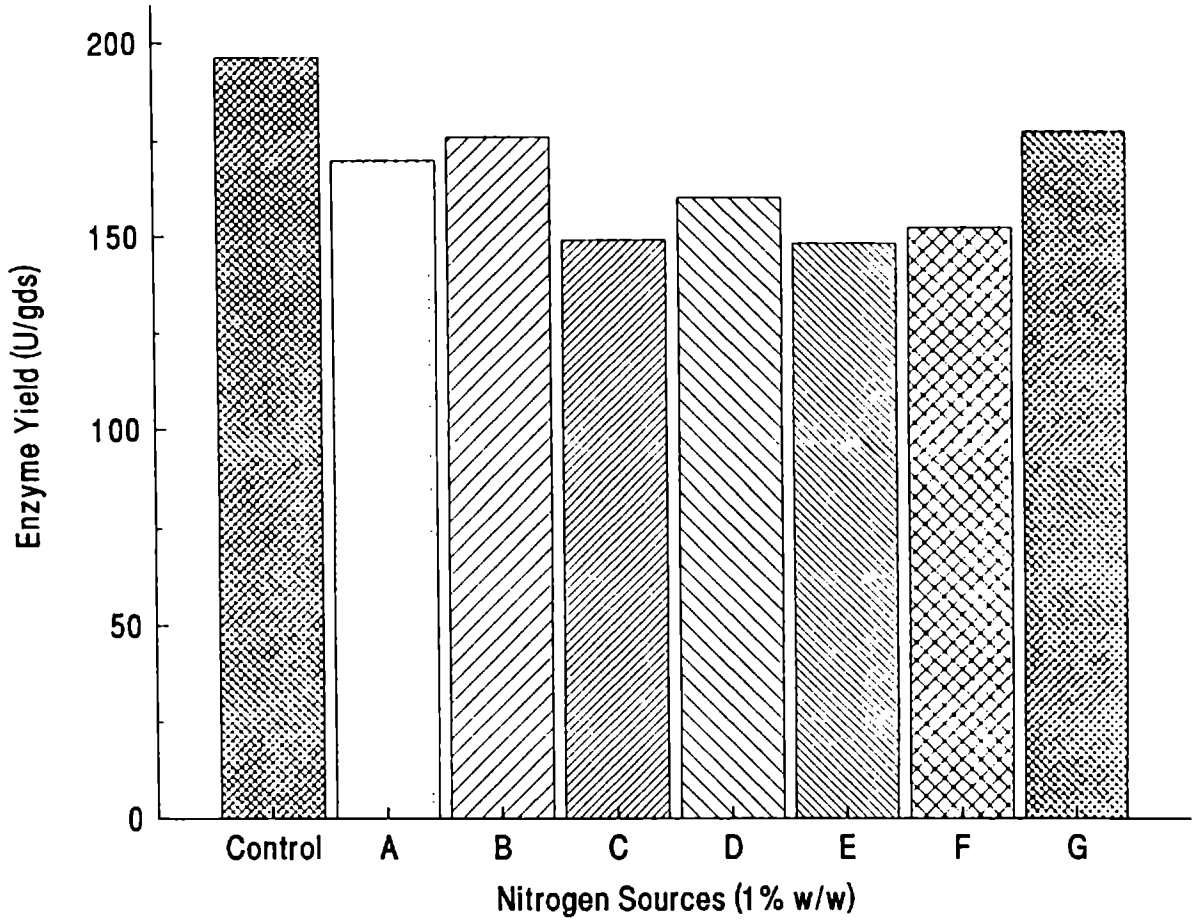
3.2.10. Effect of amino acids

The incorporation of amino acids, to the SSF medium at 1 % level led to decline in glutaminase yield when compared to the control (Fig. 17). Highest inhibition was observed with glutamic acid (37 %), followed by lysine (33 %), alanine, (24 %) cysteine (19 %), tyrosine (17 %), arginine (14 %), asparagine (13 %) and methionine respectively.

3.2.11. Effect of mineral salts

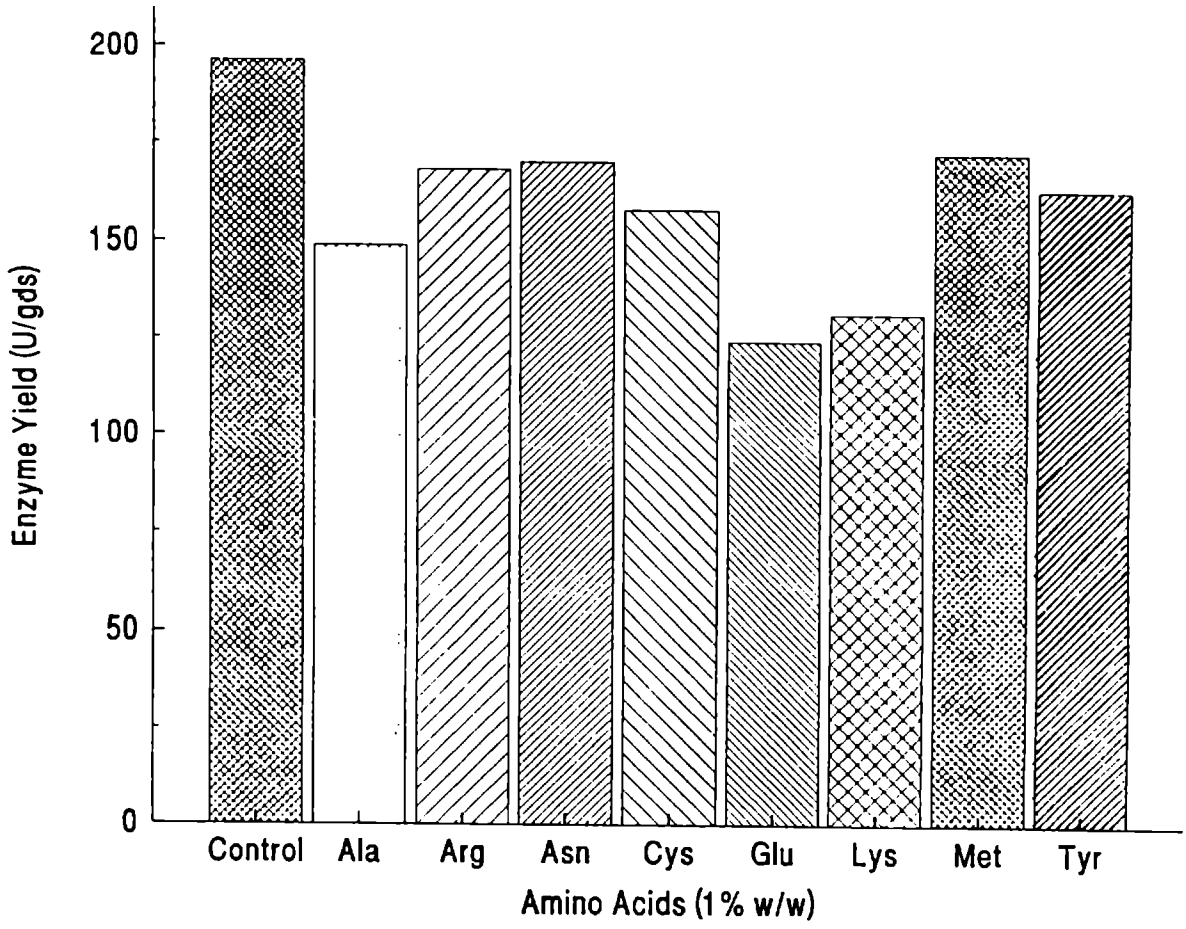
All the mineral salts tested in the present investigation enhanced glutaminase yield significantly when compared to the control (Fig. 18). KH_2PO_4 effected the maximum yield of 232 U/gds (18 % increase) when compared to the control (196 U/gds). NaH_2PO_4 and $MgSO_4$ could also enhance glutaminase production (14 % and 12 % respectively) while KCl enhanced enzyme yield by 10 %.

FIGURE 16: EFFECT OF ADDITIONAL NITROGEN SOURCES ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE



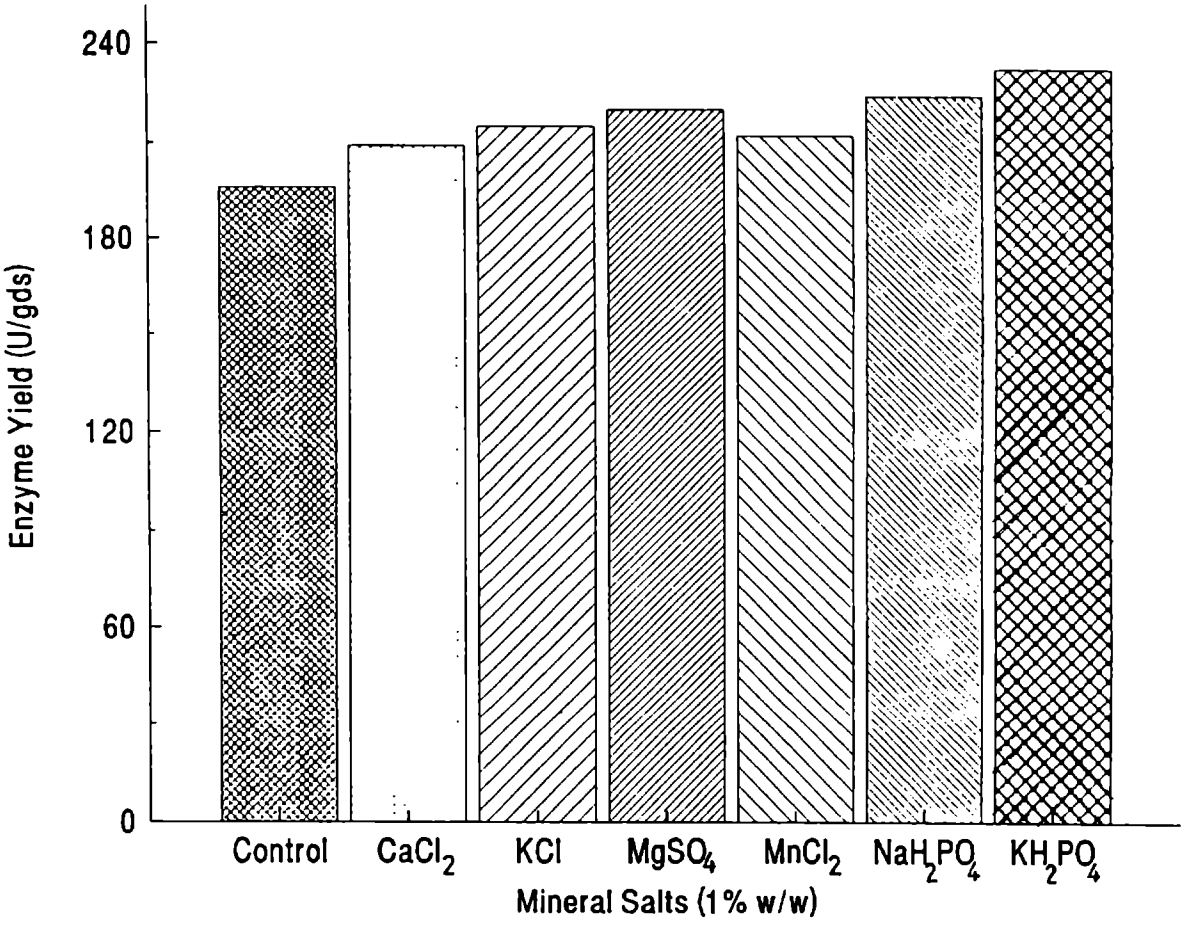
A - Yeast extract
B - Peptone
C - Urea
D - Caesine
E - Ammonium sulphate
F - Ammonium nitrate
G - Sodium nitrate

FIGURE 17: EFFECT OF AMINO ACIDS ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE



Ala - Alanine
Arg - Arginine
Asn - Asparagine
Cys - Cysteine
Glu - Glutamic acid
Lys - Lysine
Met - Methionine
Tyr - Tyrosine

FIGURE 18: EFFECT OF MINERAL SALTS ON L-GLUTAMINASE PRODUCTION BY **V. COSTICOLA** UNDER SSF USING POLYSTYRENE



3.2.12. Recovery of L-glutaminase from polystyrene SSF system

From the results given in Fig. 19, it is clear that a solid to buffer ratio of 1:4 (% w/v) with a contact time of 30 min. is ideal for the extraction of L-glutaminase from the polystyrene system, eventhough maximum levels of enzyme was recovered at the 1 5 ratio. The recovery of enzyme increased along with increase in the ratio from, 1 2 to 1 5 (143 - 230 U/gds) and a ratio of 1 10 resulted in a very dilute leachate, which is uneconomical at the industrial level.

3.2.13. Scanning Electron Microscopic studies

The SEM studies clearly evidences the ability of the bacteria to adsorb on to solid particles. Plates II and III shows polystyrene beads, uninoculated and after 24 h of incubation. The bacterial cells could be seen adsorbed on to the beads, in the inoculated beads.

3.3. ESTIMATION OF BIOMASS DURING SSF USING POLYSTYRENE

Biomass estimated in terms of cell protein and dry weight are given in Fig. 20. The results clearly indicate that maximum biomass (0.190 mg dry wt/ml and 0.309 mg/ml cell protein) was reached after 36 h. Results suggest that biomass course during SSF could be monitored satisfactorily when a polystyrene system is used.

FIGURE 19: EFFECT OF SOLID TO EXTRACTANT RATIO ON L-GLUTAMINASE RECOVERY FROM POLYSTYRENE SSF SYSTEM

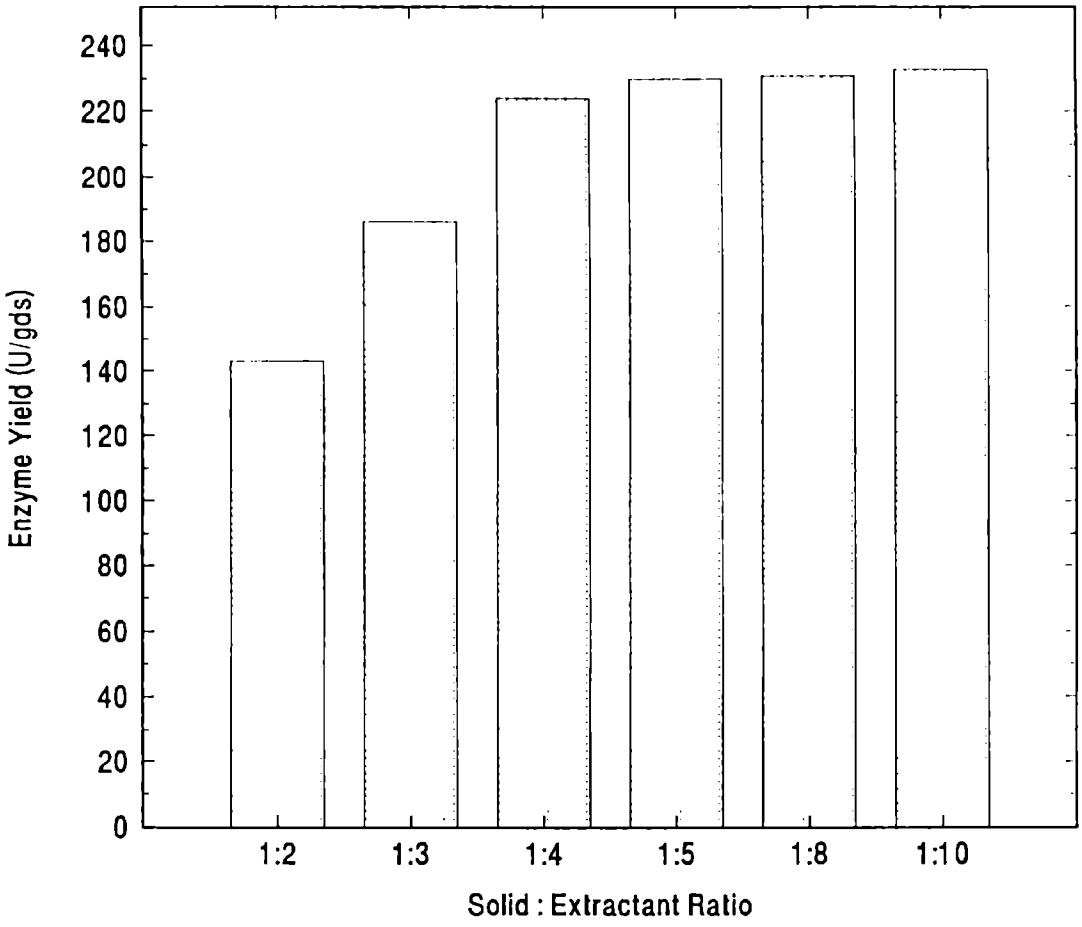


PLATE - II

SCANNING ELECTRON MICROGRAPH OF UNINOCULATED POLYSTYRENE BEAD

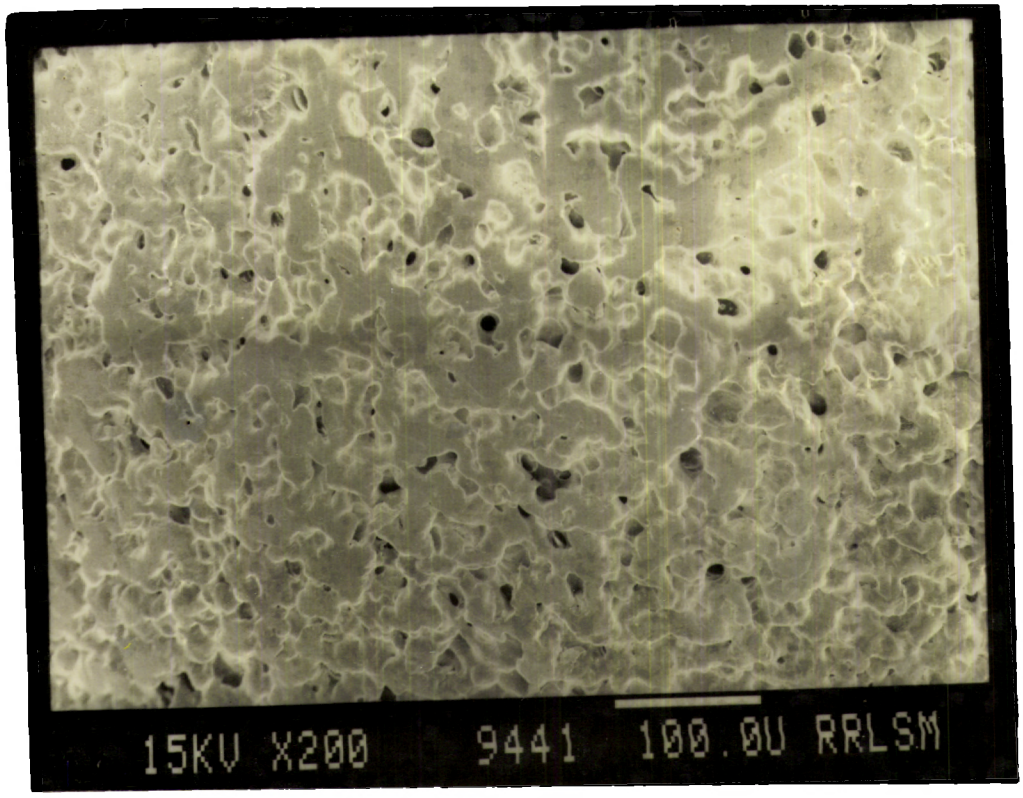


PLATE - III

SCANNING ELECTRON MICROGRAPH OF POLYSTYRENE BEAD
AFTER INOCULATION AND INCUBATION FOR 24 HOURS

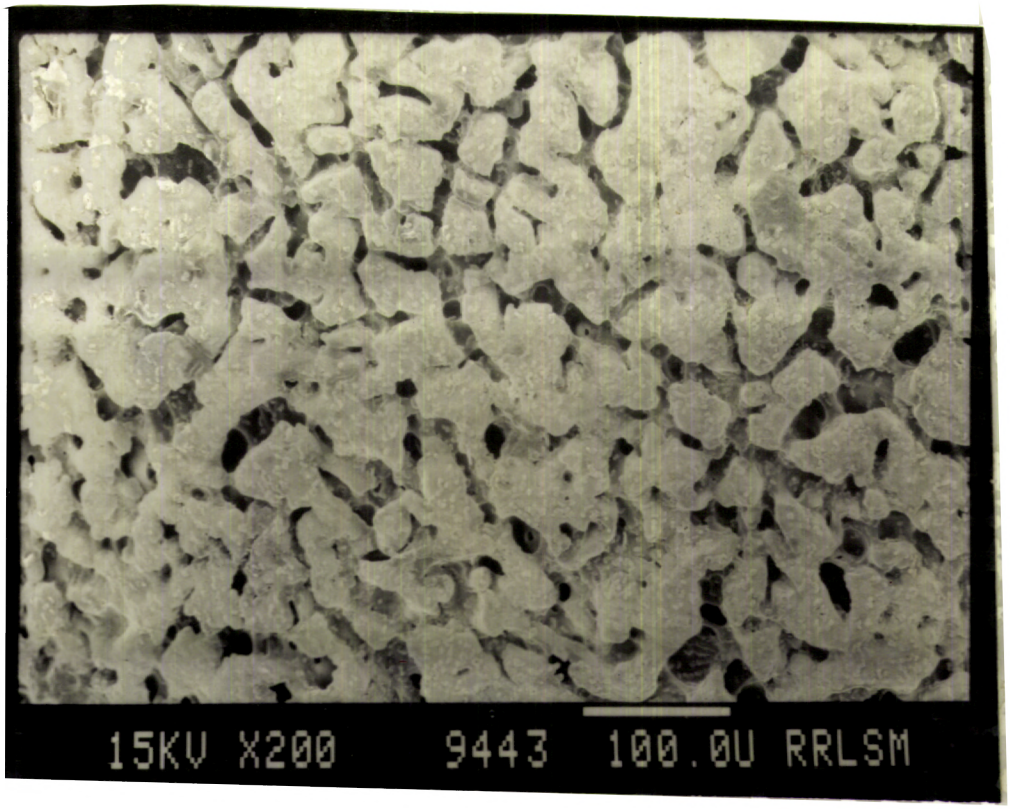
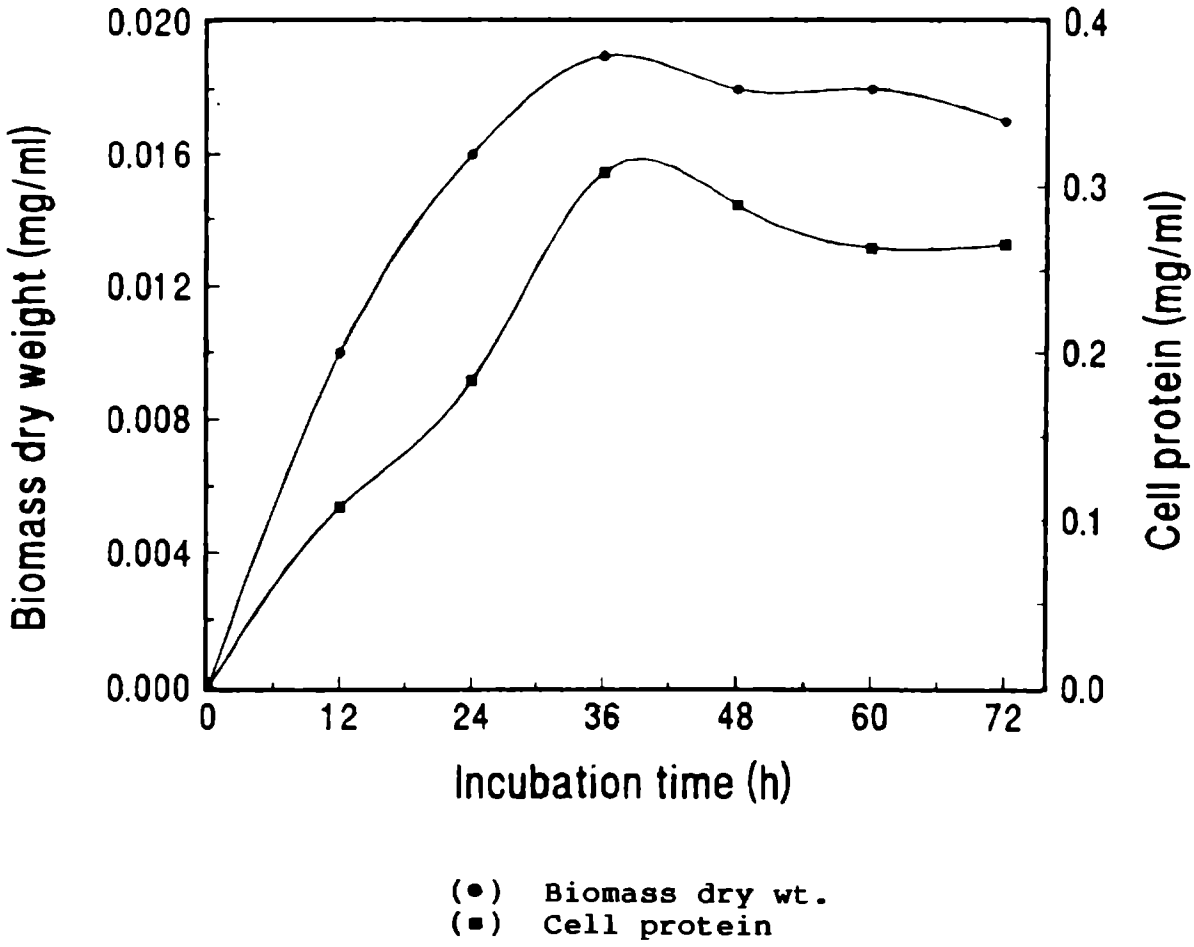


FIGURE 20: BIOMASS ESTIMATION DURING SSF USING POLYSTYRENE



3.4. L-GLUTAMINASE PRODUCTION UNDER SSF USING MIXED SUBSTRATES

A mixture of wheat bran/rice husk and polystyrene at different ratios could also support glutaminase yield when SSF was carried out at optimized conditions. However, no significant increase in enzyme yield was noticed with any of the ratios tried. From the results presented in Table 9, it is inferred that the overall yield from the mixed substrates was relatively less or at similar levels with that of individual substrates. Further, it was observed that mixtures containing higher proportions of polystyrene supported enhanced glutaminase yield, leachate with low viscosity and free from the presence of undesired proteins. A mixture of polystyrene beads to wheat bran recorded relatively higher levels of enzyme yields compared to the mixtures with rice husk. At equal proportions of polystyrene and wheat bran, the enzyme yield was less compared to other ratios tested with these two substrates. Ratios with higher polystyrene content not only recorded increased levels of enzyme yield (6.3 % more) than with higher wheat bran content (1.4 %), but also yielded leachate with lesser viscosity (1.107 Ns/m^2) and free of amylase and cellulase. Whereas, when wheat bran content was higher in proportion, not only the enzyme yield was less, but also the leachate was more viscous (1.984 Ns/m^2) and showed the presence of amylase and cellulase as contaminating enzymes.

Table - 9

COMPARISON OF ENZYME PROFILE AND VISCOSITY OF LEACHATES FROM NATURAL, INERT AND MIXED SOLID SUBSTRATE SSF SYSTEMS

Substrate	Glutaminase (U/gds)	Amylase (U/gds)	Cellulase (FPU/gds)	Viscosity (Ns/m^2)
Wheat bran (WB)	242	1150	1.6	2.072
Rice husk (RH)	235	846	1.0	1.652
Polystyrene (PS)	232	-	-	0.966
PS + WB (w/w)				
9 1	236	Trace	-	1.107
7 3	228	212	-	1.314
5 5	222	540	0.2	1.623
3 7	226	716	0.6	1.841
1 9	225	942	1.2	1.984
PS + RH (w/w)				
9 1	227	-	-	0.992
7 3	221	Trace	-	1.076
5 5	220	126	0.1	1.199
3 7	218	441	0.4	1.325
1 9	209	720	0.8	1.597

In the case of mixed substrates with polystyrene and rice husk, ratios with higher proportions of polystyrene led to a marginal increase (by 3.2 %) in enzyme yield compared to mixtures having equal proportions of both the substrates. Further, at ratios with higher proportion of rice husk and lesser polystyrene, there was a decline in enzyme yield (by 5 %). A general observation made in this study was that, higher proportions of wheat bran or rice husk in the mixture supported synthesis of amylase and cellulase besides glutaminase, unlike with that of polystyrene. It was also observed that viscosity of the leachate was comparatively lesser with mixed substrates than with wheat bran and it was at comparable levels with that of rice husk and was higher than that obtained from polystyrene alone. As the proportion of wheat bran or rice husk increased, the leachate became more viscous and showed the presence of amylase and cellulase besides glutaminase.

3.5. IMPACT OF MUTATION ON L-GLUTAMINASE PRODUCTION

The effect of UV irradiation and NTG treatment on *V. costicola* with respect to glutaminase production was studied using the polystyrene system under the same conditions optimized for the untreated organism.

Results presented in Table 10 & 11 suggest that the organism had both positive and negative impact of mutation with respect to glutaminase production, when subjected to UV irradiation and NTG treatment. Irradiation with UV rays for 80 seconds was observed to promote enzyme yield by 23.7 % over the control (Table 10). It was also noted that extended periods of exposure to UV led to decline in enzyme yield. Shorter duration upto 60 seconds did not show any significant impact on glutaminase yield.

Treatment of cells with different concentrations of NTG also resulted in induced mutation (Table 11). Thus varying levels of enzyme yield was incurred for the different concentrations of NTG tested. Enhancement of enzyme yield by 48 % over the control was recorded for an NTG concentration of 60 μg/ml compared to 50 μg/m (12 %). In general, at other concentrations, a decline in enzyme yield compared to control was observed. The stability of the mutated strains with respect to L-glutaminase production was checked repeatedly and they were found to be stable after 5 generations.

3.6. ENZYME PURIFICATION

L-Glutaminase could be purified by the methodology adopted for the study. It was observed that fractions in the range of 50–80 % saturation of ammonium sulphate contained maximum glutaminase activity. Protein precipitated at 50 % and above 80 % saturation did not possess much glutaminase activity. The active fractions were

Table - 10

IMPACT OF UV IRRADIATION ON L-GLUTAMINASE PRODUCTION BY *V. COSTICOLA* UNDER SOLID STATE FERMENTATION USING POLYSTYRENE

Exposure Time (sec)	Enzyme Yield* (U/gds)
0	215
20	216
40	220
60	229
80	266
100	212
120	198

* Mean of three sets of experiments

Table - 11

IMPACT OF NTG TREATMENT ON L-GLUTAMINASE PRODUCTION BY *V. COSTICOLA* UNDER SOLID STATE FERMENTATION USING POLYSTYRENE

NTG Concentration (µg/ml)	Enzyme Yield* (U/gds)
0	215
10	188
20	213
30	220
40	184
50	240
60	318
70	200
80	156
90	206
100	92

* Mean of three sets of experiments

pooled, dialysed against 0.01 M phosphate buffer (pH 8) for 24 h and then subjected to gel-filtration chromatography using Sephadex G-100. The specific activity of the enzyme was observed to increase after each step of purification.

The crude extract contained a total protein of 428 mg and showed total activity of 5846 Units with a specific activity of 13.66 Units/mg protein. The yield was considered to be 100 %. After ammonium sulphate fractionation the specific activity was increased to 28.13 Units/mg with 61.1 % yield. The specific activity was increased several fold after gel-filtration chromatography on Sephadex G-100. The details of each purification step are given in Table 12. Purified enzyme could be observed as single band in both SDS & native PAGE (Plates IV & V).

Electrophoretic studies show an approximate subunit molecular weight of 36,000 to 40,000 daltons and a molecular weight in the range of 140,000 to 150,000 daltons for the native protein.

3.7. ENZYME CHARACTERISTICS

3.7.1. Effect of pH on enzyme activity and stability

The enzyme was active over a range of pH 6-9 with optimum at pH 8 (Fig. 21). Glutaminase activity considerably decreased at both low (pH 5.0) and high pH (10.0). The pH stability of the enzyme also showed a similar trend in recording stability over a pH range of 6-9 (74 - 78 % residual activity) with maximal stability at pH 8.0.

Table - 12

PURIFICATION OF L-GLUTAMINASE PRODUCED BY *V. COSTICOLA*

Purification step	Protein (mg/ml)	Activity (U)	Sp.activity (U/mg)	Yield (%)	Fold Purification
Crude Extract	428	5846	13.66	100.00	1.0
Ammonium Sulphate Fraction	127	3572	28.13	61.10	2.06
Gel-filtration Chromatography Sephadex G 100	3	1790	596.67	30.62	43.68

PLATE - IV

PHOTOGRAPH SHOWING SDS-PAGE OF PURIFIED GLUTAMINASE
(Lane 1 - Crude enzyme; Lane 2 - Purified enzyme;
Lane 3 - Ammonium sulphate fraction)

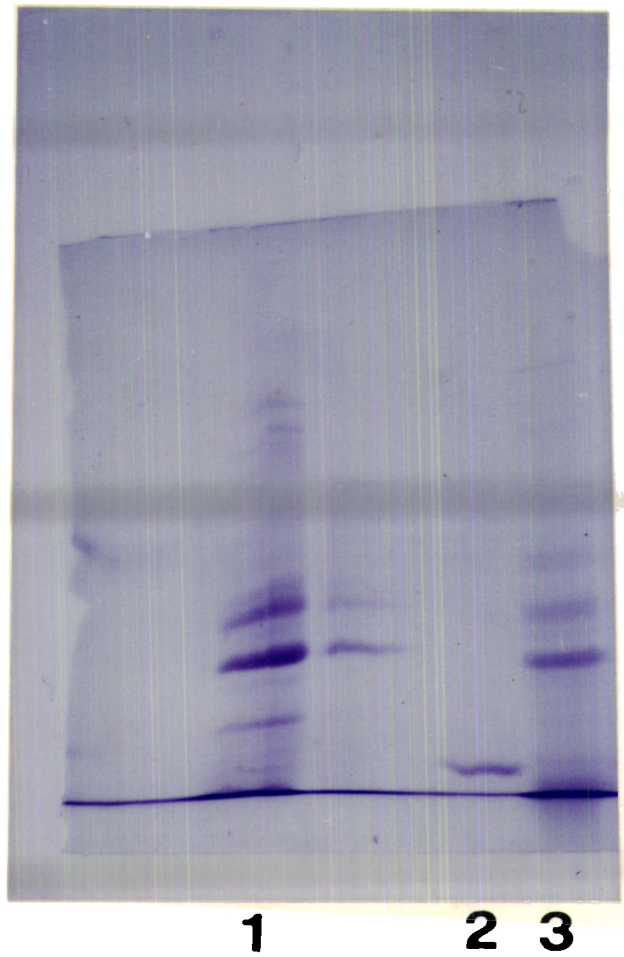
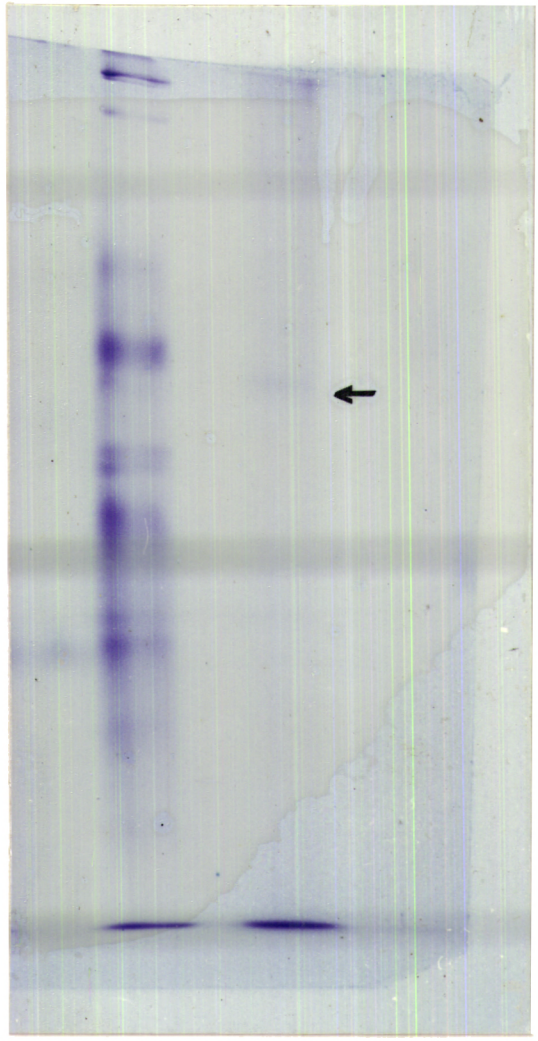


PLATE - V

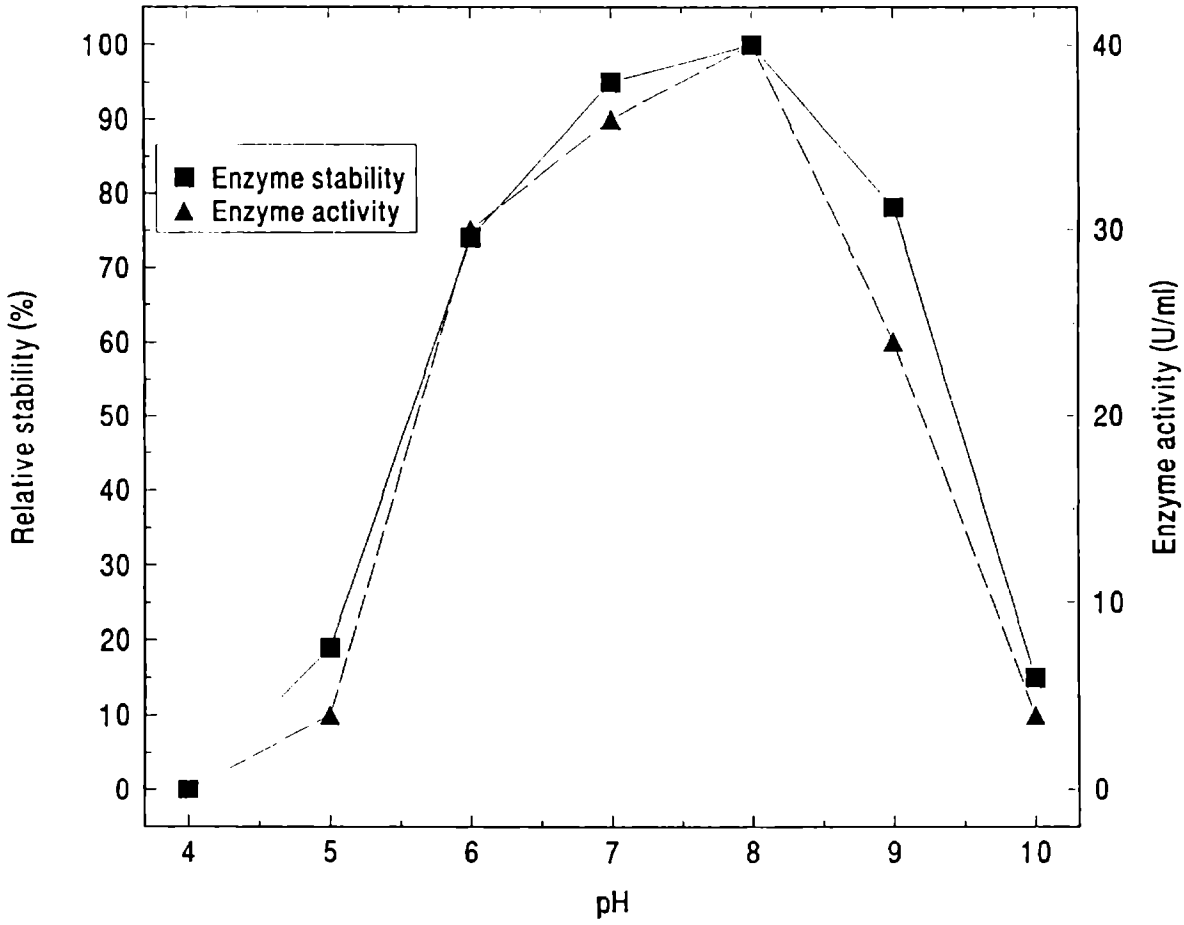
PHOTOGRAPH SHOWING NATIVE-PAGE OF PURIFIED GLUTAMINASE

(Lane 1 - Crude enzyme; Lane 2 - Purified enzyme)



1 2

FIGURE 21: EFFECT OF pH ON GLUTAMINASE ACTIVITY AND STABILITY



3.7.2. Effect of temperature on enzyme activity and stability

Results presented in Fig. 22 suggest that glutaminase prefers 40^{o}C as optimum temperature for maximal activity. However it can demonstrate significant activities (upto 85 - 86 % of the maximal enzyme activity) over a temperature range of 25 - 45^{o}C. Temperature above 50^{o}C led to a decline in enzyme activity. Temperature stability of glutaminase, also presented in Fig. 22, clearly evidence that the enzyme was stable at temperatures ranging from 25 - 40^{o}C. A loss of 5 % activity was recorded at 45 ^{o}C. Further increase in temperature led to considerable loss of activity.

3.7.3. Effect of substrate concentration on enzyme activity

L-Glutamine concentration of 0.04 M was recorded as the optimum substrate concentration for maximal activity (Fig. 23). The Km value of glutaminase was calculated to be 7.4 X 10^{-2} M.

3.7.4. Effect of NaCl concentration on enzyme activity

Results presented in Fig. 24 indicate that L-glutaminase was active over a wide range of NaCl concentration (0 - 6 %), suggesting the salt tolerant nature of the enzyme. A further increase in NaCl concentration upto 10 % led to a marginal decline in enzyme activity. NaCl concentration above 10 % however affected enzyme activity significantly.

FIGURE 22: EFFECT OF TEMPERATURE ON GLUTAMINASE ACTIVITY AND STABILITY

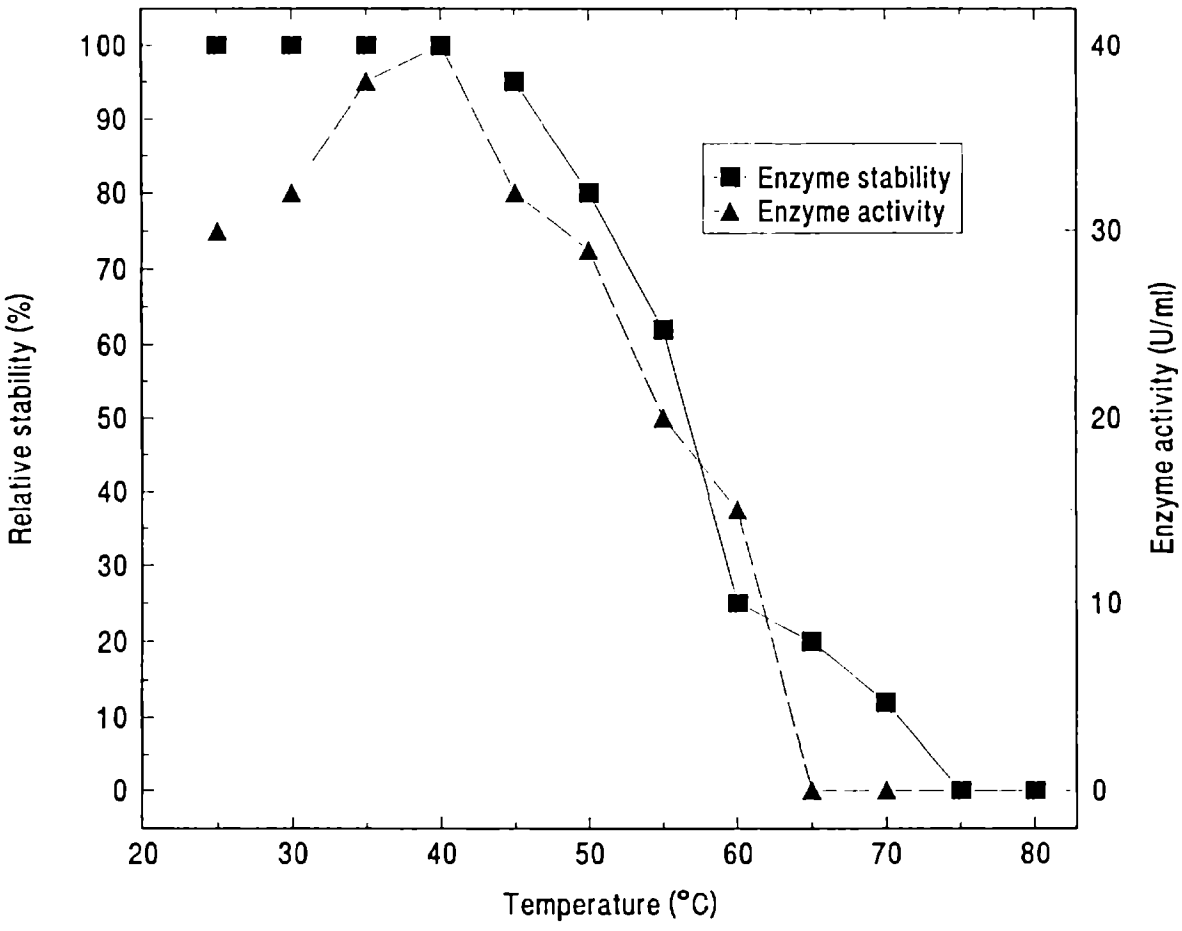


FIGURE 23: EFFECT OF L-GLUTAMINE CONCENTRATION ON GLUTAMINASE ACTIVITY

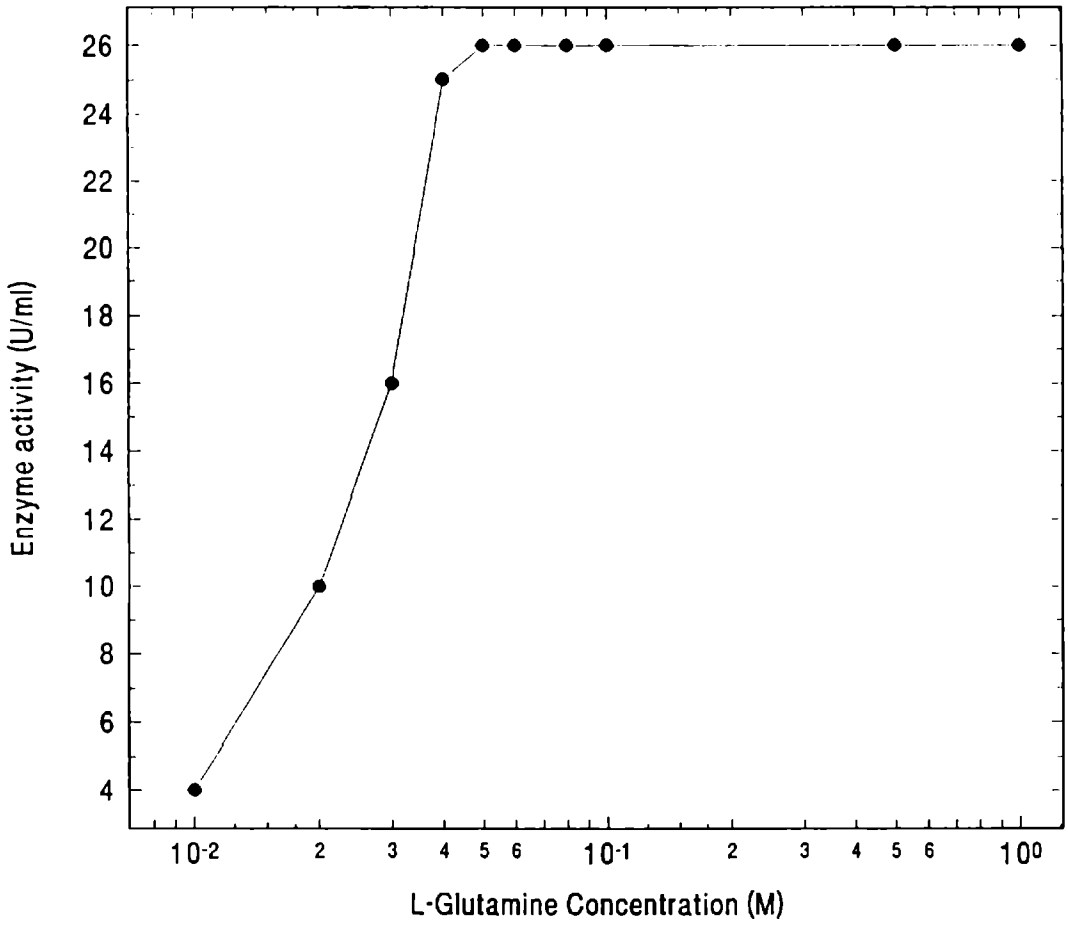
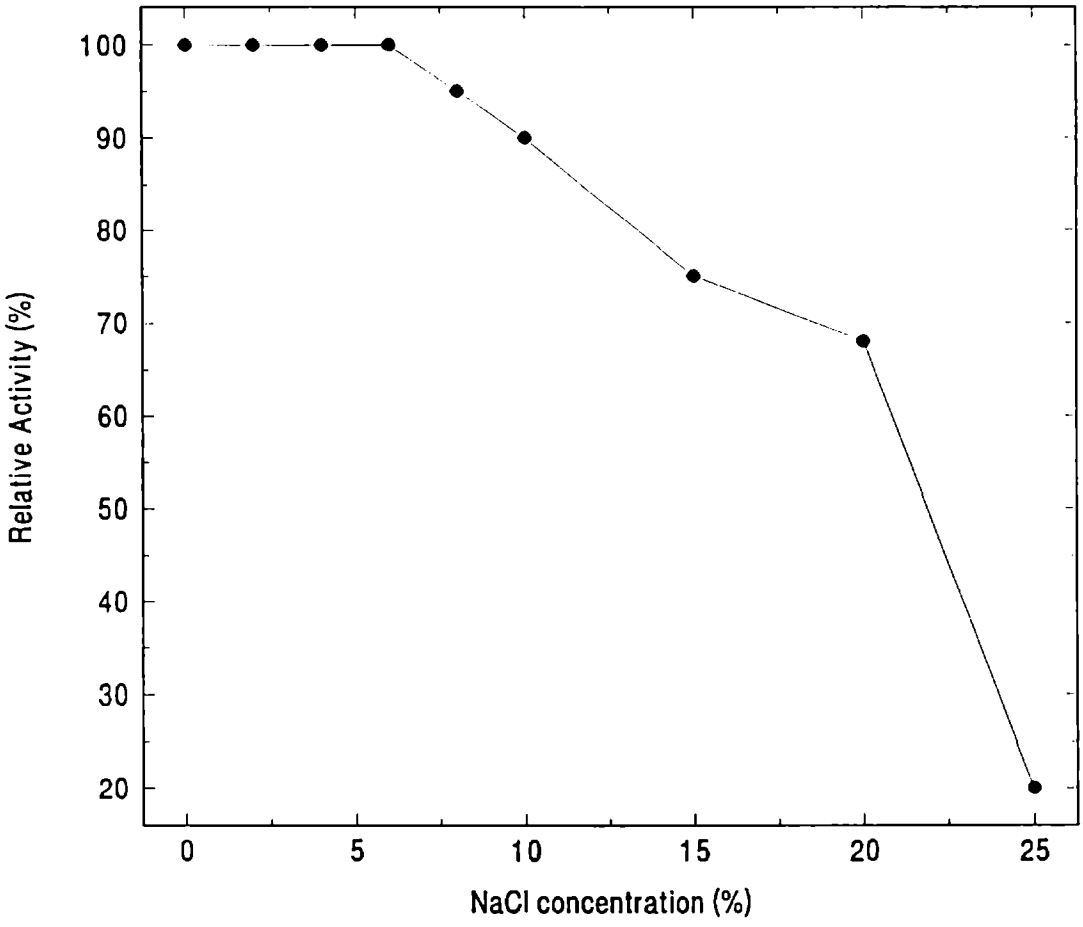


FIGURE 24: EFFECT OF NaCl ON GLUTAMINASE ACTIVITY



3.7.5. Effect of L-glutamine and NaCl on temperature stability

Presence of substrate (L-glutamine) and NaCl in the reaction mixture enhanced the temperature stability of the enzyme upto a temperature of 45°C (Fig. 25). Increase in temperature above 45°C to 50°C and 55°C, showed a marginal decrease in stability (95 - 98 % at 50°C and 89 - 93 % at 55°C). About 76 - 82 % activity was retained at 60°C in presence of 0.01 M glutamine or 10 % NaCl. Significant reduction in enzyme stability was observed at higher temperatures.

3.7.6. Substrate specificity

Results given in Fig. 26 suggest that glutaminase has high substrate specificity for L-glutamine. It showed very low activity, in the case of L-asparaginase and no activity with aspartic acid and glutamic acid. Interestingly, when a mixture of L-glutamine and L-asparagine was used as substrate, it could record only about 60 % activity compared with L-glutamine alone.

FIGURE 25: EFFECT OF L-GLUTAMINE AND NaCl ON TEMPERATURE STABILITY OF GLUTAMINASE

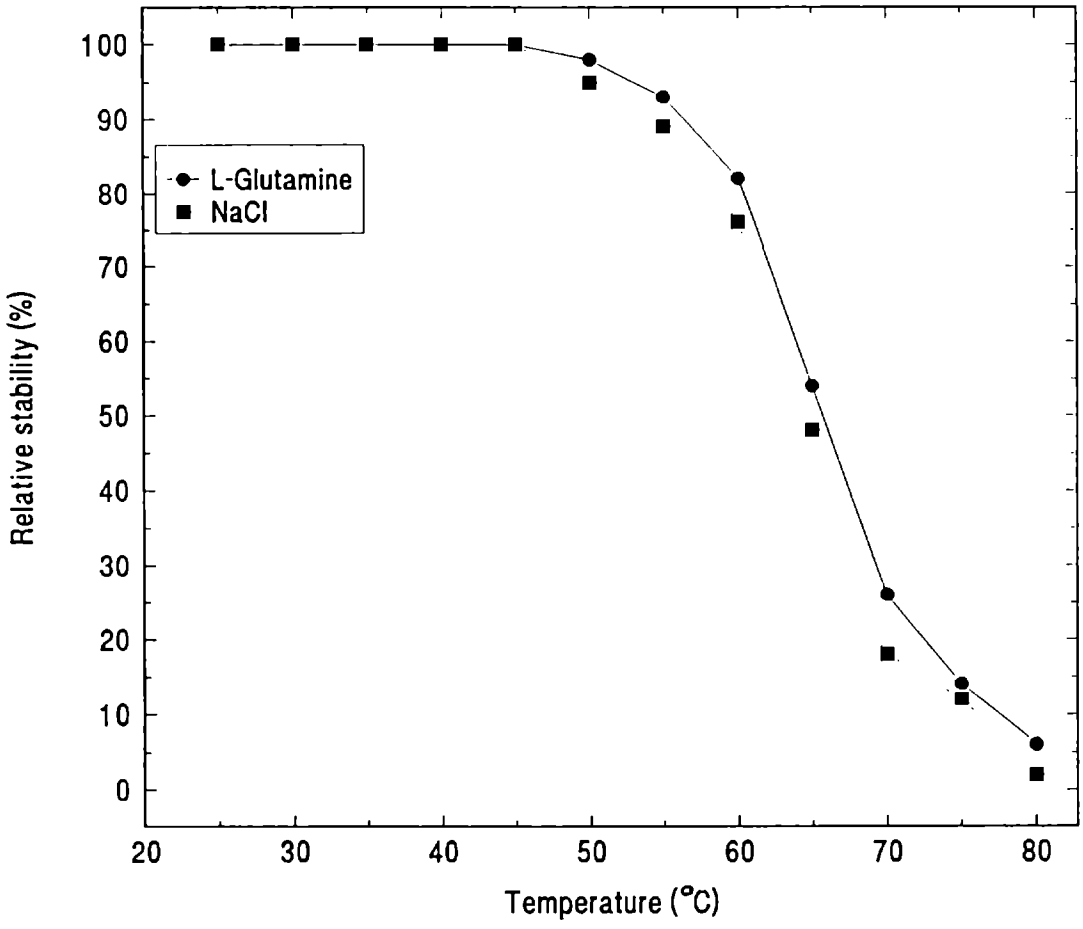
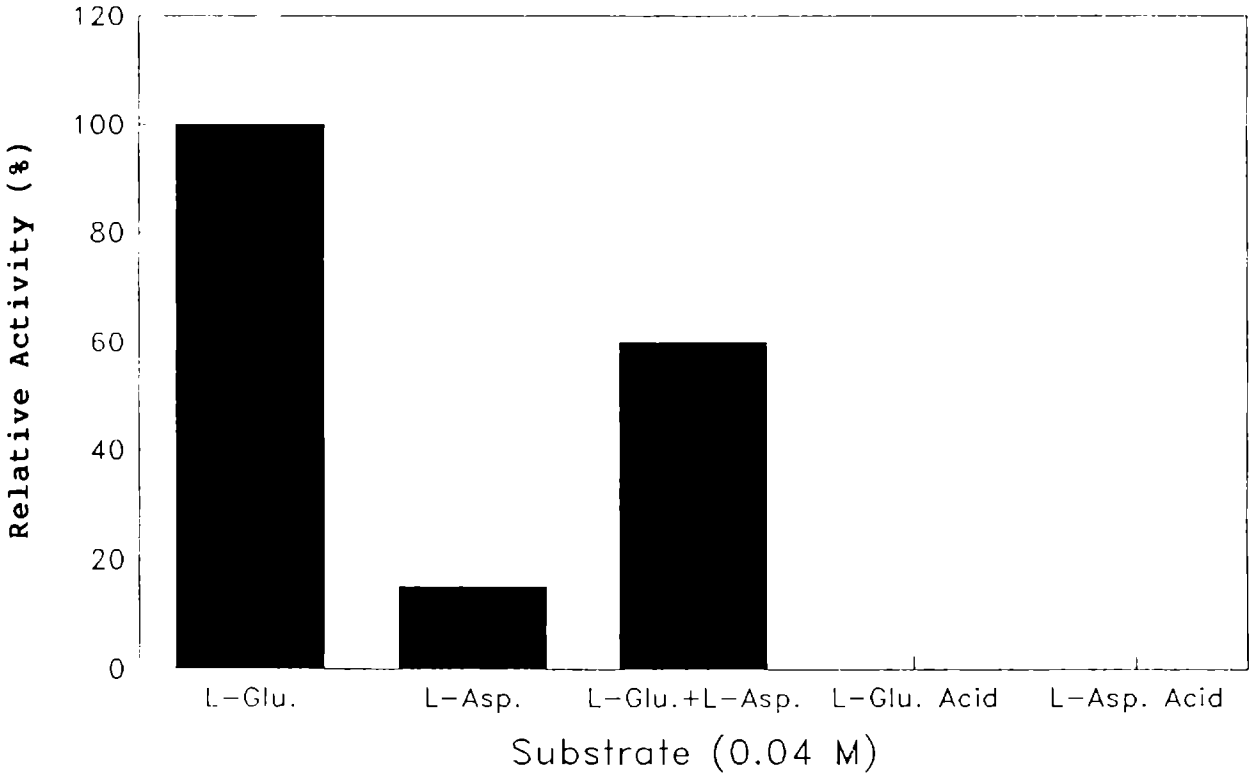


FIGURE 26: SUBSTRATE SPECIFICITY OF GLUTAMINASE PRODUCED BY **V. COSTICOLA**



L-Glu - L-Glutamine L-Asp - L-Asparagine
L-Glu.Acid - L-Glutamic acid L-Asp. Acid - L-Aspartic acid

DISCUSSION

The presence of product in dilute form in submerged fermentation (SmF) was recognized as a major obstacle in economic manufacture of microbial products, mainly due to the consequent higher costs on downstream processing and the disposal of larger volumes of waste waters. Moreover, the cost of separation of the microbial cells from the fermentation broth using centrifugation or micro filtration is reported to involve between 48 - 76 % of the total production cost of microbial metabolites by SmF. Hence more interest in solid state fermentation processes has been generated in recent years throughout the world (Hesseltine, 1972; Pandey, 1992; Lonsane, 1994).

Solid substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitrogen, minerals as well as growth factors (Pandey, 1992). The bacterial and yeast cells grow by adhering to the surface of the solid substrate particles (Lonsane & Ramesh, 1990) while the filamentous fungi are able to penetrate deep into the solid substrate particles for nutrient uptake (Lonsane *et al*, 1985; Pandey & Radhakrishnan, 1993). Wheat bran is the most commonly used and widely accepted solid substrate for SSF eventhough other substrates such as rice bran, rice husk, saw dust, sugarcane pressmud, coir pith, mango peels, lignocellulosic wastes, cabbage and banana wastes, mustard oil cake, tapioca peels, maize bran, gram bran and many more naturally occurring substances are also being tried as substrates for SSF. (Aidoo *et al*, 1982; Pandey, 1992; Satyanarayana, 1994, Krishna & Chandrasekaran, 1995, 1996).

Since the yield of product from any fermentation process, submerged or solid state, is governed by the environmental variables, it becomes mandatory to optimize these parameters in order to obtain maximum product yield. In the present study, an effort was made to optimize the important physical, chemical and nutritional parameters that influence the production of glutaminase by marine *Vibrio costicola* under SSF using natural, synthetic and mixed substrates.

4.1. L-Glutaminase production under SSF using natural substrates

Most of the process parameters tested had significant influence on glutaminase production by *V. costicola*. Interestingly, the nature of the solid substrate was observed to influence the optimal requirements of several bioprocess conditions as indicated by the results obtained in the present investigation. The initial pH of the medium, incubation temperature and inoculum concentration required for maximal glutaminase production were similar irrespective of the nature of the substrates used. Since these environmental parameters are independent in exerting their influence on the growth and performance of bacteria (Chandrasekaran *et al*, 1991), they did not vary in their optima for glutaminase yield despite the differences in the nature of the substrate used for SSF (Nagendra Prabhu & Chandrasekaran, 1996 a).

The critical importance of moisture content on microbial growth and product yield in SSF has been emphasized earlier (Ramesh & Lonsane, 1990). Microbiological activity on a substrate will progressively decrease at lower water contents finally ceasing at or near 12 %. Moisture is reported to cause swelling of the substrates thereby facilitating better utilization of substrates by the microorganism (Kim *et al*, 1985). But high moisture content led to reduced product yield, during SSF, due to reduction in interparticle spaces, decreased substrate degradation and impaired oxygen transfer (Zadrazil & Brunert, 1981; Sandhya Xavier & Lonsane, 1994). Further the physical nature and water holding capacity are important criteria for a solid substrate for its use in SSF processes (Aidoo *et al*, 1982) and the absorbency of the substrates will determine the moisture at which free water becomes apparent (Smith & Aidoo, 1988). Normally, a moisture content of 30–80 % is required for a significant level of enzyme production with an optimum between 50–60 %, depending on the material (Oriol *et al*, 1988).

In the present study, it was observed that the level of initial moisture content in the different substrates *viz.* wheat bran, rice husk, copra cake powder, groundnut cake powder and saw dust significantly influenced the rate of glutaminase synthesis and overall enzyme yield. The optimum moisture content required for maximal glutaminase yield was 60 % for wheat bran and rice husk, 70 % for saw dust and copra cake powder, and 40 % for groundnut cake powder.

Similar observations were made by earlier investigators for different substrates. While an initial moisture content of 65 % was optimal for α-amylase production by *Bacillus licheniformis* M 27 with wheat bran (Ramesh & Lonsane, 1990), a moisture content of 50 % was needed by *Aspergillus niger* for the production of citric acid using coffee husk and sugar cane press-mud (Shankaranand & Lonsane, 1993, 1994). The variation in the levels of influence of the moisture content on glutaminase yield, observed with respect to different substrates used in the present study, could be probably due to the differences in the physico-chemical nature and water holding capacity of the substrates, which normally vary from one type of substrate to another.

Initial pH of the medium is another important parameter which affects the growth and product formation by microorganisms under both SmF and SSF. Although pH is one of the critical factors, the monitoring and control of pH during fermentation is not usually attempted in SSF. Good buffering capacity of some of the substrates used in SSF help in eliminating the need for pH control during fermentation (Lonsane *et al*, 1985). This advantage is therefore exploited in the initial adjustment of the pH of the solids using the moistening media of the desired pH. However, local changes in pH of the agglomerates, produced if the organism develops in the form of a film on the solids, cannot be checked and result in low productivity

in unagitated fermenter (Knapp & Howell, 1980). The results of the present study suggest that the organism prefers an optimum pH of 7.0 for maximal enzyme yield, while the organism had a growth pH optima of 6.0 (Renu, 1991). However, significant levels of enzyme could also be recovered at pH 6.0 and 8.0.

The influence of substrate particle size, which determines the accessible surface area to the microorganism, on product formation has been emphasized (Hesseltine, 1972; Knapp & Howell, 1980). Ramesh and Lonsane (1989) used wheat bran of particle size 800 μm in order to obtain high yield of α-amylase by *B. licheniformis*. Similarly, Ofuya & Obilor (1994) used cassava peel of the size 1.0 mm to study the effect of solid state fermentation on its toxic components.

In the present study, the substrate particle size required for maximal glutaminase production varied according to the type of the substrate. Wheat bran and rice husk of 0.6 - 1.0 mm particle size supported maximum enzyme yield after 24 h compared to all other substrates, where a larger particle size of 1.0 - 1.4 mm favoured maximal enzyme yield after 36 h. The variation, among the particle size of substrates, with respect to support for maximal enzyme production may be attributed to their difference in water holding capacity and surface area for colonization by bacteria.

Temperature is directly related to the metabolic activities of the microorganism and it affects proper growth and product formation by the organism (Lonsane *et al,* 1985). Every organism has its optimum temperature at which it grows best resulting in higher yield of the desired product and hence temperature should be maintained at the optimum of the microorganism that is used for SSF. It was found that *V. costicola* used in the present study required $35^{o}C$ for maximal glutaminase production, which is also its optimal growth temperature (Renu, 1991). It is inferred that the bacteria requires its optimal growth temperature for maximal level of enzyme production.

An optimum inoculum concentration is required for obtaining maximum growth and product formation in both SmF and SSF. The inoculum is generally used at a high ratio in most solid state fermentation processes for the production of secondary metabolites, with the aim of producing the desired product in a short period (Lonsane *et al,* 1992). The inoculum must be in a metabolically active state, free from contaminants, capable of producing the desired product in subsequent culture and the success of the inoculum is judged by the productivity of the developed culture in the fermentation process (Stanbury, 1987). In the present study, maximum level of glutaminase was obtained with an inoculum concentration of 0.750 mg dry wt/10 gds irrespective of the type of solid substrate. These results suggest that enzyme yield is dependent on the initial

inoculum concentration used for SSF, and the inoculum required for obtaining maximal enzyme yield is independent of the nature of the solid substrates used.

In the present study, L-glutamine concentration needed for maximal glutaminase yield was 2 % (w/w) for *V. costicola* with all the five solid substrates. The presence of glutamine in the media showed an inducing effect, on the bacteria, for the production of extracellular glutaminase. At levels higher than the optimum value, no significant increase in enzyme yield was observed and hence it is assumed that presence of excess L-glutamine in the medium does not influence the induction of enzyme synthesis.

The variation in optimum incubation time required for maximal enzyme yield with the substrates could be attributed to the differences in their physical nature and biochemical status which consequently influence the aeration rate and nutrient availability for the bacteria during SSF. Preferential utilization of native carbon and nitrogen sources of copra cake powder and groundnut cake powder in place of glutamine may be the cause of delayed and reduced glutaminase yield compared to wheat bran and rice husk which have relatively lesser protein and carbohydrate contents. (Nagendra Prabhu & Chandrasekaran, 1996 a). Extended incubation time required for maximal enzyme production with saw dust may be accounted to its comparatively nutritionally inert and complex nature. The reduction

in glutaminase yield from all the substrates after 48 h is probably due to enzyme denaturation by the protease secreted by the bacteria (Nagendra Prabhu & Chandrasekaran, 1996 a). Similar results were obtained during α-amylase production by *B. megaterium* 16 M under SSF (Ramesh & Lonsane, 1987).

Increased product yield after incorporation of additional carbon and nitrogen sources, mineral salts and other additives during SSF was reported by Pandey *et al* (1994, 1995); Roussos *et al* (1992); Moriguchi *et al* (1994),. Whereas in the present study, incorporation of additional carbon sources enhanced the enzyme yield to a considerable level only with saw dust, compared to other substrates, where the increase in yield was not appreciable. This could be probably due to the presence of large amounts of native carbon and nitrogen sources found in the solid substrates other than saw dust which was comparatively nutritionally inert and complex in nature (Nagendra Prabhu & Chandrasekaran, 1996 a).

The addition of nitrogen sources, including organic, inorganic and amino acids, also did not have any major impact on glutaminase yield. This could also be due to the reasons mentioned above. In the case of organic nitrogen sources such as peptone, yeast extract and amino acids, there could have been a competitive preference of these sources over L-glutamine and hence a consequent reduction in glutaminase yield. On the other hand, addition of

mineral salts, enhanced glutaminase yield. Use of ammonium sulphate, sucrose, ammonium nitrate, potassium ferrocyanide and EDTA as nutritional supplements were reported to enhance the production of citric acid by SSF using different substrates (Shankaranand & Lonsane 1993, 1994).

Moriguchi *et al* (1994) have reported that the presence of 3 % NaCl in the medium resulted in highest yield of glutaminase from marine *Micrococcus luteus* K 3. Similarly, in the present study, 3 % NaCl was found to be the optimum for maximal glutaminase yield by *V. costicola.* The ability of the organism to produce significant levels of glutaminase at 0 - 5 % NaCl suggest that concentration of NaCl significantly influenced the glutaminase yield. It is obvious that since the bacteria is of marine origin, it has shown preference for the presence of adequate NaCl for enhanced enzyme yield, as it may do in its native environment.

A comparison of all the five organic solid substrates with respect to glutaminase production during SSF under optimal conditions testifies the advantages of wheat bran and rice husk over the other three substrates. Wheat bran is the most commonly used organic solid substrate for SSF processes aimed at producing microbial exoenzymes, organic acids, antibiotics, food flavours etc (Lonsane, 1994). Rice husk, saw dust and a large variety of materials have been used for various SSF experiments (Bharat Bhushan *et al*, 1994). Whereas copra

cake powder was used for lipase production and ground nut cake powder has not yet been tried as substrate for SSF (Pandey, 1992).

Viscosity of the leachate is a critical parameter which makes downstream processing difficult and expensive. Extracts from nutrient rich substrates such as wheat bran will contain lot of polymeric and gummy materials leached out along with the desired product during the extraction procedure which contributes to the viscous nature of the leachate (Ramesh & Lonsane, 1989). In the present study, viscosity of the enzyme extract was observed to be less (>2.0 Ns/m^2) with rice husk, saw dust and wheat bran compared with the others. The low viscosity of the extracts obtained with these substrates is, in fact, a desirable quality in the enzyme industry since it reduces the cost of purification. Hence, a critical analysis on the performance of the substrates clearly indicates the advantages of wheat bran and rice husk for L-glutaminase production by marine *V. costicola*. The extracts from all the substrates also showed the presence of amylase (range 126 U/gds to 1150 U/gds) and cellulase (range 0.1 FPU/gds to 1.6 FPU/gds), besides glutaminase. Further discussion on the yield of other enzymes is not held as the emphasis was made on L-glutaminase and the selection of a suitable substrate.

4.2. L-Glutaminase production under SSF using polystyrene

SSF processes using nutritionally rich substrates such as wheat bran have certain inherent problems. Use of nutritionally inert materials as supports for solid state fermentation has been recommended so as to overcome these inherent problems (Aidoo *et al*, 1982), and a variety of materials were tried as inert supports for SSF processes (Zhu *et al*, 1994; Nagendra Prabhu & Chandrasekaran, 1995, 1996 b). According to Kobayashi *et al* (1991), a fermentation system using nutritionally inert supports enables to achieve a controlled medium composition and feed rate, to separate the product readily from the inert carrier and to perform the SSF process continuously and semi-continuously.

Therefore, an effort was made, during the present investigation, to develop polystyrene as an inert solid support for L-glutaminase production by *V. costicola*. Results obtained for the various experiments, presented in the previous chapter, clearly advocates the suitability of polystyrene as an inert solid support for SSF studies.

The results of the scanning electron microscope studies presented in Plates II and III adds evidence to the fact that marine bacteria adsorb on to solid particles and colonize rapidly. It was

observed that *V. costicola* grew by adsorbing on to the polystyrene beads and utilizing the nutrients supplied in the moistening medium.

Fletcher (1976) reported that a marine *Pseudomonas* showed attachment to polystyrene by means of proteinaceous compounds. Most marine bacteria can utilize nutrients present in minute concentrations and many of them can only find sufficient food while growing as *aufwuchs*, a condition during which they remain adsorbed on to solid particles (Chandrasekaran, 1996). The growth of filamentous fungi on natural substrates such as wheat bran have been studied previously (Paredes-Lopez *et al*, 1991; Pandey & Radhakrishnan, 1993). Whereas, the present study is the first report on the bacterial attachment to solid substrates during SSF.

The growth and enzyme production by the bacterium was maximum when both mineral salts glutamine medium and aged sea water containing 1 % (w/w) L-glutamine were used, prior to optimization of SSF conditions. The aged sea water glutamine medium was selected for further studies not only due to the ease of preparation and the nonrequirement of expensive chemicals, but also it serves the requirements of a marine bacterium with respect to several trace elements and mineral salts, which are naturally present in it.

The substrate media ratio, which determines the moisture content of substrates, played a critical role in the SSF process, as observed with wheat bran and other natural substrates tried in the present study. Since the water absorbency of polystyrene was low (2 gm/100 cm^3; Brydson, 1982), increase in the proportion of media in the ratios above 1 1 (w/v; approximately 50 - 60 % moisture content) resulted in the existence of free water and a consequent reduction in the product yield (Nagendra Prabhu & Chandrasekaran, 1996 b). Zhu *et al*, (1994), in a similar study, used a moisture content of 60 % for the SSF production of nuclease P 1 by *Penicillium citrinum* using polyurethane foam as inert support.

The initial pH, incubation temperature and substrate (L-glutamine) concentration required by the bacteria with polystyrene system were very similar to that obtained in the present study with nutritionally rich substrates. As discussed earlier, these environmental parameters are independent in exerting their influence on the growth and performance of bacteria (Chandrasekaran *et al*, 1991) and were not influenced by the nature of the substrates used for SSF (Nagendra Prabhu & Chandrasekaran, 1996 a). In fact, the physico-chemical nature of polystyrene has not affected the physiology of the bacteria, but served as a substrate for surface colonization.

The effect of inoculum concentration on L-glutaminase production under SSF on polystyrene system was also very similar to that obtained with the natural substrates, recording a maximum glutaminase yield with an inoculum concentration of 0.750 mg dry wt/10 gds. The optimum incubation time was 24 h after standardization. The reason for the decrease in enzyme yield after 48 h might be attributed to the inactivation of glutaminase by the protease secreted by the bacteria (Nagendra Prabhu & Chandrasekaran, 1996 a,b). A similar observation was made during SSF on polyurethane foam for the production of nuclease P 1 by *P. citrinum*, where the nuclease yield was reduced after 3 days of fermentation due to enzyme inactivation by protease (Zhu *et al*, 1994). Ramesh and Lonsane (1987) also observed reduction in α-amylase production by *B. megaterium* under SSF on wheat bran after 52 h of incubation and postulated that this may be due to poisoning, denaturation and/or decomposition of the enzyme as a result of interaction with other components in the medium.

Incorporation of additional carbon sources enhanced the enzyme yield. Among the carbon sources tested, maltose incorporated at 1 % (w/w) level in the sea water glutamine medium could promote maximal enzyme yield compared to others. The increase in enzyme yield could be attributed to the rapid growth accomplished by the easy availability of additional carbon sources along with glutamine (Nagendra Prabhu & Chandrasekaran, 1995). On the other hand, it was observed that nitrogen sources, including amino acids, had a negative

impact on glutaminase yield. This could be due to the preferential utilization of these nutrients in place of glutamine (Nagendra Prabhu & Chandrasekaran, 1996 b).

All the mineral salts tested in the present investigation enhanced glutaminase yield significantly when compared to the control. KH_2PO_4 effected the maximum enzyme yield when compared to the control. NaH_2PO_4 followed by $MgSO_4$ could also enhance enzyme yield significantly. Results emphasize the critical role of phosphates and magnesium in the enhanced secretion of glutaminase by this bacteria. Zhu *et al* (1994) have achieved increased product yield of nuclease P 1 with polyurethane foam impregnated with a media that simulated the chemical composition of wheat bran, Whereas in the present study, maximal yield of the target product was obtained with the use of aged sea water media containing L-glutamine, maltose and KH_2PO_4, indicating enormous scope for economic production of this enzyme.

Media optimization studies in SSF could be accurately conducted only by using substrates which are nutritionally inert than with conventional substrates which contain native carbon and nitrogen sources besides other growth factors. It is difficult to optimize the exact nutritional requirements of the organism in an SSF system involving nutrient-rich substrates, because one cannot be sure about the increase/decrease in microbial growth and product formation resulted due to the added nutrient. The fermentation media must not

only meet the nutritional requirements of the microorganisms but also should be economical for an industrial process since factors such as its cost, efficiency of utilization and its effect on downstream processing are all important factors in the design of a fermentation medium (Stanbury, 1987). In this context the nutritionally inert polystyrene beads, as solid supports, has facilitated the design of an economic medium for enhanced product yield with minimal presence of undesired proteins.

4.3. Recovery of L-glutaminase from the polystyrene SSF system

An efficient extraction technique is very much essential for the recovery of products from fermented solids, in order to achieve effective commercial exploitation of the SSF processes. The optimization of extraction parameters holds paramount importance in obtaining the maximal recovery of the products from SSF and also to maintain the advantages of SSF over the SmF which include the presence of product in higher concentration in the fermented medium and the consequent reduced expenditure on downstream processing (Ghildyal *et al*, 1991; Lonsane & Krishnaiah, 1994). The volume of waste water generated is lower in SSF thus avoiding the need for intensive and economically unproductive waste treatment processes. Hence techniques of extraction of the fermented solids have received greater attention in recent years for achieving highly concentrated extracts and several methods such as percolation technique, multiple-contact

counter-current extraction, supercritical fluid extraction, repeated extraction, hydraulic pressing and pulsed plug flow column technique have been tried for product recovery from SSF using wheat bran (Ghildyal *et al*, 1991; Roussos *et al*, 1991 b; Lonsane & Krishnaiah, 1994).

At the end of fermentation, the solid substrates contain, besides the desired product, microbial cells, fermented solid substrate particles and all other concomitantly produced metabolites. The extraction process in SSF system involves the leaching of the product in a suitable solvent. The extraction efficiency is greatly affected by the properties of most natural substrates such as wheat bran which absorbs twice its dry weight of solvent (Ghildyal *et al*, 1991). Another problem encountered during extraction is the highly viscous nature of the extract which creates complications in further downstream processing and there is a need for research and development in the formulation of improved methods for reducing the viscosity of the extracts (Lonsane & Ghildyal, 1992; Lonsane & Krishnaiah, 1994).

In this context, use of synthetic, nutritionally inert supports such as polystyrene eliminates all these problems associated with product recovery, since the water absorbency of the polystyrene beads is very low (2 gm/cm^3; Brydson, 1982), and consequently very little solvent will remain absorbed to the solids. This facilitates 92 - 96 % recovery of the leachate compared to 82 - 85 % recovery

experienced with conventional SSF using natural substrates (Nagendra Prabhu & Chandrasekaran, 1995, 1996 b).

Results of the present study indicated that a solid to extractant ratio of 1 4 (w/v) is ideal for the recovery of leachate having maximal glutaminase activity, from the polystyrene system. Eventhough a slightly higher recovery was obtained at 1 5 ratio, the increase in enzyme yield over the 1 4 ratio was only 3 %. At a ratio of 1 10, the leachate was too dilute which is uneconomical from an industrial point of view.

The leachate from the polystyrene system was not only less viscous (mean viscosity 0.966 Ns/m^2) but also showed high specific activity for glutaminase. Further, the leachate was free from undesired proteins unlike that from the wheat bran or other nutritionally rich substrates which were highly viscous and contained amylase and cellulase, besides glutaminase (Nagendra Prabhu & Chandrasekaran, 1995, 1996 b). These features are highly beneficial and desirable in the enzyme industry, as they eliminate the problems caused by the viscous nature of the leachate from natural substrates (Ramesh & Lonsane, 1989).

4.4. Biomass estimation in SSF

The difficulty in estimating the microbial growth (biomass) is one of the major inherent drawbacks associated with conventional SSF processes which are carried out using nutritionally rich natural substrates (Aidoo *et al*, 1982; Zhu *et al*, 1994). In submerged fermentation, the biomass could easily be estimated by a variety of methods, after physical separation of the cells from the liquid phase of the culture. Whereas in SSF, with natural solid substrates such as wheat bran, direct measurement of biomass is impossible, since the microorganisms are intimately bound to the solid matrix and cannot be quantitatively separated from the solid medium (Durrand *et al*, 1988; Desgranges *et al*, 1991).

Though a number of methods have been developed, such as measurement of cell constituents like chitin (Aidoo *et al*, 1981), ergosterol (Seitz *et al*, 1979), nucleic acids (Bajracharya & Mudgett, 1980; Koliander *et al*, 1984), protein by Kjeldhal method; biological activity such as ATP (West *et al*, 1986), enzyme activity (Barak & Chet, 1986), respiration rate (Sakurai *et al*, 1985), immunological activity (Frankland & Bailey, 1981), and nutrient consumption (Matcham *et al*, 1984), their wider applications are limited due to a number of factors such as chemical composition of the substrate, degree of interference from the substrate and the desired degree of sensitivity. Most of the techniques are standardized with respect to one particular

substrate and hence subject to variation with other solid substrates. In most cases, the nature of the substrate dictates the choice of the method and other practical considerations like rapidity of the assay and availability of the instruments (Lekha & Lonsane, 1994). According to Mitchell (1992), these indirect methods developed for biomass estimation are of questionable reliability.

Auria *et al*, (1990) measured directly the growth of *Aspergillus niger* based on biomass dry weight, during SSF using an ion exchange resin, Amberlite IRA 900, as an inert support. Similarly a novel SSF system using polyurethane foam as inert carrier facilitated the direct estimation of biomass of fungi (Zhu *et al*, 1994).

Results of the present study clearly indicates the feasibility of polystyrene system with respect to the estimation of bacterial biomass by measuring the dry weight and/or cell protein. The polystyrene beads are nutritionally inert and does not contain any nutrients of its own and provides only surface for attachment of the bacteria during fermentation. Further all the nutritional components added in the medium are soluble and can be washed out. With the feasibility of direct biomass estimation, in this inert support system of SSF, more direct and close monitoring and control of the physiological development of the bacteria such as the growth and product formation becomes possible. The polystyrene system could be used to analyze the relationship between biomass and related

parameters and derive appropriate models to understand the similar situations of SSF on wheat bran or other substrates such as that observed with polyurethane foam (Zhu *et al*, 1994). Results of the present study clearly and strongly advocates the use of nutritionally inert solid supports for SSF studies by virtue of their suitability for easy separation of biomass from the fermentation medium and its direct estimation

4.5. L-Glutaminase production under SSF using mixed substrates

Use of mixed substrates, derived from different nutritionally rich natural sources, as solid substrates for fermentation production of enzymes have yielded varying results as documented under review of literature (section 1.2.2.4). To quote a few while enhanced yield of glucoamylase by *A. niger* was obtained using wheat bran and corn flour at a ratio of 9 1 (Pandey & Radhakrishnan, 1993), protease production by *Rhizopus oligosporus* decreased when wheat bran was mixed at various combinations with wheat flour, soy flour, soy oil, rice bran etc. (Ikasari & Mitchell, 1994). In contrast to these studies, when a mixed substrate system was prepared using nutritionally inert and rich substrates in the present study, varying degrees of glutaminase yield was recorded. Mixtures containing high proportions of polystyrene and low amounts of wheat bran/rice husk supported increase in glutaminase yield compared to mixtures having more amount of wheat bran/rice husk than polystyrene.

Unlike the earlier reports, mentioned above, the inert support was observed to favour yield of glutaminase, only at similar levels, under mixed substrate systems having low proportions of wheat bran/rice husk compared to the yield obtained when used individually. It was also observed that both wheat bran and rice husk could record significant levels of enzyme yield when used individually compared to that obtained in the mixed condition. In fact, as such, the results of the present study advocates use of inert supports alone than as mixed substrates in combination with natural substrates, when viscosity and enzyme profile of the leachate is considered important. Nevertheless, since the enzyme yield is at comparable levels with the individual substrates, the mixed substrates, which attribute nutritionally rich solid substrate media, may be preferred for glutaminase production. Further, the mixed substrate studies using other natural substrates in combination with inert supports could result in the development of a more efficient SSF system which combines the beneficial aspects of both the conventional as well as SSF systems using inert supports.

4.6. Impact of mutation on L-glutaminase production

Mutation of *Torulopsis famata*, with NTG treatment resulted in a 3 fold increase in glutaminase yield (Kakinuma *et al*, 1987). Spontaneous mutation of Streptomycin sensitive strains of *E. coli* led to a 60 % decrease in L-glutaminase and L-asparaginase activities

(Mugnetsyan & Stepanayen, 1987). Whereas in the present investigation, NTG treatment and UV irradiation of *V. costicola* yielded only 48 % and 24 % increase over the control respectively. The results indicate scope for further improvements in L-glutaminase yield through genetic manipulation. Since no reports are available on similar studies with any marine microorganism, no detailed discussion or comparison could be made.

4.7. Purification and characterization of L-glutaminase

An overall yield of 42 % was obtained after 100 fold purification of glutaminase-asparaginase from *Achromobacteraceae* (Roberts *et al*, 1972), while a 6000 fold purified glutaminase with 40% yield was obtained from *E. coli* (Prusiner *et al*, 1976). Glutaminase from *Pseudomonas sp.* was purified with an yield of 40–50 % (Roberts, 1976), whereas the enzyme from *P. acidovorans* was purified 2205 fold with 19 % yield. 4 % and 0.04 % yield of 1620 and 190 fold purified glutaminase I and II from marine *Micrococcus luteus* was reported (Moriguchi *et al*, 1994). Both intra and extra cellular glutaminase from *A. oryzae* with 6.2 % and 3.2 % recovery and 1100 and 730 fold purity was also reported (Yano *et al*, 1988). In the present study, enzyme with high specific activity (597 U/mg protein) could be recovered with 43.68 fold purification. The final yield after gel-filtration chromatography was 30.62 %. Electrophoretic studies revealed a single band upon polyacrylamide gel electrophoresis for

both SDS-PAGE and native PAGE. The subunit molecular weight was in the range of 36,000 to 40,000 daltons. The native protein had an approximate size of 140,000 to 150,000 daltons.

Glutaminases isolated from various microorganisms were reported to be active and stable over different ranges of pH. The optimum pH of *Pseudomonas* was 7.0 and it was active over a range of 5 - 9 (Roberts, 1976). The glutaminase-asparaginase from *P. acidovorans* exhibited activity over a broad range of pH. Although the pH optimum was 9.5, the enzyme retained 70 % activity at pH 7.4 (Davidson *et al*, 1977). Glutaminase from *P. fluorescence* exhibited an optimum pH range of 7.5 - 9.5 (Yokotsuka *et al*, 1987) whereas the *Cryptococcus albidus* glutaminase exhibited activity over a wide range of pH (5.5 - 8.5). The extra and intra-cellular glutaminase from *A. oryzae* recorded optimal activity and stability at pH 9.0, while *A. sojae* produced glutaminase which showed a pH optima between 7.5 - 8.5 (Yano *et al*, 1988 Yokotsuka *et al*, 1987). In a similar fashion, the purified enzyme from *V. costicola* recorded activity and stability over the pH range of 6 - 9, with an optimum at pH 8.0.

The studies on the temperature tolerance of the purified glutaminase show that the enzyme has an optimum temperature of 40^{o}C for its activity and retained 85 % activity at 45^{o}C. The enzyme was most stable at 40^{o}C and retained 95 % of the activity at 45^{o}C. The presence of substrate (L-glutamine) at 0.01 M level and NaCl at 10 %

level enhanced the thermostability of the enzyme upto 50^{o}C (95 - 98 % activity) and enabled the enzyme to retain about 80 % activity at 60^{o}C. Whereas the absence of L-glutamine or NaCl, led to a reduction in stability at 60^{o}C.

The thermal stability for both the glutaminase and asparaginase activity was identical, with both being 50 % inactivated after 10 min at 50^{o}C. But, the enzyme was significantly protected from thermal inactivation by the presence of substrate (0.01 M L-glutamine or 0.01 M L-asparagine); the temperature for 50 % inactivation was 79^{o}C (Davidson *et al*, 1977). The optimum temperature for both intra and extracellular glutaminase from *Aspergillus oryzae* was 45^{o}C and they were stable upto 37^{o}C but almost completely lost their activities at 55^{o}C (Yano *et al*, 1988). Whereas the optimum temperature for glutaminase I and II from marine *Micrococcus* was 50^{o}C (Moriguchi *et al*, 1994).

The purified enzyme demonstrated typical Michaelis-Menten kinetics at low substrate concentration. Maximal activity was obtained with 0.04 M L-glutamine and further increase in substrate concentration did not have any change in the enzyme activity. Glutaminase from *P. acidovorans* recorded a relatively low Km value of 2.2 X 10^{-5} M (Davidson *et al*, 1977). The intra and extracellular glutaminase from *A. oryzae* also had low Km values of 9.1 X 10^{-5} M and 9.6 X 10^{-5} M respectively (Yano *et al*, 1988). Whereas the glutaminase

I and II from marine *Micrococcus* had a relatively high Km value of 4.4 and 6.5 mM respectively compared to others mentioned above (Moriguchi *et al*, 1994). In the present study also the enzyme recorded a Km value of 7.4 X 10^{-2} M which is slightly higher than that reported for marine *Micrococcus luteus.*

In the present study, the enzyme from marine *V. costicola*, was found to retain 100 % activity over a wide range of NaCl concentration (0 - 6 %) and 75 % activity upto 15 % NaCl concentration, compared to the glutaminase I from marine *Micrococcus* which recorded 100 % activity at 10 - 16 % NaCl. Whereas the Glutaminase II from the *Micrococcus* was less stable (Moriguchi *et al*, 1994).

The enzyme from *Micrococcus*, *A. oryzae*, & *P. acidovorans* also showed high specificity towards L-glutamine (Davidson *et al*, 1977; Yano *et al*, 1988; Moriguchi *et al*, 1994). The enzyme from *A. oryzae* also catalyzed the hydrolysis of DL-Theanine, Glutathione and L-γ-Glytamyl-*p*-nitoanilide (Yano *et* al, 1988) and that from *P. acidovorans* exhibited affinity towards L-asparagine, L-aspartic acid-β-hydroxate and mixtures of L-asparagine + D-asparagine and L-asparagine + L-glutamine (Davidson *et al*, 1988). Whereas the enzyme obtained in the present study recorded high substrate specificity towards L-glutamine and 60 % specificity towards a mixture of L-glutamine and L-asparagine.

4.8. CONCLUSIONS

Based on the results obtained in the present study, it is concluded that marine *Vibrio costicola*, a gram negative bacterium, has immense potential as an industrial organism for large scale production of L-glutaminase which has applications in both food and pharmaceutical industries. Perhaps it is the first time that a marine bacterium has been recognized to have potential for industrial use employing the highly advantageous solid state fermentation process.

The bacteria could easily grow by adsorbing on to the solid particles and produce significant levels of L-glutaminase under solid state fermentation using natural substrates such as wheat bran, rice husk, copra cake powder, groundnut cake powder and saw dust as well as the nutritionally inert polystyrene. The present study proves that even though wheat bran is the most widely used solid substrate for exoenzyme production under SSF, other less common substrates such as rice husk, copra cake powder, groundnut cake powder and saw dust could also be used as solid supports for SSF.

A major outcome of the present investigation is the development of a new and ideal bioprocess for the production of L-glutaminase, using the polystyrene beads. The new system has facilitated the design of a simple aged sea water medium supplemented with L-glutamine, maltose and KH_2PO_4 for enhanced product yield with

minimal presence of undesired proteins. Leachate with low viscosity and high specific activity for the target product could be recovered from this system. 92 - 96 % of the leachate could be recovered from the inert support SSF system which is impossible with conventional SSF systems using natural substrates. A new protocol was also developed for the rapid and direct estimation of biomass during SSF using polystyrene. This may have tremendous application in the scaling-up studies and development of an ideal SSF system using both nutritionally rich and inert substrates.

Almost no information is available on the response of marine microorganisms to spontaneous and induced mutations. Results of the present study suggest that marine bacteria respond positively to induced mutations with respect to enzyme synthesis. Of course, detailed investigations are needed in this direction towards strain improvement for enhanced product yield.

The enzyme produced by the organism also has several beneficial properties needed for an industrial enzyme. It shows activity and stability over a wide range of pH and temperature and is not inactivated in presence of high NaCl concentrations. The substrate specificity towards L-glutamine is also very high which means that it could be used in low amounts to achieve the desired effect.

In order to sustain interest in SSF processes and to utilize its manifold advantages over the conventional SmF, the inherent problems of SSF should be overcome. Our results clearly suggest that SSF using nutritionally inert solid support materials could lead to further improvement of this already advantageous process. However, scale-up studies are needed for the development of an economic bioprocess for commercial exploitation. Further, this study conclusively proves that marine microorganisms could be exploited through solid state fermentation, for obtaining useful products such as industrial enzymes, organic acids, antibiotics and other secondary metabolites as experienced with their terrestrial counterparts. Economic exploitation of these vast untapped resources, employing solid state fermentation will yield many products beneficial for mankind and warrants serious and immediate attention.

SUMMARY

5.1. The marine *Vibrio costicola*, used in the present study, produced extracellular L-glutaminase under solid state fermentation using wheat bran, rice husk, copra cake powder, groundnut cake powder and saw dust as solid supports.

5.2. The optimum initial moisture content for maximal glutaminase yield was 60 % for wheat bran and rice husk, 70 % for copra cake powder and saw dust and 40 % for groundnut cake powder.

5.3. The optimum pH for enzyme production by the bacteria was 7.0, irrespective of the substrates tested.

5.4. Maximal glutaminase yield was obtained with substrates of particle size 0.6 - 1.0 mm for wheat bran and rice husk, and 1.0 - 1.4 mm for the others.

5.5. The organism preferred an incubation temperature of 35^{o}C for maximal glutaminase yield irrespective of the type of solid substrates used for SSF.

5.6. L-Glutamine concentration of 2 % (w/w) was needed for maximal glutaminase yield by the bacteria, with all the solid substrates.

5.7. The bacteria produced maximum enzyme titres after 24 h incubation time with wheat bran and rice husk and 36 h with copra cake powder, groundnut cake powder and saw dust.

5.8. An inoculum concentration of 0.750 mg dry wt/10 gram dry solid was found to be optimum for maximal enzyme production by the bacteria.

5.9. Addition of carbon sources at 1 % (w/w) level did not enhance glutaminase yield with the natural substrates except saw dust.

5.10. Addition of nitrogen sources including amino acids at 1 % (w/w) level led to a slight decline in glutaminase yield with all the natural substrates used.

5.11. Supplementation of mineral salts also at 1 % (w/w) level in the SSF medium resulted in enhancement of glutaminase yield by the bacterium with all the solid substrates.

5.12. The organism required 3 % (w/w) NaCl for maximal glutaminase yield, but produced significant levels of the enzyme even in the absence of NaCl.

5.13. Maximal titres of the enzyme could be obtained when wheat bran (268 U/gds) and rice husk (239 U/gds) were used as the solid substrates followed by copra cake powder (217 U/gds), groundnut cake powder (205 U/gds) and saw dust (196 U/gds) respectively.

5.14. *V. costicola* could also grow well and produce L-glutaminase under SSF using polystyrene as inert support. The organism adsorbed on to the polystyrene beads and utilized the nutrients supplied in the medium.

5.15. Aged sea water, supplemented with 1 % (w/w) glutamine supported maximal glutaminase production before optimization of the nutritional requirements.

5.16. Maximal glutaminase yield was obtained at a solid substrate to media ratio of 1 1 (w/v).

5.17. The optimum pH, temperature, L-glutamine concentration and inoculum concentration needed for maximal glutaminase production were similar to that obtained with natural substrates, ie., pH 7.0, 35^{O}C, 2 % (w/w) and 0.750 mg dry wt/10 gds, respectively.

5.18. The optimal incubation time required for maximal glutaminase production by the bacteria was 24 h with the polystyrene SSF system.

5.19. Addition of carbon sources and mineral salts at 1 % (w/w) level resulted in enhancement of glutaminase yield by the bacteria. Maximum increase was observed with maltose and KH_2PO_4.

5.20. Incorporation of nitrogen sources including amino acids at 1 % (w/w) level resulted in decline in glutaminase yield.

5.21. The enzyme could be recovered using a solid extractant ratio of 1 4 (w/v).

5.22. Direct estimation of biomass, in terms of cell protein and dry weight was achieved during SSF with polystyrene.

5.23. Use of mixed solid substrate system (Natural + Inert) did not have any major influence on L-glutaminase production by the bacterium under SSF.

5.24. Mutation of *V. costicola* using UV-irradiation and NTG treatment resulted in 24 % and 48 % increase in glutaminase yield respectively.

5.25. The enzyme could be purified to homogeneity with 30.62 % yield and 43.68 fold purification.

5.26. The subunit molecular weight of the enzyme was in the range of 36,000 - 40,000 daltons and that of the native protein was in between 140,000 - 150,000 daltons.

5.27. Optimum pH required for maximum activity and stability of the purified enzyme was 8.0.

5.28. The optimum temperature needed for enzyme activity and stability was $40^{o}C$.

5.29. L-Glutamine concentration of 0.04 M was the optimum substrate concentration needed by the enzyme for its maximal activity. The enzyme recorded a Km value of 7.4×10^{-2} M.

5.30. The enzyme retained 100 % and 75 % activity upto 6 % and 15 % NaCl concentrations respectively.

5.31. Presence of L-glutamine and NaCl resulted in increase in thermostability of the purified enzyme upto $50^{o}C$.

5.32. Purified enzyme showed high substrate specificity towards L-glutamine compared to L-asparagine, L-glutamic acid, L-aspartic acid and a mixture of L-glutamine and L-asparagine.

The results of the present study suggest that L-glutaminase, which is an industrially and therapeutically important enzyme, could be produced from marine *Vibrio costicola* under solid state fermentation using locally available natural solid substrates and the nutritionally inert polystyrene. The inert support SSF system offers several advantages over the conventional SSF systems using nutritionally rich substrates.

REFERENCES

1. Abell, C.W., & Uren, J.R. (1981). Enzymes. In: *Antitumour compounds of natural origin chemistry and biochemistry.* Aszalos, A (ed). CRC Press, Boca Raton, Florida pp. 123-153.

2. Aidoo, K.E., Hendry, R. & Wood, B.J.B. (1981). Estimation of fungal growth in a solid state fermentation system. *European Journal of Applied Microbiology and Biotechnology*, 12 6-9.

3. Aidoo, K.E., Hendry, R. & Wood, B.J.B. (1982). Solid state fermentations. *Advances in Applied Microbiology*, 28 201-237.

4. Ammon, H.L., Weber, I.T., Wlodawer, A., Harrison, R.W., Gilliland. G.L., Murphy, K.C., Sjolin, L. & Roberts, J. (1988). Preliminary crystal structure of *Acinetobacter glutaminasificans* glutaminase-asparaginase. *Journal of Biological Chemistry*, 263 150-156.

5. Arima, K., Sakamoto. T., Araki, C. & Tamura. C. (1972). Production of extracellular L-asparaginase by microorganisms. *Agricultural Biological Chemistry*, 36 356-361.

6. Auria, R., Hernandez, S., Raimbault, M. & Revah, S. (1990). Ion exchange resin, A model support for solid state fermentation of *Aspergillus niger. Biotechnology Techniques*, 4 391-396.

7. Austin, B. (1988). Marine Microbiology, Cambridge University Press, Cambridge. p 222.

8. Bajracharya, R. & Mudgett, R.E. (1980). *Biotechnology and Bioengineering*, 22 2219-2235.*

9. Barak, R. & Chet, I. (1986). Determination of flourescein dye staining of fungal viability during mycoparasitism. *Soil Biology and Biochemistry*, 18 315-319.

10. Bauer, K., Bierling, R. & Kaufman, W. (1971). *Naturwissenschaflen*, 58 526.*

11. Bharat Bhushan., Dosanjh, N.S., Kumar, K. & Hoondal, G.S. (1994). Lipase production from an alkalophilic yeast sp. by solid state fermentation. *Biotechnology Letters*, 16 841-842.

12. Bhotmange, M.G. & Shastri, P.N. (1994). SSF in relation to food enzymes. In*: Solid State Fermentation.* Pandey, A (ed) Wiley Eastern Ltd, New Delhi pp. 105-113.

13. Broome, J.D. (1961). Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. *Nature*, 191 1114-1115.

14. Brydson, J.A. (1982). Plastics based on styrene. In: *Plastics materials.* Butterworth Scientific, London pp. 386-422.

15. Cannel, E. & Moo-Young, M. (1980). Solid state fermentation systems. *Process Biochemistry*, June/July 2-7.

16. Chandrasekaran, M., Lakshmanapermalsamy, P. & Chandramohan, D. (1991). Combined effect of environmental factors on spoilage bacteria. *Fishery Technology* (India), 28 146-153.

17. Chandrasekaran, M. (1994). Economic utilization of marine microorganisms employing solid state fermentation. In: *Solid State Fermentation.* Pandey, A (ed) Wiley Eastern Ltd, New Delhi pp. 168-172.

18. Chandrasekaran, M. (1996). Harnessing marine microorganisms through solid state fermentation. *Journal of Scientific and Industrial Research*, 55 (5 & 6) 468-471.

19. Chou, C.C., Yu, R.C. & Tsai, C.T. (1993). Production of glutaminase by *Actinomucor elegans, A. taiwanensis & Aspergillus oryzae. Zhongguo Nong ye Hauxue Huizhi*, 31 (1) 78-86.*

20. Cook, W.R., Hoffman, J.H. & Bernlohr, R.W. (1981). Occurrence of an inducible glutaminase in *Bacillus licheniformis. Journal of Bacteriology*, 148 365-367.

21. Davidson, L., Russel Brear, D., Wingrad, P., Hawkins, J. & Kitto, B.G. (1977). Purification and properties of an L-glutaminase-L-asparaginase from *Pseudomonas acidovorans. Journal of Bacteriology*, 129 1379-1386.

22. Delest, P. (1995). Paper presented in Bioflavour '95 - Biocatalysts for flavour production. Dijon, France 14-17 February.

23. Desgranges, C., Vergoignan, C., Georges, M. & Durand, A. (1991). Biomass estimation in solid state fermentation I & II. *Applied Microbiology and Biotechnology*, 35 200-209.

24. Distastio, J.A., Niederman, R.A., Kaflewitz, D. & Goodman, D. (1976). *Journal of Biological Chemistry*, 251 6929.*

25. Durand, A., Broise, D de la., Blachere, H. (1988). Laboratory scale bioreactors for solid state process. *Journal of Biotechnology*, 8 59-62.

26. Ehrenfeld, E., Marble, S.J. & Meister, A. (1963). *Journal of Biological Chemistry*, 238 3711.*

27. Faulkner, D.J. (1986). Marine natural products. *Natural Products Reports*, 3 1-33.

28. Fletcher, M. (1976). The effects of proteins on bacterial attachment to polystyrene. *Journal of General Microbiology*, 94 400-404.

29. Fletcher, M. (1980). Adherence of marine microorganisms to smooth surfaces. In: *Receptors and recognition*, Series B, Vol. 6. Beachey. E.H (ed) Chapman & Hall pp. 354-374.

30. Fogarty, W.M. & Kelly, C.T. (1990). Microbial Enzymes and Biotechnology. Elsevier Science Publishers. London.

31. Frankland, J.C. & Bailey, A.D. (1981). Development of an immunological technique for estimating mycelial biomass of *Mycorhyzopus* in leaf litter. *Soil Biology and Biochemistry*, 13 87-92.

32. Fujishima, T., Uchida, K. & Yoshino, H. (1972). Enzyme production by molds in sponge culture. *Journal of Fermentation Technology*, 50 724-730.

33. Fukushima, Y. & Motai, H. (1990). Continuous conversion of glutamine to glutamate by immobilized glutaminase-producing yeast. *Journal of Fermentation and Bioengineering*, 69 189-191.

34. Furuya, T., Ishige, M., Uchida, K. & Yoshino, H. (1983). *Journal of Agricultural Chemical Society of Japan*, 57 1.*

35. Furuya, T., Noguchi, K., Miyagushi, K. & Uchida, K. (1985). *Nippon Nogeikagaku Kaishi*, 59 605.*

36. Ghildyal, N.P. (1991). Industrial scale methodology for solid state fermentation. *Manual for Short Term course on SSF* - conducted by CFTRI, Mysore, pp. 4.1-4.7.

37. Ghildyal, N.P., Ramakrishna, M., Lonsane, B.K. & Karanth, N.G. (1991). Efficient and simple extraction of mouldy bran in a pulsed column extractor for recovery of amyloglucosidase in concentrated form. *Process Biochemistry*, 26 235-241.

38. Greenstein, J.P. & Winitz, M. (1961) In: *Chemistry of amino acids*, Vol. 12, John Wiley & Sons Inc. New York. pp. 1929.

39. Hacking, A.J. (1986). Economic aspects of Biotechnology. Cambridge University Press, Cambridge.

40. Halevy, S. (1990). Drugs from the sea. In: *Microbial Applications in Food Biotechnology.* Nga. B.H & Lee Y.K (eds). Elsevier Applied Science pp. 123-134.

41. Hames, B.D. (1990). One dimensional polyacrylamide gel electrophoresis. In *Gel electrophoresis A Practical Approach* Hames. B.D & Rickwood. D (eds), IRL Press, Oxford. pp. 1-147.

42. Harayama, F. & Yusuhira, H. (1991). Effect of glutaminase in rice koji and from *B. subtilis* Gt strain on miso fermentation. *Journal of Brewing Society of Japan*, 86 529-535.

43. Hartman, S.C. (1968). Glutaminase of *Escherichia coli.* Purification and general catalytic properties. *Journal of Biological Chemistry*, 243 853-863.

44. Herbert, D., Phipps, P.J. & Strange, R.E. (1971). Chemical analysis of microbial cells. *Methods in Microbiology*, 5 B 209-344.

45. Hermanson, M. & Marshall, K.C. (1985). Utilization of surface localized substrate by non-adhesive marine bacteria. *Microbial Ecology*, 11 91-105.

46. Hesseltine, C.W. (1972). Solid state fermentation. *Biotechnology and Bioengineering*, 14 517-532.

47. Holcenberg, J.S., Teller, D.C., Roberts, J. & Dolowy, W.C. (1972). Physical properties of *Acinetobacter* glutaminase - asparaginase with antitumour activity. *Journal of Biological Chemistry*, 245 3708-3715.

48. Holcenberg, T.S., Roberts, J. & Dolowy, W.C. (1973). In: *Enzymes of glutamine metabolism.* Prusiner. S & Stadtman. E.R (eds). Academic Press, Ney York. pp. 277.

49. Holcenberg, J.S., Teller, D.C. & Roberts, J. (1976). *Journal of Biological Chemistry*, 251 5375.*

50. Holcenberg, J.S. & Kien, C.L. (1985). The effects of protein or amino acid intake on the nitrogen balance and antitumour activity of glutaminase treatment. *Current Topics in Cell Regulation*, 26 395-402.

51. Hughes, D.E. & Williamson, D.H. (1952). Some properties of glutaminase of *Clostridium welchii*. *Biochemical Journal*, 51 45-55.

52. Ikasari, L. & Mitchell, D.A. (1994). Protease production by *Rhizopus oligosporus* in solid state fermentation. *World Journal of Microbiology and Biotechnology*, 10 320-324.

53. Imada, A., Igarasi, S., Nakahama, K. & Isono, M. (1973). Asparaginase and glutaminase activities of microorganisms. *Journal of General Microbiology*, 76 85-99.

54. Iwaasa, T., Fujii, M. & Yokotsuka, T. (1987). On the glutaminase produced by *Cryptococcus albidus* ACTT 20293. *Journal of Japanese Soysauce Research Institute*, 13 205-210.

55. Jayaraman, K. & Jayaraman, R. (1979). Laboratory Manual in Molecular Genetics. Wiley Eastern Ltd, New Delhi.

56. Jwanny, E.W., Rashed, M.M. & Abdu, H.M. (1995). Solid state fermentation of agricultural wastes into food through pleurotus cultivation. *Applied Biochemistry and Biotechnology*, 50 71-78.

57. Kakinuma, T., Fujii, S., Iwaasa, T. & Yokotsuka, T. (1987). *Journal of Japanese Soysauce Research Institute*, 13 116.*

58. Katzumata, H., Katzumata, R., Abe, T., Takenaka, O .& Inada, Y. (1972). *Biochemia et Biophysica Acta*, 289 405.*

59. Kien, C.L., Anderson, A.J. & Holcenberg, J.S. (1985). Tissue nitrogen-sparing effect of high protein diet in mice with or without ascites tumour treated with *Acinetobacter* glutaminase-asparaginase. *Cancer Research*, 45 (10) 4876-4882.

60. Kikuchi, M., Hayashida, H., Nakano, E. & Sugimori, T. (1969). *Journal of Fermentation Technology*, 47 693.*

61. Kim, J.H., Hosobuchi, M., Seki, T. & Ryu, D.D.Y. (1985). Cellulase production by a solid state culture system. *Biotechnology and Bioengineering*, 22 1445-1450.

62. Knapp, J.S. & Howell, J.A. (1980). Solid substrate fermentation. In: *Topics in Enzyme and Fermentation Technology*. Wiseman. A (ed). Ellis Horwood Ltd. Chicester. pp. 85-143.

63. Kobayashi, T., Ozawa, S., Sato, K., Nagamune, T. & Endo, I. (1991). Production of glucoamylase by solid state fermentation using urethane foam carrier as a semi-solid medium. *Kagaku Kogaku Ronbunshi* (Japan), 17 (3) 491-496.*

64. Koliander, B., Hampel, W. & Roehr, M. (1984). Indirect estimation of biomass by rapid ribonucleic acid determination. *Applied Microbiology and Biotechnology*, 19 272-276.

65. Kozlov, E.A., Kovalenko, N.A. & Mardashev, S.R. (1972). *Biochimiya*, 37 56.*

66. Krishna, C. & Chandrasekaran, M. (1995). Economic utilization of cabbage wastes through solid state fermentation by native flora. *Journal of Food Science and Technology*, 32 (3) 199-201.

67. Krishna, C. & Chandrasekaran, M. (1996). Banana waste as substrate for α-amylase production by *Bacillus subtilis* (CBTK 106) under solid state fermentation. *Applied Microbiology and Biotechnology*, (in press).

68. Kumagai, H., Kashima, N., Torii, H., Yamada, H., Enei, H. & Okumura, S. (1972). *Agricultural Biological Chemistry*, 36 472.*

69. Kunitz, M. (1946). Crystalline soybean trypsin inhibitor-II. *Journal of General Physiology*, 30 291-310.

70. Kuroshima, E., Oyama, Y., Matsuo, T. & Sugimori,Y. (1969). *Journal of Fermentation Technology*, 47 693.*

71. Lekha, P.K. & Lonsane, B.K. (1994). Biomass estimation on solid state fermentation. In: *Solid State Fermentation*. Pandey A (ed). Wiley Eastern, New Delhi. pp. 38-43.

72. Lonsane, B.K., Ghildyal, N. P., Budiatman, S. & Ramakrishna, S.V. (1985). Engineering aspects of solid state fermentation. *Enzyme Microbial Technology*, 7 258-265.

73. Lonsane, B.K. & Ramakrishna, M. (1989). Microbial enzymes in food processing industry Present status and future prospects in India. *Indian Food Industry*, 8 15-31.

74. Lonsane, B.K. & Ramesh, M.V. (1990). Production of bacterial thermostable alpha amylase by solid state fermentation: A potential tool for achieving economy in enzyme production and starch hydrolysis. *Advances in Applied Microbiology*, 35 1-56.

75. Lonsane, B.K. & Ghildyal, N. P. (1992). Exoenzymes. In: *Solid substrate cultivation.* Doelle. H.W, Mitchell. D.A & Rolz. C (eds). Elsevier Science Ltd. London. pp. 191-209.

76. Lonsane, B.K., Castaneda, G.S., Raimbault, M., Roussos, S., Gonzalez, G.V., Ghildyal, N.P., Ramakrishna, M. & Krishnaiah, M. M. (1992). Scale-up strategies for solid state fermentation systems. *Process Biochemistry*, 27 259-273.

77. Lonsane, B.K. (1994). Resurgence of interest in solid state fermentation Reasons and justifications. In: *Solid State Fermentation.* Pandey. A (ed). Wiley Eastern Ltd. New Delhi. pp. 11-20.

78. Lonsane, B.K. & Krishnaiah, M. M. (1994). Leaching of product from fermented solids Methodology appraisal. In: *Solid State Fermentation.* Pandey. A (ed). Wiley Eastern Ltd. New Delhi. pp. 173-179.

79. Lowry, O.H., Rosebrough, N.N., Farr, A.L. & Randall, R. Y. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193 265-275.

80. Lubkowski, J., Wlodawer, A., Ammon, H.L., Copeland, I.D. & Swain, A.L. (1994). Structural characterization of *Pseudomonas* 7 A glutaminase-asparaginase. *Biochemistry*, 33 10257-10265.

81. Luh, B. S. (1995). Industrial production of soy sauce. *Journal of Industrial Microbiology*, 14 (6) 467-471.

82. Madamwar, D., Patel, S. & Parikh, H. (1989). Fermentation for cellulose and β - glucosidase production by *Aspergillus niger*. *Journal of Fermentation and Bioengineering*, 47 424-426.

83. Mandels, M., Medeiros, J.E., Andreotti, R. & Bisset, F.H. (1981). Enzymatic hydrolysis of cellulose Evaluation of cellulase culture filtrates under use conditions. *Biotechnology and Bioengineering*, 23 2009-2026.

84. Marshall, K.C., Stout, R. & Mitchell, R. (1971). Mechanism of initial events in the sorption of marine bacteria to surfaces. *Journal of General Microbiology*, 68 337-348.

85. Matcham, S.E., Jordan, B.R. & Wood, D.A. (1984). Methods for assessment of fungal growth on solid substrates. *Society for Applied Biotechnology Series*, 19 5-18.

86. Mardashev, S.R., Eremenko, V. V. & Nikolaev, A. Y. (1970). *Microbiology*, 39 6.*

87. McGregor, W.G. & Roberts, J. (1989). Glutaminase enhances therapeutic effectiveness of glutamine antimetabolites against human and murine solid tumours *in vivo*. *Proceedings of Annual Meeting of American Association for Cancer Research*, 30 A 2302.

88. Medda, S. & Chandra, A.K. (1980). New strains of *Bacillus licheniformis* and *Bacillus coagulans* producing thermostable alpha amylase active at alkaline pH. *Journal of Applied Bacteriology*, 48 47-58.

89. Meister, A. (1955). In: Methods in Enzymology. Vol. 2. Mosback. K (ed). Academic Press. pp. 380.

90. Meyrath, J. (1966). Citric acid production. *Process Biochemistry*, 1 (4) 234-238.

91. Mitchell, D.A. (1992). Biomass determination in solid state cultivation. In: *Solid substrate cultivation.* Doelle. H.W, Mitchell. D.A, Rolz. C.E (eds) Elsevier Applied Science, London pp. 53-63.

92. Montedoro, G.F. (1995). Paper presented in Bioflavour '95. Biocatalysts for flavour production. Dijon, France, 14-17 February.

93. Moo-Young, M., Moreira, A.R. & Tangerdy, R.P. (1983). Principles of solid state fermentation. In *The Filamentous Fungi* Vol. 4 J.E. Smith, D.R.Berry & B. Kristiansen (eds) Edward Arnold, London pp. 117-144.

94. Moriguchi, M., Sakai, K., Tateyama, R., Furuta, Y. & Wakayama, M. (1994). Isolation and characterization of salt tolerant glutaminase from marine *Micrococcus luteus* K-3. *Journal of Fermentation and Bioengineering*, 77 621-625.

95. Mugnetsyan, S. N. & Stepanayen, K. P. (1987). *Biol. Zh. Arm.*, 40 325.*

96. Nagendra Prabhu, G. & Chandrasekaran, M. (1995). Polystyrene- an inert carrier for L-glutaminase production by marine *Vibrio costicola* under solid state fermentation. *World Journal of Microbiology and Biotechnology*, 11 683-684.

97. Nagendra Prabhu, G. & Chandrasekaran, M. (1996 a). L-Glutaminase production by marine *Vibrio costicola* under solid state fermentation using different substrates. *Journal of Marine Biotechnology*, (in press).

98. Nagendra Prabhu, G. & Chandrasekaran, M. (1996 b). Impact of process parameters on L-glutaminase production by marine *Vibrio costicola* under solid state fermentation using polystyrene as an inert support. *Process Biochemistry* (Communicated).

99. Nair, P.M. (1994). Biotechnology and high technology in food production, processing and preservation - industrial and export opportunities. *Indian Food Industry*, 13 18-24.

100. Nakadai, T. & Nasuno, S. (1989). Use of glutaminase for soy sauce made by koji or a preparation of protease from *Aspergillus oryzae*. *Journal of Fermentation and Bioengineering*, 67 158-162.

101. Nasuno, S. & Ohara, T. (1972). *Seasoning Science*, 19 41.*

102. Novak, E. K. & Phillips, A. W. (1974). *Journal of Bacteriology*, 117 593.*

103. O'Dwyer, P.J., Alonso, M.T. & Jones, B.L. (1984). Acivicin A new antagonist in clinical trials. *Journal of Clinical Oncology*, 2 1064-1071.

104. Ofuya, C.O. & Obilor, J.N. (1994). The effect of solid state fermentation on the toxic components of cassava peel. *Process Biochemistry*, 29 25-28.

105. Oriol, E., Raimbault, M., Roussos, S. & Gonzalez, B.V. (1988). Water and water activity in the solid state fermentation of cassava starch by *Aspergillus niger*. *Applied Microbiology and Biotechnology*, 27 498-503.

106. Oshima, M., Yamamoto, T. & Soda, K. (1976). Further characterization of glutaminase isozymes from *Pseudomonas aeruginosa*. *Agricultural Biological Chemistry*, 40 2251-2256.

107. Pal, S. & Maity, P. (1992 a). Antineoplastic activities of purified bacterial glutaminase on transplanted tumour system. *Indian Journal of Cancer Chemotherapy*, 13 73-76.

108. Pal, S. & Maity, P. (1992 b). Investigation on glutamine amidohydrolase (EC. 3.5.1.2) and glutamine aminotransferease (EC. 2.5.1.15) activity in liver and plasma of EAC-bearing mice following glutaminase therapy. *Cancer Letters*, 66 225-231.

109. Pal, S. & Maity, P. (1994). Antitumour efficacy of glutaminase-copper-ATP combination in mice bearing Ehrlich ascites carcinoma. *Anticancer Drugs*, 5 (1) 57-63.

110. Pandey, A. (1992). Recent process developments in solid state fermentation. *Process Biochemistry*, 27 109-117.

111. Pandey, A. & Radhakrishnan, S. (1993). The production of glucoamylase by *Aspergillus niger* NCIM 1245. *Process Biochemistry*, 28 305-309.

112. Pandey, A., Selvakumar, P. & Ashakumary, L. (1994). Glucoamylase production by *Aspergillus niger* on rice bran is improved by adding nitrogen sources. *World Journal of Microbiology and Biotechnology*, 10 348-349.

113. Pandey, A., Ashakumary, L., Selvakumar, P. & Vijayalakshmi, K.S. (1995). Effect of yeast extract on glucoamylase synthesis by *Aspergillus niger* in solid state fermentation. *Indian Journal of Microbiology*, 35 335-338.

114. Peredes-Lopez, O., Gonzalez, C.T. & Trejo, C.A.J. (1991). Influence of solid substrate fermentation on the chemical composition of chick pea. *Journal of Fermentation and Bioengineering* 71 58-62.

115. Peterson, R.G., Handshumacher, R.E. & Mitchell, M.S. (1974). Immunological responses to L-asparaginase. *Journal of Clinical Investigation*, 50 1080-1090.

116. Prusiner, S. (1973). In: *Enzymes of glutamine metabolism.* Academic Press, New York pp. 293.

117. Prusiner, S., Davis, J.N. & Stadtman, E.R. (1976). Regulation of glutaminase B in *E. coli. Journal of Biological Chemistry*, 251 3447-3456.

118. Raha, S. K., Roy, S.K., Dey, S. K. & Chakrabarty, S. L. (1990). Antitumour activity of L-asparaginase from *Cylindrocarpon obtusisporum* MB-10 and its effect on the immune system. *Biochemistry International*, 21 (6) 1001-1011.

119. Ramadan, M.E.A., El-asmar, F.A. & Greenberg, D.M. (1964). Purification and properties of glutaminase and asparaginase from a *Pseudomonad* I Purification & physical chemical properties. *Archives of Biochemistry and Biophysics*, 108 143-149.

120. Ramesh, M. V. & Lonsane, B. K. (1987). Solid state system for production of α-amylase by *Bacillus megaterium* 16 M. *Biotechnology Letters*, 9 (5) 323-328.

121. Ramesh, M. V. & Lonsane, B. K. (1989). Purification of thermostable alpha amylase produced by *Bacillus licheniformis* M 27 under solid state fermentation. *Process Biochemistry*, 24 176-178.

122. Ramesh, M. V. & Lonsane, B. K. (1990). Critical importance of moisture content of the medium in alpha amylase production by *Bacillus licheniformis* M 27 in a solid state fermentation system. *Applied Microbiology and Biotechnology*, 33 501-505.

123. Reitzer, L. J., Wice, B. M. & Kennel, D. (1979). Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *Journal of Biological Chemistry*, 254 2669-2676.

124. Renu, S. (1991). L-Glutaminase production by marine bacteria. Ph.D Thesis, Cochin University of Science & Technology, Cochin, India.

125. Renu, S. & Chandrasekaran, M.(1992 a). Extracellular L-glutaminase production by marine bacteria. *Biotechnology Letters*, 14 471-474.

126. Renu, S. & Chandrasekaran, M. (1992 b). Impact of operational parameters on glutaminase production by *Vibrio costicola* in solid state fermentation. Proceedings of the 8th Carbohydrate Conference, Trivandrum, India, Nov. 18-20. pp. 169-172.

127. Roberts, J., Holcenberg, J.S. & Dolowy, W.C. (1970). Antineoplastic activity of highly purified bacterial glutaminase. *Nature*, 227 1136-1137.

128. Roberts, J., Holcenberg, J.S. & Dolowy, W.C. (1972). Isolation, crystallization and properties of *Achromobacteraceae* glutaminase-asparaginase with antitumour activity. *Journal of Biological Chemistry*, 247 84-90.

129. Roberts, J. (1976). *Journal of Biological Chemistry*, 251 2119-2123.*

130. Roberts, J. & Holcenberg, J.S. (1981). Enzymes as drugs. John-Wiley & Sons Inc. New York.

131. Roberts, J. & McGregor, W.G. (1989). Glutaminase has potent anti retroviral activity *in vivo*. *Proceedings of the Annual Meeting - American Association for Cancer Research*, 30 A 1806.

132. Rolz, C.E. (1984). Microbial biomass from renewables. A second review of alternatives. *Annual Reports of Fermentation Proceedings*, 8 213-355.

133. Roussos, S., Raimbault, M., Gonzalez, G. V., Castaneda, G. S. & Lonsane, B. K (1991 a). Scale-up of cellulases production by *Trichoderma harzianum* on a mixture of sugar cane bagasse and wheat bran in solid state fermentation system. *Micol. Neotrop. App*, 4 83-98.

134. Roussos, S., Raimbault, M., Castaneda, G. S. & Lonsane, B. K. (1991 b). Efficient leaching of cellulases produced by *Trichoderma harzianum* in solid state fermentation. *Biotechnology Techniques*, 6 (5) 429-432.

135. Rudman, S. & Meister, A. (1953). *Journal of Biological Chemistry*, 200 591.*

136. Sandhya Xavier, Lonsane, B. K. (1994). Factors influencing fungal degradation of total soluble carbohydrates in sugar cane pressmud under solid state fermentation. *Process Biochemistry*, 29 295-301.

137. Satyanarayana, T. (1994). Production of bacterial extracellular enzyme by solid state fermentation. In: *Solid State Fermentation.* Pandey. A (ed). Wiley Eastern Ltd. New Delhi. pp. 122-129.

138. Seitze, L.M., Saver, D. B., Burroughs, R., Mohr, H. E. & Hubbard, J. D, (1979). *Phytopathology*, 69 1202-1203.*

139. Schmidt, A. & Roberts, J. (1974). *Cancer Chemotherapy Reports*, 58 829. *

140. Shikata, H., Yasui, T., Ishigami, Y. & Omori, K. (1978). Studies on the glutaminase of shoyu koji. I Properties of the insoluble glutaminase. *Nippon Shoyu Kenkyusho Zasshi*, 4 48-52.*

141. Shankaranand, V. S. & Lonsane, B. K. (1993) Sugar-cane pressmud as a novel substrate for production of citric acid by solid state fermentation. *World Journal of Microbiology and Biotechnology*, 9 377-380.

142. Shankaranand, V. S. & Lonsane, B. K. (1994) Coffee husk an inexpensive substrate for production of citric acid by *Aspergillus niger* in a solid state ferementation system. *World Journal of Microbiology and Biotechnology*, 10 165-168.

143. Shimazu, Y., Ueyama, A. & Goto, K. (1991). Purification and characterization of glutaminase from *Bacillus subtilis* GT strain. *Journal of Brewers Society of Japan*, 86 441-446.

144. Smith, J. E. & Aidoo, K. E. (1988). Growth of fungi on solid substrates. In: *Physiology of Industrial Fungi.* Berry. D.R (ed). Blackwell Scientific Publications, Oxford. pp. 249-269.

145. Soda, K., Oshima, M. & Yamamoto, T. (1972). Purification and properties of isozymes of glutaminase from *Pseudomonas aeruginosa. Biochemistry Biophysics Research Communications*, 46 1278-1284.

146. Stanbury, P. (1987) Culturing of microorganisms for production. In: *Biotechnology:. The Biological Principles.* Trevan, M.D., Boffey, S., Goulding, K.H. & Stanbury, P (eds). Open University Press, England. pp. 61-108.

147. Tanaka, S., Robinson, E.A., Appella, E., Miller, M., Ammon, H.L., Roberts, J., Weber, I.T. & Wlodawer, A (1988). Structures of amidohydrolases. Amino acid sequence of a glutaminase-asparaginase from *Acinetobacter glutaminasificans* and preliminary crystallographic data for an asparaginase from *Erwinia chrysanthemi. Journal of Biological Chemistry*, 263 (18) 8583-8591.

148. Teramoto, A., Ishige, M., Furuya, T., Uchida, K. & Yoshino, H. (1985). *Nippon Nogei Kogaku Kaishi*, 50 245. *

149. Tomita, K., Yano, T., Kumagai, H. & Tochikura, T. (1988). Formation of γ-glutamyl glycylglycine by extracellular glutaminase of *Aspergillus oryzae. Journal of Fermentation Technology*, 66 299-304.

150. Ushijima, S. & Nakadai, T. (1984). *Aspergillus sojae* improvement by protoplast fusion. Annual Meeting of Agricultural and Chemical Society, Osaka, Japan.*

151. Wade, H.E., Robinson, H.K. & Phillips, B.W. (1971). Asparaginase and glutaminase activities of bacteria. *Journal of General Microbiology*, 69 299-312.

152. Wealth of India (1950) Raw materials. Vol. 2. Council of Scientific and Industrial Research, New Delhi. pp. 276-277.

153. West, A.W., Ross, D. J., Cowling, J. C. (1986). Changes in microbial C, N, P and ATP contents, number and respiration on storage soil. *Soil Biology and Biochemistry*, 18 141-148.

154. Wiseman, A. (1978). Topics in enzyme and fermentation biotechnology. John Wiley, London. pp. 180.

155. Yamamoto, S. (1974). Asian Food Conference, Food Science and Technology in Industrial Development. Bangkok, October 24-26. pp. 8.*

156. Yamamoto, S. & Hirooka, H. (1974). Production of glutaminase by *Aspergillus sojae*. *Journal of Fermentation Technology*, 52 564-569.

157. Yano, T., Ito, M., Tomita, K., Kumagai, H. & Tochickura, T. (1988). Purification and properties of glutaminase from *Aspergillus oryzae*. *Journal of Fermentation Technology*, 66 137-143.

158. Yokotsuka, T. (1985). Fermented protein foods in the Orient, with emphasis on shoy and miso in Japan. In: *Microbiology of Fermented Foods.* Wood. B.J.B (ed) Elsevier Applied Science, London. pp. 197-247.

159. Yokotsuka, T. (1986). Soy sauce biochemistry. In: *Advances in Food Research.* Chichester. C.O, Mrak. E.M & Schweigertt. B.S (eds). Academic Press, Orlando, Florida. pp. 195-329.

160. Yokotsuka, T., Iwaasa, T., Fujii, S. & Kakinuma, T. (1987). Studies on temperature digestion of shoyu koji. Part I. *Journal of Japanese Soysauce Research Institute*, 13 18-23.

161. Zadrazil, F. & Brunert, H. (1981). Investigation of physical parameters important for solid state fermentation on straw by white rot fungi. *European Journal of Applied Microbiology and Biotechnology*, 11 183-188.

162. Zobell, C.E. & Allen, E.C. (1935). The significance of marine bacteria in the fouling of submerged surfaces. *Journal of Bacteriology*, 29 239-251.

163. Zhu, Y., Smits, J.O., Knol, W. & Bol, J. (1994). A novel solid state fermentation system using polyurethane foam as inert carrier. *Biotechnology Letters*, 16 643-648.

* Originals not referred.

LIST OF PUBLICATIONS

I. In Journals

1) Polystyrene - an inert carrier for L-glutaminase production by marine *Vibrio costicola* under solid state fermentation.

World Journal of Microbiology and Biotechnology
11, 683-684 (1995)

2) L-Glutaminase production by marine *Vibrio costicola* under solid state fermentation using different substrates.

Journal of Marine Biotechnology
(in press, 1996)

3) Impact of process parameters on L-glutaminase production by marine *Vibrio costicola* under solid state fermentation using polystyrene as inert support.

Process Biochemistry
(Communicated, February, 1996)

4) Cobalt(II) and Copper(II) complexes of 1-nitrobenzyl 2-nitrophenylbenzimidazole: Synthesis, Characterization and Antibacterial activity studies.

Synthesis and Reactivity in Inorganic and Metal-Organic Chemistry
(in press, 1996)

II. Papers presented in Seminars/Symposia:

1) Characterization of L-glutaminase from marine *Vibrio*.

16th International Congress of Biochemistry and Molecular Biology,
New Delhi, September, 19-22, 1994.

2) Production of L-glutaminase by marine *Vibrio costicola* ACMR 267 using different substrates under solid state fermentation.

Microbiology International Conference (MICON - 94)
Mysore, November, 9-12, 1994.

3) Simultaneous production of Polysaccharases and L-glutaminase by marine *Vibrio costicola* ACMR 267 during solid state fermentation.

Microbiology International Conference (MICON - 94)
Mysore, November, 9-12, 1994.

4) L-Glutaminase production by marine *Vibrio costicola* using copra cake under solid state fermentation.

National Symposium on
The Relevance of Biotechnology in Industry,
Cochin, March, 4-5, 1995.