L-GLUTAMINASE PRODUCTION
BY
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CERTIFICATE

Certified that the work presented in this thesis is based on the bona fide work done by Miss Renu, S under my guidance in the Department of Applied Chemistry, Cochin University of Science and Technology and that no part thereof has been included in any other thesis submitted previously for the award of any degree.

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DECLARATION

I hereby declare that the work presented in this thesis is based on the original work done by me under the guidance of Dr.M.Chandrasekaran, Head, Centre for Biotechnology, Cochin University of Science and Technology (formerly Reader in Microbiology, Department of Applied Chemistry) and that no part of this thesis has been included in any other thesis submitted previously for the award of any degree.

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ABSTRACT

L-glutaminases (L-glutamine amidohydrolase EC.3.5.1.2) is proposed as a prospective candidate for enzyme therapy of cancer and also as an important additive during enzymatic digestion of shoyu koji since it could enhance glutamate content of soysauce. Commercial production of glutaminase could make possible its wide application in these areas, which would demand availability of potential sources and suitable fermentation techniques.

The present investigation highlighted marine environment as a potential source of efficient glutaminase producing bacteria mainly species of Pseudomonas, Aeromonas, Vibrio, Alcaligenes, Acinetobacter, Bacillus, and Planococci. Among them Pseudomonas fluorescens ACMR 171, P.fluorescens ACMR 43, Vibrio costicola ACMR 267 and V. cholerae ACMR 347 were chosen as the ideal strains for glutaminase production. They could grow and produce maximal glutaminase as extracellular enzymes under submerged condition at a wide range of parameters ranging from pH 5 to 8, 25-40°C, NaCl concentration of 0-5%, substrate concentration 0.5-3% within 18-24 hours of incubation. Glucose, at 0.5% level, enhanced significant level of enzyme production in all strains

except V.costicola ACMR 267 which preferred 1% glucose concentration for the same. Beef extract, followed by lysine, peptone and glutamic acid were observed to enhance the level of enzyme production. The strains were found to possess longer generation time in mineral media than in nutrient broth except V.cholerae ACMR 347 exhibited the opposite trend. Extracellular glutaminase fraction from all strains were in higher titres than intracellular enzymes during growth in mineral media, nutrient broth and nutrient broth added with glutamine.

Glutaminase from all strains were purified employing $(NH_4)_2SO_4$ fractionation followed by dialysis and ion exchange chromatography. The purified glutaminase from all strains were observed to be active and stable over a wide range of pH and temperature. Glutaminases purified from the strains possessed a Km of 1.0×10^{-4} M for P.fluorescens ACMR 171, 4.6×10^{-5} M for P.fluorescens ACMR 43, 9.54×10^{-5} M for V.costicola ACMR 267 and 1.5×10^{-5} M for V.cholerae ACMR 347. Enzymes from all strains were observed to be highly salt tolerant upto 25% NaCl. Heavy metals viz., Hg, Fe, Ca, Mn, Pb, Co, Cu and Zn affected the enzyme activity adversely. Glutamic acid, aspartic acid and EDTA had not influenced the activity of glutaminase

while phosphate ion and tris ion enhanced the activity of glutaminase. Glutamine was identified as the preferential substrate for glutaminase from all strains and a combination of L-asparagine and L-glutamine resulted in a reduced enzyme activity.

Production pattern of glutaminase by these strains in SSF was also examined. There too a wide range of operational parameters such as moisture content of 40-60%, pH 4-10, temperatures 15-45°C, 0-5% NaCl concentrations could effect significant levels of enzyme production. Interestingly V.cholerae ACMR 347 produced maximal enzyme production in the absence of NaCl in both SmF and SSF. Extraction parameters for maximal recovery of glutaminase from SSF was also standardised. A comparative analysis for enzyme production in both SmF and SSF showed that SSF process can yield many fold enzymes. Glucose enhanced the level of enzyme production by all the strains in SmF while reducing the enzyme production in SSF except in P.fluorescens ACMR 171. Optimization studies of environmental variables that normally influence the yield of glutaminase indicated that the optimal requirements of these bacteria for maximal glutaminase production remained stable irrespective of the medium, they are provided with for enzyme production. However, solid state fermentation technique was observed to be the most suitable process for the production of glutaminase.

CONTENTS

1. INTRODUCTION

1.1 PREFACE

L-Glutaminase (L-Glutamine amidohydrolase EC. 3.5.1.2) the enzyme deamidating L-glutamine plays a major role in the cellular nitrogen metabolism of both prokaryotes and eukaryotes. L-glutamine constitutes a large proportion of the available free aminonitrogen of tissues, blood and of the metabolic nitrogen pool and is an important non-toxic, temporary reservoir of ammonia nitrogen in microorganisms which can be drawn upon for synthetic purposes (Owen & Robinson, 1963; Shalhoub et al., 1963; Squires et al., 1970). It also acts as a direct precursor for glutamic acid in the metabolism of certain tumors which consequently furnish the carbon for the partial operation of tricarboxylic acid cycle from and α-ketoglutarate to oxaloacetate (Roberts & Simonsen, 1960). Reactions catalysed by glutamine constituted the primary mechanism of ammonia production in the body and it plays an important role in the acid base control of body fluids (Pitts, 1971).

In microorganisms the intracellular levels of glutamine are determined by rates of enzymatic synthesis and degradation (Prusiner et al., 1976). Glutamine synthetase

catalyses the synthesis while glutaminase catalyses the hydrolytic degradation of glutamine and splits off the γ-amide of glutamine as ammonia.

1) L-glutamate + NH_3 + ATP $\xrightleftharpoons{\text{Glutamine Synthetase}}$ L-glutamine + ADP + Pi

2) L-glutamine + H_2O $\xrightleftharpoons{\text{Glutaminases}}$ L-glutamate + NH_3

The action of glutaminase directly opposes that of glutamine synthetase, so their coupling would result in a futile cycle of amide synthesis and degradation (Meister et al., 1955; Prusiner & Stadtman, 1971).

Ability of the enzyme to bring about degradation of glutamine poses it as a possible candidate for enzyme therapy which may soon replace or combined with L-asparaginase in the treatment of acute lymphocytic leukaemia. It is found that administration of L-glutaminase will deplete L-glutamine which is required for asparagine synthesis in the body of patient thereby inhibiting asparagine dependent protein synthesis and eventually the synthesis of DNA and RNA. However, the large scale application of glutaminase in cancer chemotherapy is still under experimental conditions and not much information is available.

Most of the basic flavour components of fermented condiments are aminoacids produced by enzymatic degradation of protein contained in raw materials. The unique flavour of fermented soysauce or shoyu is mainly due to glutamic acid (concentrations of 0.7 to 0.8% per total nitrogen) (Yokotsuka, 1988a). Activity of glutaminase, which is responsible for the synthesis of glutamic acid makes it an important additive during enzymatic digestion of shoyu koji. Attempts to increase the glutamate content of soysauce using salt tolerant and heat stable glutaminase has drawn large attention.

Solid state fermentation (SSF), a cultivation technique for microorganisms involves growth and metabolism of the culture in a moist solid substrate in the absence of any freewater (Lonsane et al., 1982, 1985). One of the most successful exploitation of SSF technique is for the commercial production of different exoenzymes such as pectinases, fungal alpha amylases, amyloglucosidases and cellulases since it offers many advantages over submerged fermentation (SmF) (Lonsane & Karanth, 1990).

While glutaminase is widely distributed in animal tissues and microbes, commercial production is mostly based

on extraction from animal tissues. Microbes as sources of this enzyme has not been attempted sufficiently and there exists a dearth of knowledge on the production patterns of the enzyme by bacterial sources in general and more specifically from marine environments which normally harbours heterotrophic bacteria of unknown potentials.

L-glutaminase produced by bacteria from marine environments may hold more potential in the treatment of leukaemia unlike L-asparaginase which is reported to cause allergic reactions. Further their commercial production using marine bacteria could make possible its wide application in cancer chemotherapy besides their use in food industry. Hence the production pattern of these enzyme producing bacteria in SSF and SmF were studied for selection of a suitable fermentation technique for the commercial production of glutaminase by these strains.

1.2 REVIEW OF LITERATURE

1.2.1 Glutaminase-occurrence and distribution

Glutaminase activity is widely distributed in plants, animal tissues and microorganisms including bacteria, yeast and fungi (Meister, 1956; Roberts, 1960; Varner, 1960; Imada et al., 1973; Yokotsuka et al., 1987).

No attempt was made to review the glutaminase of plants and animals as they are out of scope of the present study and hence only the literature available on micro-organisms are presented here.

Glutaminase from microorganisms

Several species of microorganisms including bacteria, yeast and fungi are reported to produce L-glutaminase and L-asparaginase which have probable therapeutic applications.

Earlier reports indicate that glutaminase activity is widely distributed among bacteria (Wade et al., 1971; Imada et al., 1973; Yokotsuka et al., 1987).

Although glutaminase have been detected in several bacterial strains, the best characterized were from members of Enterobacteriaceae family. Among them E.coli glutaminase have been studied in detail (Hughes, 1949; Meister et al., 1955; Hartman, 1968; Hammer & Hartman, 1968; Prusiner & Stadtman, 1971; Prusiner, 1973; Prusiner et al., 1976). However, other members such as Proteus morganii, P.vulgaris, Xanthomonas juglandis, Erwinia carotovora, E.aroideae, Serratia marcescens, Enterobacter cloacae, Klebsiella aerogenes and Aerobacter aerogenes (McIlwain 1948; Wade et al., 1971; Imada et al., 1973; Novak & Philips, 1974).

Among other groups of bacteria, Pseudomonads are well recognised for the production of glutaminase (EL-Asmar and Greenberg, 1966; Katsumata et al., 1972; Abe et al., 1974; Holcenberg et al., 1976; Roberts, 1976; Smirnova et al., 1977) especially Pseudomonas aeruginosa (Greenberg et al., 1964; Soda et al., 1966a,b; Mardashev et al., 1970; Ohshima 1976), P.aureofaciens (Imada et al., 1973) P.aurantiaca (Kabanova et al., 1985, 1986; Lebedeva et al., 1986) P.boreopolis (Pekhov et al., 1985) P.fluorescens (Yokotsuka et al., 1987). Aeromonas hydrophila, A.liquefaciens, Rhodopseudomonas spheroides (Wade et al., 1971; Imada et al., 1973) were also reported to possess glutaminase activity.

Among the members of Achromobacteriaceae Acinetobacter glutaminasificans (Roberts et al., 1972; Schrek et al., 1971; Holcenberg et al., 1972; 1978; Wlodawer et al., 1975; 1977). Flavobacterium flavescens, F.heparinum, Alcaligenes faecalis (Imada et al., 1973) and Achromobacter sp. (Spiers & Wade, 1979) were reported to possess significant levels of glutaminase activity.

Significant levels of glutaminase was also produced by Clostridium welchii (Hughes, 1949; Hughes &

Williamson, 1952; Mardashev et al., 1966; Kovalenko et al., 1970; 1971; Kozlov et al., 1972), Bacillus circulans (Kikuchi et al., 1971), B.licheniformis (Imada et al., 1973; Cook et al., 1981), B.subtilis, B.megaterium, B.pumilus (Wade et al., 1971; Imada et al., 1973).

Other major species of bacteria which were shown to possess considerable levels of glutaminase activity include Azotobacter agilis (Ehrenfeld et al., 1963; Imada et al., 1973), Spirillum lunatum, Brevibacterium flavum, B.ammoniagenes, Micrococcus glutamicus, M.lysodeikticus, Staphylococcus aureus, Corynebacterium equi, Agrobacterium tumiefaciens (Imada et al., 1973).

Apart from this many of the bacterial asparaginases which have been characterized, displayed significant levels of glutaminase activity (Miller & Balis, 1969; Hakimi & Bosman, 1979) especially asparaginases from E.coli, several strains of Erwinia carotovora (Wade et al., 1971) and serratia marcescens (Novak and Philips, 1974).

Among yeasts, species of Hansenula, Cryptococcus, Rhodotorula, Candida scottii (Imada et al., 1973) especially Cryptococcus albidus (Abdumalikov & Nikolaev, 1967;

Imada et al., 1973; Yokotsuka et al.,1987; Nakadai & Nasuno, 1989; Fukushima & Motai, 1990) Cryptococcus laurentii, Candida utilis and Torulopsis candida (Kakinuma et al., 1987) were observed to produce significant levels of glutaminase.

Species of Tilachlidium humicola, Verticillum malthoasei and Fungi Imperfecti were recorded to possess glutaminase activity (Imada et al., 1973). Glutaminase activity of soysauce fermenting Aspergillus sojae and A.oryzae were also reported (Kuroshima et al., 1969; Yamamoto and Hirooka, 1974a,b; Shikata et al., 1978; 1979; Furuya et al., 1985; Teramoto et al., 1985; Tomita et al., 1988, Yano et al., 1988).

Studies on the qualitative and quantitative distribution of glutaminase activity among the members of bacteria are rather limited.

Wade et al., (1971) studied the distribution of glutaminase activities among 46 strains from 13 species of bacteria which included Pseudomonas fluorescens, Rhodopseudomonas spheroides, Xanthomonas juglandis, Aeromonas liquefaciens,

Alcaligenes metalcaligenes, Escherichia coli, Aerobacter aerogenes, Serratia marcescens, Proteus vulgaris, Micrococcus lysodeikticus, Bacillus megaterium and B.subtilis

L-asparaginase and L-glutaminase activities were detected in many microorganisms (Imada et al., 1973). Among 464 species of bacteria, the activities occured in many gram negative bacteria and in a few gram positive bacteria and also a large proportion of Pseudomonads exhibited L-glutaminase activity. Glutaminase activity was also observed in several species of Streptomyces such as S.californicus, S.netropsis, S.olivochromogenes and in Nocardia sp.

Yokotsuka et al., (1987) examined 194 strains isolated from soil including bacteria, actinomycetes, yeasts and molds for their ability to produce glutaminase active at high temperatures in the presence of salt and in acidic conditions. Among them E.coli, Cryptococcus albidus, Pseudomonas fluorescens were found to produce considerable amount of heat and salt tolerant glutaminase.

A critical scrutiny of available reports on the occurrence of glutaminase producing bacteria with reference

to environments clearly indicate that so far they had been isolated only from terrestrial environment, that too from soil (Roberts et al., 1972; Roberts, 1976 and Yokotsuka et al., 1987). In recent years majority of investigations on this enzyme were dedicated to its occurrence in mammalian tissues and elucidiation of its structure and kinetic properties. Of course but for the report on the distribution of glutaminase activity in marine sediments (Dharmaraj et al., 1977), aquatic environments, both fresh water was well as marine, has been neglected in this respect so far as per the available literature. Marine environment by virture of its unique characteristics could contribute potential glutaminase producers which could be fruitfully exploited for pharmaceutical as well as industrial purposes if appropriately studied.

1.2.2 Synthesis, Isolation and Purification of glutaminase

Assay methods of glutaminase and asparaginase have been documented in the earlier years itself (Meister, 1955). Further, an extensive review of earlier literature is also available on the purification, specificity, inhibition by heavy metals and other compounds effect of physicochemical parameters and mechanism of action of glutaminase (Hartman, 1971).

Glutaminases were reported to be produced both as extracellular and intracellular by bacteria.

Arima et al., (1972) observed significant levels of extracellular glutaminase activity in the culture broth of P.dacunhae, P.ovalis, P.schuylkilliensis, P.aureofaciens and P.chlororaphis.

Imada et al., (1973) reported the presence of intracellular glutaminase in Pseudomonas fluorescens, P.aureofaciens, P.schuylkilliensis, Spirillum metamorphum and Brevibacterium sp. Among them P.aureofaciens, P.schuylkilliensis also produced glutaminase extracellularly into the culture filtrate. Among fungi Tilachildium humicola, Verticillium malthoasei and Penicillium urticae were able to produce extracellular glutaminase.

Furuya et al., (1985) described intracellular distribution of koji glutaminases of Aspergillus oryzae and their characteristics. While Yano et al., (1988) reported that Aspergillus oryzae produced two fold extracellular fractions higher than intracellular glutaminase.

The substrates generally used for the production of glutaminase by bacteria included glucose, yeast extract, peptone, casein hydrolyzate, meat extract (Hughes, 1949; Hughes & Williamson, 1952; Kozlov et al., 1972; Novak & Philips, 1974; Prusiner et al., 1976), L-glutamic acid (Ramadan et al., 1964a; Soda et al., 1966a; 1972; Roberts et al., 1972; Roberts, 1976; Smirnova et al., 1977) and L-glutamine (Katsumata et al., 1972; Cook et al., 1981). For fungal production of extracellular glutaminase, wheat bran was used as solid substrate (Tomita et al., 1988; Yano et al., 1988). According to Smirnova et al., (1977) Pseudomonas sp. especially P.aeruginosa, P.boreopolis showed highest activity of glutaminase - asparaginase when cultivated in a meat peptone broth.

Glutaminase production by E.coli was found to be independent of growth medium used (Hartman, 1968; Prusiner et al., 1976) whereas for Acinetobacter glutamic acid must be an essential component of growth medium for the maximal production of glutaminase (Roberts et al., 1972).

Wade et al., (1971) reported that a low concentration of glucose (0.1%) was enough to enhance glutaminase and asparaginase production by bacteria while higher concentra-

tions of carbohydrate were repressive. Further, glucose partially prevented the induction of glutaminase production by glutamine in Bacillus licheniformis (Cook et al., 1981) and also inhibited glutaminase production in Pseudomonas 7A (Roberts, 1976). The addition of yeast extract and tryptone to the growth medium was resulted in inhibition of glutaminase production by Acinetobacter glutaminasificans (Roberts et al., 1972).

Optimal temperature for maximal glutaminase production ranged from 25-30°C for Pseudomonas sp. (Katsumata et al., 1972; Soda et al., 1972; Roberts, 1976) whereas in Acinetobacter sp. enzyme production decreased at temperatures above 25°C (Roberts et al., 1972). Glutaminase production by E.coli required 37°C as their optimal temperature (Hartman, 1968; Prusiner et al., 1976).

Maximal glutaminase production was reported to occur at the late exponential phase in Pseudomonas and in Bacillus licheniformis (Roberts, 1976; Cook et al., 1981) and in early stationary phase in E.coli (Hartman, 1968; Prusiner et al., 1976).

The glutaminase produced by bacteria were subjected to extensive purification by many investigators. Glutaminase from Acinetobacter sp., Pseudomonas sp., Clostridium sp., and E.coli were purified by employing all or few of the following procedures viz., $(NH_4)_2SO_4$ fractionation, protamine treatment, streptomycin precipitation, heat treatment at 55°C, chromatography upon DEAE cellulose, sephadex, electrophoresis and crystallization (Hughes & Williamson, 1952; Ramadan et al., 1964a; Hartman, 1968; Kozlov et al., 1972; Soda et al., 1972; Katsumata et al., 1972; Roberts, 1976; Prusiner et al., 1976). Glutaminase of fungal origin were also purified by same procedures mentioned above (Tomita et al., 1988; Yano et al., 1988).

Hughes and Williamson (1952) and Kozlov et al., (1972) obtained 40% yield of purified glutaminase from Clostridium welchii. A homogeneous form of glutaminase having a specific activity of 1520 μ moles per min.per mg. of protein was obtained from E.coli (Hartman, 1968). Later E.coli was reported to contain two glutaminases A and B with their pH optima below pH 5 and above pH 7 respectively (Prusiner et al., 1976) and glutaminase B has been purified 6000 fold with a yield of 40%.

Roberts et al., (1972) obtained after purification a glutaminase-asparaginase from Acinetobacter sp. with an overall yield of 40-60% and a specific activity of 160 IU/mg of protein.

Glutaminase from Pseudomonas were purified to homogeneous condition employing treatment with butanol, ammonium sulphate and zone electrophoresis and observed that it had also asparaginase activity (Ramadan et al., 1964a). Homogeneous preparations of isozymes of glutaminase A and B from P.aeruginosa purified 200 and 170 fold respectively after ultracentrifugation and disc gel electrophoresis (Soda et al., 1972) crystalline, homogeneous preparations of glutaminase were obtained from Pseudomonas with specific activities of 36 IU/mg of protein (Katsumata et al., 1972) and 160 IU/mg of protein (Roberts, 1976).

Novak and Philips, (1974) purified and separated L-glutaminase enzyme with L-asparaginase activity from Serratia marcescens by DEAE cellulose chromatography and found that both activities were due to the same enzymatic site.

Glutaminase of soysauce fermenting Aspergillus strains were partially purified by earlier workers (Kuroshima et al., 1969; Yamamoto & Hirooka, 1974a,b; Shikata et al., 1978; 1979; Teramoto et al., 1985) and purification procedures such as ammonium sulfate fractionation, ion exchange chromatography and polyacrylamide gel electrophoresis were employed (Yano et al., 1988) for isolation of glutaminase from A.oryzae with a 730 fold purification and 6.2% of recovery for the intracellular fraction and a 1100 fold purification with a recovery of 3.2% for the extracellular fraction.

Characterization of physicochemical properties like pH, temperature, kinetics, substrate specificity and inhibition of the purified glutaminase were studied by many investigators (Ramadan et al., 1964b; Hartman, 1968; Katsumata et al., 1972; Roberts et al., 1972; Soda et al., 1972; Prusiner et al., 1976; Roberts, 1976).

Glutaminase from Pseudomonas were reported to be active over a pH range of 5-9 with optimal activity near pH 7 (Ramadan et al., 1964b; Roberts, 1976) whereas optimal activities of glutaminase A and B of P.aeruginosa were more active at alkaline range, at pH 7.5-9 and 8.5 respectively

(Soda et al., 1972). Contrastingly, glutaminase from Clostridium welchii showed a preference for acidic pH range at 5-5.2 (Hughes & Williamson, 1952) and at pH 4.5 (Kozlov et al., 1972).

E.coli had two glutaminases A and B, one with pH optima below 5 and another requiring pH above 7.1-9.0 (Prusiner et al., 1976). Enzyme from Acinetobacter sp. showed a high degree of glutaminase and asparaginase activities over the pH range 6-9 with near optimal activity at pH 7 (Roberts et al., 1972). Whereas B.circulans produced two different peptidoglutaminases with optimum pH around 7.5 (Kikuchi et al., 1971).

Both extracellular and intracellular glutaminase from Aspergillus oryzae were most active and stable at pH 9 (Yano et al., 1988). According to Yokotsuka et al., (1987) intracellular glutaminase from Cryptococcus albidus, although showed an optimal pH of 5.5-8.5 was most stable at pH 5.0-7.0.

Glutaminase from Pseudomonas were maximally active at 37°C and unstable at high temperatures (Ramadan et al., 1964b) whereas glutaminase from Clostridium welchii was

inactivated beyond 60°C (Kozlov et al., 1972). Enzymes from E.coli were inactivated by cooling and activated by warming (Prusiner et al., 1976). While the optimum temperature for activity of both extracellular and intracellular glutaminase from A.oryzae was 45°C, their thermal stability was upto 37°C and resulted in the loss of activities at 55°C. Whereas glutaminase from Cryptococcus albidus retained 77% of its activity at 70°C even after 30 min. of incubation (Yokotsuka et al., 1987).

Glutaminase activity decreased in the presence of NaCl and glutaminase from E.coli, P.fluorescens, Cryptococcus albidus and Aspergillus sojae recorded only 65, 75, 65 and 6% respectively of their original activity in the presence of 18% NaCl (Yokotsuka et al., 1987). NaCl also decreased the activity of glutaminase of Cryptococcus albidus, Candida utilis, Torulopsis candida (Kakinuma et al., 1987). Activity of both intra and extracellular glutaminases from A.oryzae were reduced in the presence of NaCl and were inhibited about 50% with 5% NaCl (Yano et al., 1988).

Glutaminase activity in the clayey sediments showed two pH optima of 5.6 and 8.4 and unaffected by NaCl

upto a 10% concentration and were higher than asparaginase activity in all marine sediment samples collected from different biotopes (Dharmaraj et al., 1977).

Several investigations have been conducted on the kinetics of glutaminase from bacteria.

The substrate saturation curve of glutaminase B from E.coli was elucidated by Prusiner et al., (1976) and Km for its different substrates such as glutamine, glutamyl methylamide, glutamyl hydrazide, glutamyl hydroxamic acid and glutamic acid have been studied by Hartman, (1968) and it is shown to hydrolyze glutamic acid with a Km of 2.9 mM (Hammer & Hartman, 1968).

While L-glutaminase - L-asparaginase from Acinetobacter sp. recorded a Km of $5.8 \pm 1.5 \times 10^{6}$ for L-glutamine, those from Clostridium welchii registered a Km of 10^{-3} M for L-glutamine (Kozlov et al., 1972). Glutaminase A and B from Pseudomonas aeruginosa were reported to possess Km of 1.1×10^{-4} M and 1.8×10^{-4} M for L-glutamine respectively (Soda et al., 1972). Phosphate influenced the Km of glutaminase from Pseudomonas where in the presence of phosphate, Km was found to be 7×10^{-3} M, and in its absence

it was 8 x 10^{-3} M (Ramadan et al., 1964a). The average Km values of Pseudomonas 7A glutaminase-asparaginase was 4.6 ± 0.4 x 10^{-6} M for L-glutamine (Roberts, 1976).

Glutaminase from Pseudomonas sp. was shown to be comparatively a smaller protein with a molecular weight of 26,300 to 25,800 (Ramadan et al., 1964a) whereas glutaminase A and B of P.aeruginosa were estimated to possess a molecular weight of 1,37,000 and 67,000 respectively (Soda et al., 1972). Glutaminase from Pseudomonas p.210 had a molecular weight of 1,22,000 ± 10,000 (Katsumata et al., 1972) which was later reported to be composed of four identical subunits of molecular weight of 36,400 each and possess on apparently higher molecular weight of 1,46,000 (Abe et al., 1974). The specific activity of this enzyme decreased rapidly during incubation in aqueous solution, probably due to the cleavage of the enzyme by the contaminant traces of proteases that attack on the liable peptide bonds of the enzyme. Glutaminase-asparaginase of Pseudomonas 7A was observed to have four subunits with a molecular weight of 36,000 ± 500 which showed polymerization in the presence of substrate (Holcenberg et al., 1976).

The glutaminase of Acinetobacter was observed to be dissociated into four subunits of molecular weight of 33,000 and 1,38,000 altogether. It was suggested that the dissociation of the enzyme into active smaller fragments could increase its effectiveness by increasing the distribution of the enzyme in the animal host (Roberts et al., 1972). Sedimentation equilibrium studies on glycosylated preparations of glutaminase-asparaginase from Acinetobacter glutaminasificans showed mixtures of molecular weight from 60,000 to 1,80,000 (Holcenberg et al., 1975). Whereas glutaminase A of E.coli recorded a molecular weight of 1,10,000 (Hartman, 1968) and that of B had 90,000 (Prusiner et al., 1976). In the case of obligate anaerobe, Clostridium welchii the molecular weight ranged from 1,10,000-1,40,000 (Kozlov et al., 1972). Both intracellular and extracellular glutaminase from Aspergillus oryzae were reported to have a molecular weight of about 1,13,000 (Yano et al., 1988).

Isoelectric point of glutaminase varied for different organisms. Thus it was 5.5 for Clostridium welchii (Kozlov et al., 1972), 5.4 for E.coli (Prusiner et al., 1976), 8.43 for Acinetobacter (Roberts et al., 1972), 5.8 for Pseudomonas (Holcenberg et al., 1976), 7.6 for

another species of Pseudomonas (Katsamata et al., 1972), and 3.94-4.09 Cryptococcus albidus (Yokotsuka, 1987).

Aminoacid composition of glutaminase have not been analyzed by many investigators. No cysteine was detected in glutaminase-asparaginase from Pseudomonas (Holcenberg et al., 1976). Cysteic acid was well below the level of any other aminoacid in the glutaminase-asparaginase from both Pseudomonas and Acinetobacter sp. (Roberts et al., 1972; Roberts, 1976). Although the aromatic and basic aminoacids in the glutaminase of Pseudomonas were considerably lower than the enzymes of Acinetobacter sp. glutamic acid content was higher (Katsumata et al., 1972). Different catalytic properties possessed by glutaminases of Acinetobacter glutaminasificans and Pseudomonas 7A prompted comparative studies on the aminoacid sequence of diazo 5 oxo L-norleucine (DON) binding site of these enzymes. The results indicated that DON binding site on the enzymes of both species is also a part of catalytic site for glutamine (Holcenberg & Ericsson, 1976; Holcenberg et al., 1978).

Among a large number of structurally related compounds tested, glutaminase from E.coli bound only with substances that had an unsubstituted L-glutamyl acylportion

and a substituent in the γ-position (Hartman, 1968). Studies on the exchange of oxygen between water and substrates of glutaminases of E.coli revealed no catalytic exchange between γ-carbonyl oxygen atoms of glutamine and water (Hammer & Hartman, 1968). Glutaminase A and B from E.coli exhibited a high degree of substrate specificity catalyzing only the deamidation of L-glutamine or formation of γ-glutamyl hydroxamate from L-glutamine (Prusiner et al., 1976).

Glutaminase from Pseudomonas catalyzed the hydrolysis of L-glutamine and D & L-asparagine (Ramadan et al., 1964b), those from P.aeruginosa catalyzed in addition, the formation of hydroxamates and hydrolysis of theanine and γ-glutamyl derivatives (Soda et al., 1966a,b; Ohshima, 1976). Studies have indicated that there exists a competition by both glutamine and asparagine for the same activity site of the enzyme from Pseudomonas (Roberts, 1976). Glutaminase-asparaginase from Acinetobacter catalyzed the hydrolysis of glutamine and asparagine (Roberts et al., 1972). While the peptidoglutaminases from Bacillus circulans catalyzed the deamidation of free L-glutamine poorly (Kikuchi et al., 1971), Clostridium welchii glutaminases catalyzed the hydrolysis of L-glutamine and γ-methyl L-glutamate (Kozlov et al., 1972).

Both intra and extracellular glutaminases from Aspergillus oryzae hardly catalysed the hydrolysis of D-glutamine or L & D-asparagine but were active towards L-glutamine and γ-glutamyl derivatives ie., DL-theanine and glutathione (Yano et al., 1988). The extracellular glutaminase possessed a considerable γ-glutamyl transpeptidase activity which catalyzed the formation of γ-glutamyl-glycyl glycine from L-glutamine and glycyl glycine (Tomita et al., 1988).

Glutaminase activity has been reported to be inhibited by various substances and heavy metals. Cetavlon, while accelerating glutaminase of Clostridium welchii, E.coli and Proteus morganii in crude extracts and intact cells (Hughes, 1949; 1950), inhibited purified extracts (Hughes & Williamson, 1952). Glutaminase from E.coli was found to be sensitive to heavy metals (Hartman, 1968) and Acinetobacter glutaminase-asparaginase was inactivated by glutamine analog 6-diazo 5-oxo L-norleucine even at very low concentration while unaffected by EDTA, NH_3, L-glutamate or L-aspartate (Roberts et al., 1972).

Various investigations have shown that glutaminase from Pseudomonas was activated by certain divalent anions

and cations while inhibited by monovalent anions and by certain competitive inhibitors like NH_3, D & L-glutamic acid and 6-diazo-5-oxo L-norleucine (Ramadan et al., 1964b; Soda et al., 1972; Roberts, 1976). In the case of fungi, both extra and intracellular glutaminase from Aspergillus oryzae were inhibited by Hg, Cr and Fe but were not affected by sulfhydroxyl reagents (Yano et al., 1988).

Crystallization of purified glutaminase from Pseudomonas aeruginosa (Soda et al., 1972) and Acinetobacter glutaminasificans (Roberts et al., 1972) are reported. Two crystal forms of glutaminase-asparaginase were prepared from Acinetobacter glutaminasificans (Wlodawer et al., 1975) and compared with the crystals of enzyme from Pseudomonas (Wlodawer et al., 1977).

Few reports are available on the mutation of glutaminase producing microorganisms. A mutant of Torulopsis famata was reported to produce threefold glutaminase activity higher than the mother strain through NTG (N-Me-N^1-NO_2-N) nitrosoguanidine) treatment (Kakinuma et al., 1987). Mugnetsyan and Stepanayan (1987) examined L-glutaminase and L-asparaginase activities in streptomycin sensitive and resistant strains of E.coli and found that

streptomycin sensitive strains possessed comparatively high activities of both enzymes while spontaneous and induced mutants of these strains showed a decrease in amidase activities upto 60% along with an increase in streptomycin resistance.

1.2.3 Solid State Fermentation (SSF)

One of the most successful exploitation of SSF technique is for the commercial production of different exoenzymes. Diverse kinds of enzyme koji preparations are manufactured which contain specific exoenzymes such as alpha and beta amylase, protease, maltase, isomaltase, sucrase, lipase, phosphatases and cellulases (Lonsane & Karanth, 1990). SSF technique was used to produce amylases using fungi (Alazard & Raimbault, 1981) and bacteria B.licheniformis (Ramesh & Lonsane, 1987a,b; 1989; Ramesh, 1989) lipases (Aunstrup, 1979; Godfrey, 1983; Munoz et al., 1991) and cellulases (Chahal, 1983).

Whereas reports on glutaminase production is limited to soysauce fermenting Aspergillus strains (Kuroshima et al., 1969; Yamamoto & Hirooka, 1974a,b; Shikata et al., 1978; 1979; Teramoto et al., 1985). Yano et al.,(1958) observed that extracellular glutaminase from

A.oryzae by SSF was two fold higher than intracellular fraction. Tomita et al., (1988) reported that extracellular glutaminase of A.oryzae possessed considerable γ-glutamyl transpeptedase activity and catalyzed the production of γ-glutamyl glycylglycine in SSF which is significant from the view point of glutamic acid content. However, no reports are available for the production of glutaminase from bacteria by solid state fermentation techniques.

1.2.4 Glutaminase and treatment of cancer

An exciting breakthrough in the enzymatic treatment of cancer resulted from the discovery of a metabolic difference between certain tumor and host cell (Sizer, 1972). Only a limited number of microbially produced enzymes that deplete nutritionally essential aminoacids or nonessential aminoacids such as asparaginase (Adamson & Fabro 1968; Burchenal & Kranofsky, 1970; Wade & Rutter, 1970; Chang, 1971; Roberts et al., 1976; Sudha, 1981), glutaminases (Roberts et al., 1970, 1971) streptodornase (Nuzhina, 1970), lysozyme (Oldham, 1967), serine dehydratases (Wade & Rutter, 1970) and carboxy-peptidase (Bertino et al., 1971) have been suggested for the treatment of human leukaemias and solid tumors.

L-asparaginases and L-glutaminases have received greater attention with respect to their antitumor effect (Capizzi et al., 1970; Cooney & Handschumacher, 1970; Wriston 1971; Broome, 1971; Wriston & Yellin, 1973; Cooney & Rosenbluth, 1975; Abell & Uren, 1981; Flickinger, 1985). Considerable attention has been paid to the enzyme L-asparaginase since Broome (1961) showed that it was responsible for the antitumor activity of guinea pig serum (Kidd, 1953). Unlike most normal tissue cells some neoplastic cells are unable to survive in the absence of L-asparagine. It is used for treating leukaemias and disseminating cancer which require asparagine for growth (Mauer & Simone, 1976).

A parallel interest in L-glutaminase has arisen from demonstrations that microbial glutaminases also exhibit antitumour activity (Levintow, 1954; Roberts & Simonsen, 1960; Greenberg et al., 1964; Knox et al., 1969; Broome, 1971; Roberts et al., 1970; 1971). A number of lines of evidence motivated the treatment of neoplasms by glutaminase. Certain tumor cells grown in tissue culture required glutamine at a level which is tenfold or greater than any other aminoacid (Eagle, 1955; Eagle et al., 1956) and the dual requirement of Walker carcinosarcoma 256

invitro for asparagine and glutamine (Newman & McCoy, 1956). El.Asmar and Greenberg (1966) investigated the mechanism of inhibition of tumor growth by glutaminase. The glutamine analogs, asazerine, 6-diazo 5-oxo L-norleucine and azotomycin have also been shown to possess antineoplastic activity (Jacobs et al., 1969; Tarnowski et al., 1969; 1970; Catane et al., 1979). Riley (1970) observed complete regression by L-asparaginase, of a mouse leukaemia, could be obtained only under conditions in which the circulating L-glutamine was depleted.

Roberts et al., (1970) observed that glutaminase preparations purified from different bacteria, one from a gm positive coccus and other three from gm negative rods, with considerably lower km values resulted in marked inhibition of an Ehrlich ascites carcinoma when given one day after tumor implantation. According to them glutaminase-asparaginase preparations showed greater antitumor effect than the enzyme with only glutaminase activity. Roberts et al., (1971) demonstrated for the first time the induction of complete prolonged regression of a seven day established asparaginase resistant Ehrlich carcinoma by glutaminase and found that a combination of glutaminase with asparaginase did not produce a better therapeutic effect than glutaminase

alone. Holcenberg et al., (1971) reported that leukaemic lymphocytes from 6C3HED lymphoma were killed directly or indirectly by L-glutaminase, usually at the level of 1.7 IU/ml and also found that the decrease of glutamine in the media of incubated cells killed leukaemic but not normal lymphocytes in vitro. Hersh (1971) reported that L-glutaminase from E.coli inhibited, on continuous exposure, response of human lymphocytes to phytohaemagglutinin and streptolysin O and addition of L-glutamine resulted in a complete reversal of inhibition.

Greenberg et al., (1964) reported that a glutaminase-asparaginase preparation from Pseudomonas aeruginosa with a relatively high Km for glutamine decreased the initial rate of growth of a number of tumors including an Ehrlich ascites carcinoma but caused no significant increase in the survival time of tumor bearing animals. Another glutaminase-asparaginase isolated from Pseudomonas 7A that has a longer plasma half life was found to be effective against both ascites and solid tumors (Roberts, 1976) and a purified preparation of Pseudomonas glutaminase was reported to possess an antitumor activity (Iwasa et al., 1972; 1987).

According to Puntus et al., (1979) glutaminase-asparaginase from P.aurantiaca IBFM V-14 possessed greater

cytotoxic activity than E.coli deamidase preparation in asparagine dependent cultures like mouse leukosis LTL and Burkitts lymphoma. However, all enzymic preparations were equally active in asparagine dependent cultures like mouse leukosis 1-210 and human ovarian cancer.

An amidase from species of Xanthomonas has a greater activity towards L-glutamine and its extremely high affinity for both the substrates ie., glutamine and asparagine made it very effective against tumors (Broome, 1971).

Glutaminase-asparaginase from Acinetobacter glutaminasificans demonstrated a broader spectrum of antitumor activity towards both mouse transplantable tumors and human leukaemic cells in vitro than E.coli asparaginase (Holcenberg et al., 1972, 1973; Schmid & Roberts, 1977) L-glutaminase-asparaginase from Acinetobacter glutaminasificans and a succnylated derivative of the same enzyme that has a longer plasma half life have both received preliminary trials with respect to human pharmacology and toxicology in acute leukaemia (Holcenberg et al., 1979a,b; Warell et al., 1980). Tissue nitrogen sparing effect of high protein diet in mice with or without ascites tumor treated with Acinetobacter glutaminase-asparaginase was studied by Kien et al., (1985).

Shrek et al., (1971) observed that Achromobacteriaceae glutaminase-asparaginase selectively killed human leukaemic leukocytes in tissue culture at about one hundredth the effective concentration of E.coli asparaginase. Roberts et al., (1972) described a glutaminase-asparaginase from Achromobacteriaceae with potent antineoplastic activity and established criteria for selection of a glutaminase for testing of antitumor activity which included optimal activity, stability under physiological conditions, low km values, slow clearance from blood and low endotoxic activity. Achromobacter glutaminase-asparaginase have also received attention with respect to human pharmacology, toxicology and activity in acute leukaemia (Spiers & Wade, 1979). Immunosuppressive properties and circulatory life of glutaminase-asparaginase from Achromobacter covalently attached to polyethylene-glycol in man was reported by Abuchowski (1981). Asparaginase and glutaminase from Achromobacter persisted in the circulation of rat after undergoing chemical modifications like reaction with aminospecific reagents (Blazek & Benbough, 1981). Other modifications have also been reported to increase the persistence of glutaminase including deamidation (Wagner et al., 1969), glycosylation and succnylation (Holcenberg et al., 1975; Marsh et al., 1977).

Baskerville et al., (1980) reported that on administration of chemically modified microbial glutaminase at various doses to rhesus monkeys, marmosets, rabbits and mice; the enzyme induced diarrohea and dysentry and specifically the minimal doses caused illness which was fatal within 10 days. Hambleton et al., (1980) studied clinical and biochemical aspects of microbial glutaminase toxicity in rabbits and rhesus monkeys. According to them treatment with chemically modified glutaminases was lethal to rabbits and rhesus monkeys and lesions were produced in kidney, liver and intestine while treatment with unmodified glutaminase induced similar changes in rabbits but not in rhesus monkeys. Influence of glutamine on the growth of human glioma and medulloblastoma and a combination chemotherapy in vitro exploiting glutamine metabolism was discussed by Dranoff et al., (1985a,b).

1.2.5 Industrial use of glutaminase

Yokotsuka et al., (1974) digested shoyu koji mixed with 10-70% of heat denatured defatted soybean grits with and without addition of glutaminase from Cryptococcus albidus. They observed that the addition of glutaminase raised glutamic acid contents of test shoyu 20% more than the control. The current fermented soysauce or shoyu in Japan is

manufactured from a mixture of defatted soybean grits and wheat kernals of almost equal amounts (Yokotsuka, 1986a). Good quality genuine fermented shoyu contains 1.5-1.8% (w/v) total nitrogen, 3-5% reducing sugar, 2-2.5% ethanol, 1-1.5% polyalcohol, 1-2% organic acid and 16-18% NaCl. In order to ensure a palatable taste about one half of the nitrogeneous compounds present must be of free aminoacids and over 10% free glutamic acid (Yokotsuka, 1986 ; 1987).

About 75% of shoyu manufacturers in Japan are using A.oryzae but about 50% of shoyu is made by utilizing A.sojae. During shoyu mash fermentation glutamic acid and glutamine are separated from peptides by the action of peptidases and the glutamine is then converted into glutamic acid by the action of glutaminase (Yokotsuka, 1988a).

The raw materials of shoyu contain the source of glutamic acid equivalent to 1.2-1.4% against 1% total nitrogen while the actual content of glutamic acid ranges from 0.3% to 1%. The difference is due to the insufficient amount of glutaminase produced by koji making and heat labile nature of koji glutaminases and a gap in the optimal pH values of koji glutaminases and shoyu mash. So it proves to be effective to add heat and salt tolerant glutaminase during

enzymatic digestion of shoyu koji, especially when conducted with reduced salt concentrations and high temperatures (Yokotsuka et al., 1972; Yokotsuka, 1987).

Kakinuma et al., (1987) reported Cryptococcus, Candida and Torulopsis as the major producers of glutaminases among the yeasts tested and their glutaminase activity was found to be less impaired by salt enabling their use in shoyu mash fermentation. Nakadai and Nasuno (1989) tried to increase the glutamic acid content of soysauce by salt tolerant glutaminase from Crytococcus albidus.

Yokotsuka et al., (1987) selected three strains, E.coli, Pseudomonas fluorescens 30-21 and Cryptococcus albidus IAM 4830 from 292 strains of bacteria and 450 strains of yeasts as producers of heat and salt tolerant glutaminase, and among them enzyme from P.fluorescens was more salt tolerant and Cryptococcus albidus was more heat resistant. They also observed that glutaminase from E.coli produced more glutamic acid/total nitrogen ratio compared to glutaminase from P.fluorescens and C.albidus in an experimental 150 day shoyu mash fermentation.

Glutaminase from soysauce fermenting Aspergillus strains were partially purified and characterized (Kuroshima et al., 1969; Yamamoto & Hirooka, 1974a,b; Shikata et al., 1978; 1979; Teramoto et al., 1985). Yano et al., (1988) isolated Aspergillus oryzae MA 27 - IM from a commercial koji seed for soysauce fermentation and extra and intracellular glutaminase purified to be applied in the brewing of high quality soysauce. A glutaminase with γ-glutamyl transpeptidase activity was also isolated from a wheat bran koji of A.orysae and the peptide was identified and purified with a view to improve the glutamic acid content of the food (Tomita et al., 1988).

Yokotsuka (1987; 1988a;b) reviewed high temperature enzymatic liquifaction of raw materials, factors contributing to flavor quality and productivity and discussed advances in raw material cooking, koji making and mash fermentation in shoyu manufacture.

Attempts have been made to improve koji molds with respect to production of protease (Nasuno & Ohara, 1972; Furuya et al., 1983; 1985) and glutaminase (Yamamoto, 1974) by induced mutation. Ushijima and Nakadai (1983) employed protoplast fusion among the same species of A.sojae to induce significant levels of both protease and glutaminase production.

Kakinuma et al., (1987) obtained a mutant of Torulopsis famata which had threefold glutaminase activity as that of mother strain after treatment with nitrosoguanidine.

Eventhough glutamic acid is the most important aminoacid in food manufacture for delicious taste (O'Mahony & Ishi, 1987) practically no attempt have been made to produce glutamate by immobilizing glutaminase or glutaminase producing microorganisms. However, one report is available on the continuous conversion of glutamine to glutamate by immobilizing salt tolerant glutaminase producing yeast, Cryptococcus albidus on silicagel and aliginate-silicagel complex with hydrolyzed wheat bran as a substrate and obtained a continuous production of 10 mg/ml of glutamic acid (Fukushima & Motai, 1990).

1.3 OBJECTIVES OF THE PRESENT STUDY

From the review of literature it is clear that no work has been done in India on glutaminase producing bacteria and their application. Hence in the present study it was decided to screen glutaminase producing bacteria from marine and estuarine environments of Cochin.

Main objectives of the present study included the following:

1. To isolate glutaminase producing bacteria from marine and estuarine environments of Cochin.

2. To select potential strains that produce maximal level of glutaminase.

3. To identify the selected strains of Pseudomonas and Vibrio to the species level.

4. To characterize the organisms for their growth and enzyme production with respect to various environmental variables in submerged fermentation (SmF).

5. Purify glutaminase from Pseudomonas and Vibrio and characterize them.

6. To study the production pattern of glutaminase by selected strains in solid state fermentation (SSF) with respect to various environmental variables.

7. To standardize the extraction parameters for the maximal recovery of glutaminase from solid state fermentation.

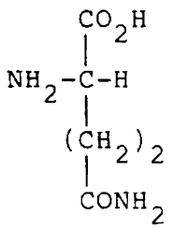
8. To compare glutaminase production in SmF and SSF.

2. MATERIALS AND METHODS

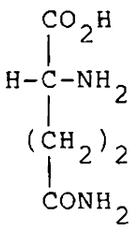
2.1 SUBSTRATE

L-Glutamine (HI-Media) was used as the substrate for growth and enzyme production by L-glutaminase producing bacteria.

Chemical names of glutamine are 2-amino 4-carbomylbutanoic acid and α-amino γ-carbamidobutryic 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 gluten hydrolyzate and beet sugar molasses are two natural sources of glutamine.

2.2 ISOLATION OF GLUTAMINASE PRODUCING BACTERIA FROM MARINE ENVIRONMENT

2.2.1 Samples

Both water and sediment samples of marine and estuarine environments of Cochin were screened for isolation of L-glutaminase producing bacteria over a period of six months from July 1988 to December 1988.

2.2.2 Collection of samples

Water samples were collected using sterilized glass containers from the surface region. Sediment samples were collected using Peterson grabs from 1 m depth of water and middle portion of the sediment was aseptically transferred to sterile polythene bags using sterile spatula. The samples were immediately taken to the laboratory (within a period of 2 hours) and processed for bacteriological analysis.

2.2.3 Preparation of media

Since no direct media was reported in literature for the direct isolation of glutaminase producing bacteria

from natural environments, an attempt was made to develop a mineral salts basal medium supplemented with L-glutamine as the sole carbon source.

The composition of the Mineral Salts Glutamine Agar medium (MSGA) after standardisation is as below:

KH_2PO_4	:	0.10 g
$MgSO_4 7H_2O$	:	0.05 g
$NaNO_3$	:	0.01 g
$CaCl_2$	:	0.01 g
$FeCl_3$	:	0.01 g
L-Glutamine	:	1.00 g
NaCl	:	1.00 g
Agar	:	2.00 g
Distilled water	:	100 ml
pH	:	7 ± 0.2

Medium was autoclaved and used.

2.2.4 Plating procedures

Serially diluted water and sediment samples were plated on the Mineral Salts Glutamine Agar (MSGA) medium

under aseptic conditions employing pourplate technique. Plates were incubated at room temperature (28 ± 2°C) for a period of 3-7 days and colony counts were made on the 3rd, 5th and 7th days. All the bacterial colonies developed on MSGA medium were assumed as glutaminase producers since glutamine was the only carbon source in the medium and utilization of glutamine required the presence of glutaminase. L-glutaminase producing bacterial populations are (GPB) expressed in terms of No. ml^{-1} of water and No. g^{-1} of dry weight of sediment.

2.2.5 Isolation and maintenance of cultures

Isolates were subcultured on nutrient agar and after repeated purification, one set of subcultures were stocked under mineral oil (sterilized liquid paraffin was used). Another set of subculture was used as working cultures for further studies. They were maintained at room temperature and subcultured once in two weeks. At regular intervals of one month their purity was checked.

2.2.6 Identification of bacteria

All the isolates were assigned to various genera based on their morphological and biochemical characters

outlined in the Bergey's Manual of Systematic Bacteriology (Bucchanan & Gibbons, 1974). The selected strains of Pseudomonas and Vibrio which were used in the later studies were further identified upto their species level based on the schemes suggested in Bergey's Manual of Systematic Bacteriology (Kreig & Holt, 1984).

2.3 SELECTION OF POTENTIAL L-GLUTAMINASE PRODUCING BACTERIA FOR FURTHER STUDIES

Selection of potential L-glutaminase producing bacteria was carried out in two stages. Initially, all the strains were rechecked for their efficiency to grow in a mineral medium containing L-glutamine as the sole source of carbon. This was performed by inoculating a loopful of 12 hours old agar slope culture into 10 ml of MSG broth (MSGB) (Section 2.2.3) without agar and incubated for 24 hours at room temperature. Turbidity resulted due to the growth of bacteria was measured in terms of OD at 660 nm using a UV-visible spectrophotometer (Hitachi Model 200).

In the second stage of selection, 100 strains, that recorded higher levels of growth in MSG broth were further tested for their enzyme production as detailed below:

2.3.1 Media

Mineral Salts Glutamine Agar (MSGA) medium mentioned earlier (Section 2.2.3) was used as a broth (MSGB) without agar. Fifty ml of MSGB (Mineral Salts Glutamine Broth) taken in 250 ml Erlenmeyer conical flasks were autoclaved and used.

2.3.2 Preparation of inoculum and inoculation procedures

Inoculum for secondary screening was prepared as follows:

1. Initially a loopful of 24 hours old agar slope culture was transferred to 10 ml of NBG (Nutrient Broth added with Glutamine) and grown for 24 hours at room temperature (28 ± 2°C).

2. One ml of the cultured broth was then aseptically transferred into another 50 ml of NBG media and incubated for 24 hours in a rotary shaker (150 rpm) at room temperature (28 ± 2°C).

3. Cells were harvested by centrifugation (MB centrifuge model MB 20) at 5000 rpm for 20 min.

4. The harvested cells were made upto 10 ml volume using physiological saline (0.85% NaCl) after repeated washing with the same.

5. The prepared cell suspension was used as inoculum at 1% level for further inoculation of 50 ml MSGB.

6. All the flasks were uniformly inoculated and incubated on rotary shakers (150 rpm) for a period of 24 hours at room temperature (28 ± 2°C).

2.3.3 Measurement of growth

The growth of bacteria in the MSG broth was followed by estimating the turbidity of the broth by taking the absorbance at 660 nm in a UV-Visible Spectrophotometer (Hitachi Model 200).

2.3.4 Enzyme production

Enzyme production was measured by following the procedure of Imada et al., (1973) with slight modifications. The reaction mixture containing 0.5 ml of enzyme preparation plus 0.5 ml of phosphate buffer (0.2 M) pH 8 (unless

otherwise mentioned) plus 0.5 ml of 0.04 M L-glutamine and distilled water (0.5 ml) to a total volume of 2.0 ml was incubated for 30 min. at 37°C and the reaction was arrested by the addition of 0.5 ml of 1.5 M trichloroacetic acid. The precipitated protein was removed by centrifugation at 10,000 rpm for 20 min. To 0.1 ml of this supernatent, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added and the color developed after 10 min. was measured at 450 nm in a UV-Visible Spectrophotometer. Enzyme and substrate blanks were separately included in each assay.

Enzyme production was assessed in terms of enzyme activity which is expressed in terms of international units. One international unit of the enzyme is defined as the amount of enzyme that liberates 1 μ mol of ammonia under optimal assay conditions.

2.3.5 Determination of enzyme protein

Enzyme protein was measured according to the method of Lowry *et al.*, (1951) using Folin Ciocaltaeu's reagent

2.3.6 Selection of strains

All the 100 strains were ranked in terms of their level of enzyme production and the top four ranked strains were used for further studies.

2.4. GROWTH STUDIES

Growth studies were carried out for the four selected strains of bacteria belonging to the species of Pseudomonas and Vibrio (two each) as detailed below:

2.4.1 Optimization of growth conditions for maximal enzyme production by bacteria

Optimal conditions required for maximal growth and L-glutaminase production by the selected strains were determined by subjecting them to various incubation temperatures, different levels of pH, substrate concentrations, NaCl concentrations, additional carbon and nitrogen sources, glucose concentrations, inoculum concentrations in the growth media and different incubation periods.

2.4.1.1 Media

Mineral salts glutamine broth with the composition mentioned earlier (Section 2.2.3) was used for these studies

unless otherwise stated. The prepared media were dispensed in 100 ml aliquots in 250 ml Erlenmeyer conical flasks, autoclaved and used for optimization studies.

2.4.1.2 Preparation of inoculum and inoculation procedures

The procedure described in preveious section (2.3.2) was followed for the preparation of inoculum and inoculation of media. Concentration of inoculum was at 1% level unless otherwise stated.

2.4.1.3 Measurement of growth

Growth was measured according to the procedures mentioned in section 2.3.3.

2.4.1.4 Enzyme production

Enzyme production in the media was estimated in terms of enzyme activity as per procedures mentioned in section 2.3.4 except for the incubation temperature as 40°C and incubation time as 15 min. instead of 30 min. since it was observed that maximal amounts of enzyme units are obtained under these conditions. pH of the buffer varied from 6 to 8 according to the optimum pH of enzyme from each organism.

2.4.1.5 pH

Optimal pH for maximal growth and enzyme production was determined by subjecting the organisms to various pH levels (pH ranging from 4-11) adjusted in the culture broth (MSGB) using 1 N NaOH or 1 N HCl. After inoculation and incubation for 24 hours at room temperature (28 ± 2°C), the culture broths were centrifuged and growth and enzyme production were determined as per the procedures described under sections 2.3.3 and 2.4.1.4.

2.4.1.6 Temperature

Optimal temperature for maximal growth and enzyme production was estimated by incubating the MSG broth inoculated with the test strains at various temperatures (15, 25, 30, 35, 45 and 55°C) for a total period of 24 hours. Growth and enzyme production were determined as per the procedures mentioned earlier (Section 2.3.3 and 2.4.1.4).

2.4.1.7 Substrate concentration

Optimal substrate concentration that favours growth and enzyme production of the strains was checked by growing them in MSG broth supplemented with different

glutamine concentrations (0.25, 0.5, 1, 2, 3%). After 24 hours of incubation at room temperature (28 ± 2°C) the growth and enzyme production were estimated as per the procedures mentioned under sections 2.3.3 and 2.4.1.4).

2.4.1.8 NaCl concentration

Optimal NaCl concentration that promotes maximal growth and enzyme production of the organisms was determined by subjecting them to different NaCl concentrations (0, 1, 3, 5, 7 and 10%) adjusted in the MSG broth. After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production were analyzed according to the procedures described under sections 2.3.3 and 2.4.1.4).

2.4.1.9 Carbon sources

Requirement of additional carbon sources other than glutamine for enhanced enzyme yield was tested by the addition of glucose, galactose, starch, maltose, lactose, Na_2CO_3 and trisodium citrate in the MSG broth at 1% level. After 24 hours of incubation at room temperature (28 ± 2°C), growth and enzyme production were estimated (Sections 2.3.3 and 2.4.1.4).

2.4.1.10 Nitrogen sources

Requirement of additional nitrogen sources other than glutamine for enhanced growth and enzyme production was estimated by the addition of various nitrogen sources viz., peptone, beef extract, yeast extract, glutamic acid, lysine, $NaNO_3$ and KNO_3 at 1% concentration in the MSGB. After 24 hours of incubation at room temperature growth and enzyme production were estimated as mentioned earlier (Section 2.3.3 and 2.4.1.4).

2.4.1.11 Glucose concentration

Since glucose was found to enhance enzyme production during the studies, optimal requirement of glucose level in the culture medium was estimated by incorporating different concentrations of glucose (0.5, 1, 2, 3%) along with 1% glutamine in the MSGB. After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production were estimated (Section 2.3.3 and 2.4.1.4).

2.4.1.12 Inoculum concentration

Optimal inoculum size that yields maximal growth and enzyme production was determined in MSG broth at their

optimal growth conditions determined earlier by inoculating the broths with various levels of the prepared medium (1-7%). After 24 hours of incubation at room temperature (28 ± 2°C) growth and enzyme production in the media were estimated according to the procedures mentioned under sections 2.3.3 and 2.4.1.4).

2.4.1.13 Incubation time

Optimal incubation time that leads to maximal growth and enzyme production of the strains was estimated by incubating culture flasks for various incubation periods upto a maximum of 48 hours. Growth and enzyme production in the broths were estimated according to the procedures described under sections 2.3.3 and 2.4.1.4.

2.4.2 Growth curve

Growth curve studies for all the four strains of bacteria were carried out in nutrient broth (HI-media) and MSG broth at the optimal conditions standardized earlier. The prepared media were dispersed in 50 ml aliquots in 250 ml conical flasks, autoclaved and inoculated with 0.5 ml of the prepared inoculum and incubated at room temperature (28 ± 2°C). Samples were drawn at regular intervals and

growth was determined by measuring the turbidity at 660 nm in a UV-Visible Spectrophotometer besides enumeration of TVC on plates employing pourplate technique. From the results obtained growth curve was constructed.

2.5 COMPARISON OF INTRACELLULAR AND EXTRACELLULAR GLUTAMINASE PRODUCTION BY BACTERIA

A comparative study was made, on the extracellular and intracellular glutaminase production by the strains, by cultivating them in nutrient broth (HI-media) with and without the addition of glutamine and in MSG broth (composition mentioned earlier under section 2.3.1). Inoculation and incubation procedures were as detailed earlier under section 2.3.2. After 24 hours of incubation the cells were harvested by centrifugation at 5000 rpm for 20 min. and the supernatent was partially purified by $(NH_4)_2SO_4$ fractionation followed by dialysis and analyzed for extracellular glutaminase activity as per the procedures mentioned earlier under section 2.4.1.4.

Intracellular glutaminase production by cells was tested as stated below. The harvested cells were subjected to osmotic shock using 30% sucrose solution (Cedar & Schwartz, 1967) which resulted in cell lysis and liberation

of enzymes. The lysed cell suspension was centrifuged as mentioned above and the supernatent was fractionated with $(NH_4)_2SO_4$ followed by dialysis and later tested for glutaminase activity as mentioned under section 2.4.1.4.

2.6 ENZYME STUDIES

2.6.1 Isolation of enzymes

2.6.1.1 Media

The enzyme production medium (EPM) was designed based on the data obtained from the studies conducted for optimization of growth conditions for maximal enzyme production in MSG broth. The final composition of EPM after standardisation is as follows:

KH_2PO_4	:	0.10 g
$MgSO_4 7H_2O$	:	0.05 g
$NaNO_3$	:	0.01 g
$CaCl_2$	:	0.01 g
$Na_3C_6H_5O_7 2H_2O$	:	0.01 g
NaCl*	:	1 g
Glucose	:	0.5%
Glutamine	:	1%
pH	:	6 ± 0.2
Distilled water	:	100 ml

* For V.cholerae, no NaCl was used

Prepared medium was autoclaved and used.

2.6.1.2 Preparation of inoculum

a) A loopful of 18 hours old slope culture was transferred aseptically to 10 ml of EP medium and incubated for 24 hours at room temperature (28 ± 2°C).

b) One ml of the 18 hours old culture was then transferred to 50 ml of EP medium and incubated on a rotary shaker (150 rpm) at room temperature (28 ± 2°C) for a further period of 24 hours.

c) Cells were harvested by centrifugation at 5000 rpm for 20 min. washed repeatedly with physiological saline and suspended in 10 ml of the same saline.

d) The prepared cell suspension was adjusted to a concentration of 1×10^6 cells ml^{-1} and used as inoculum.

2.6.1.3 Enzyme production in the medium

Hundred ml of EP medium taken in 1000 ml conical flask was inoculated with 3 ml of the prepared inoculum aseptically and incubated on a rotary shaker (150 rpm) at 35 ± 2°C for 18 hours. Later the culture broth was centrifuged at 5000 rpm for 20 min. at 4°C and the cell free extract was used as the crude enzyme for further studies.

2.6.2 Enzyme assays

2.6.2.1 Buffers

Different buffers with various pH ranges were used for the assay of glutaminase. They included acetate buffer (0.2 M) with a pH ranging from 4 to 5, phosphate buffer (0.2 M) pH 6 to 8 and glycine-NaOH buffer (0.2 M) of pH 9-11.

2.6.2.2 Determination of enzyme activity

Enzyme activity was measured according to the procedure mentioned earlier (Section 2.4.1.4).

2.6.2.3 Determination of enzyme protein

Enzyme protein was estimated as per the procedure outlined under section 2.3.5.

2.6.3 Purification of enzymes

Enzyme purification was carried out following the methods suggested by Hartman (1968) and Roberts (1976). The cell free extract obtained after centrifugation (Section 2.6.1.3) was purified by $(NH_4)_2SO_4$ fractionation followed by dialysis and ion exchange chromatography.

2.6.3.1 $(NH_4)_2SO_4$ fractionation

The enzyme was purified by $(NH_4)_2SO_4$ precipitation. It was done by adding $(NH_4)_2SO_4$ (Sisco-enzyme grade) slowly and increasing the concentration upto 50-80% saturation along with continuous stirring using a magnetic stirrer, at 4°C, in an ice bath. The precipitate obtained for each saturation was removed by centrifugation at 5000 rpm for 30 min. and dissolved in phosphate buffer (0.2 M) of pH 6 or 8, (varied with organism). Enzyme activity and protein content of each fraction was determined according to the procedures mentioned in sections 2.4.1.4 and 2.3.5.

2.6.3.2 Dialysis

The precipitate obtained after $(NH_4)_2SO_4$ fractionation was dissolved in phosphate buffer (0.2 M) (pH 6 or 8) and dialyzed against the same buffer extensively at 4°C for 24 hours. Enzyme activity and protein content of the dialyzate were determined according to the procedures mentioned under sections 2.4.1.4 and 2.3.5.

2.6.3.3 Chromatography upon seralite SRA-400

The dialyzate was further purified by anion exchange chromatography, upon seralite SRA-400.

1. A strongly anionic exchange resin (seralite SRA-400, SRL) was packed in a column (5 x 30 cm) and kept at 4°C.

2. The packed column was equilibrated with sodium phosphate buffer of pH 8 (0.2 M) containing 1 mM EDTA.

3. The dialyzate was adjusted to the pH 8 with dilute NaOH (0.1 M) and added to the column.

4. The column was washed with sodium phosphate buffer of pH 8 (0.2 M) containing 1 mM EDTA.

5. Elution was carried out using the sodium phosphate buffer (0.2 M) of pH 8 containing different concentrations of NaCl (0.1, 0.2, 0.3, 0.4 M) and 1 mM EDTA in a sequential manner along with increasing concentration of NaCl in buffer. Flow rate was adjusted to 5 ml/10 min. and the fractions of 20 ml were collected using Redifrac fraction collector (Pharmacia). Each fraction was analyzed for enzyme activity and protein as mentioned earlier (Sections 2.4.1.4 and 2.3.5). The fractions with higher specific activities were pooled and concentrated using ammonium sulphate (50-60%) at 4°C (Section 2.6.3.1). The precipitate obtained was dissolved in a minimal volume of phosphate buffer of pH 6 to 8 (0.2 M) and dialyzed as per the procedure mentioned in section 2.6.3.2.

2.6.3.4 Chromatography upon seralite SRA-120

The dialyzate obtained after anion exchange chromatography was further purified by cation exchange chromatography using seralite SRA-120.

1. A cationic exchange resin (Seralite SRA-120, SRL) was packed into a column (5x30 cm) and kept at 4°C.

2. The packed column was equilibrated with sodium phosphate buffer (pH 6), 0.2 M containing 1 mM EDTA.

3. The dialyzate was adjusted to pH 6 with dilute HCl (0.1 N) and added to the column.

4. The column was washed with sodium phosphate buffer of pH 6 (0.2 M) containing 1 mM EDTA.

5. Column was eluted with sodium phosphate buffer (0.2 M) of pH 6 containing 1 mM EDTA and various concentrations of NaCl (viz., 0.1, 0.2, 0.3, 0.4 M) in a sequence of increasing concentration of NaCl. Flow rate was adjusted to 5 ml/10 min. and 20 ml fractions were collected. Each fraction was analyzed for enzyme activity and protein

according to the procedures mentioned under sections 2.4.1.4 and 2.3.5. Active fractions were pooled, and concentrated and stored at 4°C and used for further characterization studies.

2.6.4 Characterization of glutaminase

The purified enzyme was characterized for its activity at various pH, temperature, substrate concentration, incubation time, NaCl concentration, substrate specificity and inhibition by heavy metals and other substances. The stability of the enzyme at different pH and temperatures was also studied.

2.6.4.1 Effect of pH on activity and stability of the enzyme

Effect of pH on the activity of the enzyme was studied as per the following procedure.

A mixture containing 0.5 ml of purified enzyme and 0.5 ml of distilled water and 0.5 ml of 0.04 M glutamine was incubated with 0.5 ml of buffers of different pH ranging from pH 4-10 (prepared using acetate buffer pH 4-5), phosphate

buffer (pH 6-8) and glycine--NaOH buffer (pH 9-10) for 15 min. at 40°C. Later the enzyme activity in the reaction mixture was determined as mentioned earlier (Section 2.4.1.4).

Stability of the enzyme at various pH (viz., pH 4-10) was determined by incubating 1 ml of enzyme with 1 ml of buffers for 1 hour (prepared with acetate buffer pH 4-5, phosphate buffer pH 6-8, glycine--NaOH buffer pH 9-10).

A mixture containing 0.5 ml of this treated enzyme solution, 0.5 ml of distilled water, 0.5 ml of phosphate buffer of pH 6 or 8 (0.2 M) was incubated with 0.5 ml of 0.04 M glutamine for 15 min. at 40°C. Enzyme activity remained in the reaction mixture was determined according to the procedure mentioned under section 2.4.1.4.

2.6.4.2 Effect of temperature on activity and stability of enzyme

Effect of temperature on the activity of the enzyme was estimated according to the following procedure.

0.5 ml of purified enzyme added with 0.5 ml of distilled water and 0.5 ml of phosphate buffer of appropriate

pH (pH 6 or 8), (0.2 M) was incubated with 0.5 ml of 0.04 M glutamine at different temperatures (30, 40, 50, 60 and 70°C) for 15 min. Later the enzyme activity in the reaction mixture was determined according to the procedures mentioned under section 2.4.1.4.

The stability of the enzyme at various temperatures was determined by incubating 0.5 ml of the purified enzyme with 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8), (0.2 M) at different temperatures of 30, 40, 50, 60 and 70°C for 1 hour.

The treated enzyme buffer mixture was later incubated with 0.5 ml of distilled water and 0.5 ml of glutamine for 15 min. at 40°C. Enzyme activity remaining in the reaction mixture was estimated following the procedures mentioned under section 2.4.1.4.

2.6.4.3 Effect of substrate concentration on activity of the enzyme

Effect of substrate concentration on the activity of purified enzyme was determined by incubating 0.5 ml of enzyme with 0.5 ml of distilled water and 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8, 0.2 M) with

different concentrations of glutamine (viz., 0.01 M, 0.02 M, 0.03 M, 0.04 M, 0.05 M, 0.06 M, 0.08 M and 1 M) for 15 min. at 40°C. Reaction mixture was analyzed for glutaminase activity as per the procedures mentioned under the section 2.4.1.4). Michaelis - Menten constants (Km) of enzymes were evaluated from Line weaver - Burk plots of the data.

2.6.4.4 Effect of incubation time on activity of the enzyme

Effect of incubation time on the rate of hydrolysis of glutamine by purified enzyme was determined according to the following procedure.

Different aliquots of reaction mixture containing 0.5 ml of purified enzyme and 0.5 ml of distilled water and 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8, 0.2 M) was incubated with 0.5 ml of 0.04 M glutamine at 40°C for different time intervals of 3, 5, 10, 15, 20 and 30 min. Enzyme activity in the reaction mixture was estimated as per the procedures mentioned under section 2.4.1.4.

2.6.4.5 Effect of NaCl concentration on activity of the enzyme

Effect of NaCl concentration on the activity of enzyme was estimated by incubating the reaction mixture

containing 0.5 ml of purified enzyme and 0.5 ml of distilled water and 0.5 ml of phosphate buffer of pH 6 or 8 (0.2 M) and different concentrations of NaCl (viz., 0, 1, 3, 5, 7, 10, 15, 20 and 25%) for 15 min. at 40°C. Glutaminase activity in the reaction mixture was determined as per the procedures mentioned under section 2.4.1.4.

2.6.4.6 Determination of substrate specificity

0.5 ml of purified enzyme solution was incubated with 0.5 ml of distilled water, 0.5 ml of phosphate buffer (0.2 M) of appropriate pH (pH 6 for all strains except P.fluorescens ACMR 171 for which pH 8 was used. Buffer with pH 7.5 was used for all strains when tested with asparagine) and 0.04 M of asparagine, glutamine, glutamine plus asparagine separately for 15 min. at 40°C. Later enzyme activity was estimated following the procedures mentioned under section 2.4.1.4.

2.6.4.7 Effect of heavy metals on the activity of enzyme

0.5 ml of purified enzyme was incubated with 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8, 0.2 M) and 0.5 ml of glutamine and 0.5 ml of 1 mM of heavy metals such as Pb, Co, Mn, Hg, Cu, Fe, Ag, Ca and Zn separately

at 40°C for 15 min. Glutaminase activity in the reaction mixture was measured according to the procedures mentioned under section 2.4.1.4.

2.6.4.8 Effect of other substances activity of enzyme

Effect of other substances including EDTA, phosphate, borate and tris ions, aspartic acid, glutamic acid, phenolphthalein, bromocresol purple and α-keto glutarate on the activity of the enzyme was determined by the following procedure.

0.5 ml of purified enzyme was incubated with 0.5 ml of phosphate buffer of appropriate pH (pH 6 or 8, 0.2 M) plus 0.5 ml of the above mentioned substances at different concentrations (phosphate at 0.4 M; borate and tris ion at 0.2 M; glutamate and aspartate at 30 mM, EDTA at 0.1 mM, α-ketoglutarate at 2 mM, phenolphthalein and bromocresol purple at 1 mM) and 0.5 ml of 0.04 M glutamine at 40°C for 15 min. Enzyme activity was estimated as per the procedures mentioned under section 2.4.1.4.

2.7 FERMENTATIVE PRODUCTION OF GLUTAMINASE BY SOLID STATE FERMENTATION (SSF)

2.7.1 Preparation of solid substrate

Commercially available wheat bran of <0.24 cm particle size was used in the solid state fermentation (SSF)

studies for the production of glutaminase. However the exact size of particles were optimised first and later that sized particles were used in all the later studies. Composition of wheat bran is shown in Table 1. The wheat bran medium for SSF was prepared as detailed below: (Ramesh, 1989).

Hundred gram of wheat bran taken in 1000 ml Erlenmeyer conical flask was thoroughly mixed with MSG solution (Mineral Salts Glutamine Solution - composition as given under section 2.3.1 but for the volume of distilled water. Volume of the water was separately standardised to give 60% moisture content to the final wheat bran medium) and the flask was autoclaved for 60 min. and allowed to cool down to room temperature (28 ± 2°C).

2.7.2 Inoculation and incubation

The prepared inoculum (Section 2.6.1.2) was adjusted to a ratio of 20 mg dry cell equivalent/100 g wheat bran (Ramesh, 1989) and added to the sterilized moist wheat bran in the flasks. The contents were mixed thoroughly and the flasks were incubated in a slanting position at 35°C in an incubator with 65-70% relative humidity (Ramesh & Lonsane, 1987a) for 24-28 hours.

2.7.3 Extraction and recovery of enzyme

2.7.3.1 Optimization of extraction parameters

Extraction of glutaminase from bacterial wheat bran (BWB) after SSF was optimized for maximal enzyme recovery which included drying temperature of BWB, extraction medium, buffer system, pH of extraction, ratio of bran to buffer, extraction temperature and contact time were optimized (Kumar & Lonsane, 1987) as detailed below:

2.7.3.1.1 Drying temperature

Effect of drying temperature on enzyme recovery was studied by drying the BWB at various temperatures of 30°C, 40°C and 50°C for 1 hour.

Later the enzyme was extracted under arbitrarily selected conditions including 0.2 M phosphate buffer of appropriate pH (pH 6 or 8, varied with the organism) in a 1 : 5 ratio (bran to buffer) and a contact time of 90 min. at 30°C. It was carried out in two stages. In the first stage the BWB was contacted with 300 ml of buffer for 60 min. with occasional stirring. Then the slurry was squeezed through a dampened cheese cloth. In the second stage the left over solids were again contacted with another

200 ml of the same buffer for another 30 min. and later the slurry was squeezed as mentioned earlier. The extracts were pooled and centrifuged for 20 min. at 8000 rpm in a refrigerated centrifuge (Ramesh & Lonsane, 1990). The cell free clear extract obtained after centrifugation was used for estimation of enzyme activity (Section 2.4.1.4).

2.7.3.1.2 Extraction medium

Appropriate medium which shall yield maximal enzyme recovery on extraction was determined using ethanol, distilled water, distilled water plus 1% NaCl, tap water and 0.2 M phosphate buffer of appropriate pH (pH 6 or 8). Enzyme extraction and estimation of enzyme activity were carried out as per the procedures mentioned earlier under sections 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.3 pH of extraction media

Effect of pH of the buffer used for extraction were determined by using buffers of pH ranging from 5-8 (Acetate buffer, 0.2 M of pH 5 phosphate buffer 0.2 M, of pH 6-8). Enzyme recovery and estimation of enzyme activity were done according to the procedures mentioned under section 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.4 Different buffer systems

Influence of different buffer systems on the enzyme recovery was analyzed by using 0.2 M acetate buffer of pH 6, phosphate buffer of pH 6, phosphate buffer of pH 8 (0.2 M), borate buffer of pH 8 (0.2 M) according to the optimum pH. Enzyme extraction and enzyme activity estimation were carried out as per the procedures mentioned in sections 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.5 Ratio of bran to buffer

Effect of bran to buffer ratio on the enzyme recovery was determined by adding buffer to fermented BWB in different ratios of 1:3, 1:5 and 1:10. Enzyme recovery and estimation of enzyme activity were carried out as per the procedures mentioned under section 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.6 Effect of contact time

Effect of contact time of buffer with fermented BWB was tested by contacting the BWB with buffer for various time intervals of 30, 60, 90, 120 min. Enzyme recovery and estimation of enzyme activity were done according to the procedures mentioned in sections 2.7.3.1.1 and 2.4.1.4.

2.7.3.1.7 Effect of contact temperature

Effect of contact temperature on enzyme recovery was determined by keeping BWB and buffer in contact at different temperatures of 25, 35, 45°C. Enzyme recovery and estimation of enzyme production were performed by following the procedures mentioned under sections 2.7.3.1.1 and 2.4.1.4.

2.7.4 Effect of operational parameters on enzyme production by SSF

Effect of pH, temperature, moisture content, substrate concentration, NaCl concentration, inoculum size, particle size of wheat bran and various incubation time on enzyme production by SSF was studied as detailed below:

2.7.4.1 Measurement of enzyme production

Enzyme production was determined according to the procedures mentioned earlier under section 2.4.1.4.

The enzyme units were calculated according the methods suggested by Ramesh (1989) for the total volume of the extract after centrifugation divided by the initial weight of wheat bran (in g) gives the units of enzyme per gram of commercial wheat bran. The enzyme units are expressed per gram of commercial wheat bran.

2.7.4.2 Effect of particle size of wheat bran

The effect of particle size of wheat bran on enzyme production during SSF was determined by using wheat bran of different particle size. Commercial wheat bran was graded into various fractions of their particle size using sieves of mesh size ranging from 7-14. The fractions were having particle size of greater than 2.41 mm, 1.41-2.06 mm and less than 1.20 mm. Inoculation and incubation, enzyme recovery and enzyme estimation were carried out as mentioned earlier. (Sections 2.7.2, 2.7.3.1.1 and 2.4.1.4).

2.7.4.3 Effect of moisture content of WB medium

Hundred gram of commercial wheat bran of optimised particle size was weighed and distributed in 1000 ml Erlenmeyer conical flasks. The moisture content of the WB medium was adjusted to various levels ranging from 20-60% (w/w) by varying the water content of the MSG solution. Inoculation and incubation were carried out as mentioned earlier (section 2.7.2). After 48 hours of incubation the contents in the flasks were subjected to analysis for estimation of enzyme production (Sections 2.7.3.1.1 and 2.4.1.4)

2.7.4.4 Effect of pH

Effect of pH on the enzyme production by SSF was determined by subjecting the organisms to various pH levels adjusted in the MSG solution from pH 4-10 (Section 2.3.1). Inoculation, incubation, enzyme extraction and estimation of enzyme activity were carried out following the procedures mentioned earlier under sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

2.7.4.5 Effect of temperature

Effect of temperature on enzyme production by SSF was determined by incubating the inoculated wheat bran medium (Section 2.7.1) at different temperatures of 25, 35, 45 and 55°C. Inoculation, incubation, enzyme recovery and estimation of enzyme production were done according to the procedures mentioned in section 2.7.2, 2.7.3.1.1, and 2.4.1.4.

2.7.4.6 Effect of substrate concentration

Effect of substrate concentration on enzyme production by SSF was determined at different substrate concentrations (0.25, 0.5, 1, 2, 3%) in the MSG solution added to the wheat bran. Inoculation, incubation, enzyme recovery and estimation of enzyme production were carried out according to the procedures mentioned in sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

2.7.4.7 Effect of NaCl concentration

Effect of NaCl concentration on enzyme production by SSF was carried out by adjusting the NaCl concentration of MSG solution to various levels of 0, 1, 3, 5, 7% and subjecting the organisms for enzyme production. Inoculation, incubation, enzyme extraction and estimation of enzyme production were done according to the procedures mentioned earlier in sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

2.7.4.8 Effect of carbon sources

Effect of additional carbon sources on enzyme production by SSF was determined by incorporating carbon sources viz., glucose, galactose, maltose, starch, lactose, Na_2CO_3 and trisodium citrate at 1% concentration level to the WB medium (Section 2.7.1). Inoculation, incubation, enzyme extraction and estimation of enzyme production were done according to the procedures mentioned under sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

2.7.4.9 Effect of nitrogen sources

Influence of extra nitrogen sources on enzyme production by SSF was determined by the addition of beef extract, yeast extract, peptone, glutamic acid, lysine, KNO_3

and $NaNO_3$ at 1% level separately to the wheat bran medium (Section 2.7.1). Inoculation, incubation, enzyme recovery and estimation of enzyme production were carried out as per the procedures mentioned earlier (Sections 2.7.2, 2.7.3.1.1 and 2.4.1.4).

2.7.4.10 Effect of inoculum concentration

Effect of inoculum concentration on enzyme production by SSF was determined by using different levels of inoculum. Inoculum ratio was adjusted to 10, 20, 30 and 40 mg dry cell equivalents/100 g of wheat bran. Inoculation, incubation, enzyme recovery amd estimation of enzyme production were carried out according to the procedures mentioned earlier (Sections 2.7.2, 2.7.3.1.1 and 2.4.1.4).

2.7.4.11 Effect of incubation time

Effect of incubation time on enzyme production by SSF was determined by incubating the inoculated wheat bran medium (Section 2.7.1) for a total period of 48 hours and estimating the enzyme production at regular intervals of 6 hours. Inoculation, incubation, extraction of enzyme and enzyme production were estimated according to the procedures mentioned under sections 2.7.2, 2.7.3.1.1 and 2.4.1.4.

3. RESULTS

3.1 ENUMERATION AND ISOLATION OF L-GLUTAMINASE PRODUCING MICROORGANISMS

Bacteria producing L-glutaminase were quantitatively and qualitatively enumerated in water and sediment samples of both marine and estuarine environments of Cochin. Results obtained for the quantitative distribution of L-glutaminase producing bacterial population (GPB) associated with water and sediment samples are presented in Table 2.

In general, GPB varied from $1.0x10^5$/g to $4.5x10^7$/g in the sediments and from $1.0x10^7$/ml to $5.5x10^7$/ml in the water samples of marine environments and from $1.7x10^6$/ml to $3.0x10^7$/ml and $2.5x10^6$/g to $4.5x10^7$/g in water and sediment samples respectively in the estuarine environments during the period of analysis carried out over six months. Marine environments, especially seawater harboured maximal glutaminase producers compared to the estuarine environments.

Qualitative distribution of L-glutaminase producing bacteria present in various marine and estuarine environments are presented in terms of percentage of

occurrence in Table 3. It is evident from the table that, among the total 500 isolates identified, gram negative, forms were dominant over gram positive groups. In general, gram positive forms were more in sediment samples than in water. L-glutaminase producers were distributed only among the species of Pseudomonas, Aeromonas, Vibrio, Alcaligenes, Acinetobacter, Bacillus and Planococci. Of them Pseudomonas sp. were dominant in marine samples (42% in water and 41.94% in sediment) while Acinetobacter sp. (35%) and Bacillus (33.33%) were dominant in water and sediment samples in estuarine environment respectively. However, in general, Pseudomonas was a prominent group in all environments except in estuarine water and all others included the lesser dominant groups.

3.2 SELECTION OF POTENTIAL L-GLUTAMINASE PRODUCING BACTERIA

Selection of potential strains for glutaminase production was carried out on the basis of quantitative determination of growth and glutaminase production in a mineral salts media supplemented with 1% glutamine as the sole carbon source. Results obtained for the analysis of glutaminase production by 100 strains were individually ranked from 1 to 100 and the first four ranked strains were selected. (Two Pseudomonas sp. isolated from marine water,

(ACMR 43); and marine sediment, (ACMR 171) and two Vibrio sp., isolated from estuarine water, (ACMR 267) and estuarine sediment, (ACMR 347)) for further studies.

3.3 IDENTIFICATION OF SELECTED STRAINS

All the four potential strains selected after secondary screening were tentatively identified as Pseudomonas fluorescens (ACMR 171), Pseudomonas fluorescens (ACMR 43), Vibrio costicola (ACMR 267) and Vibrio cholerae (ACMR 347) based on their morphological, biochemical and physiological characters (Table 4a and 4b) according to the schemes outlined in Bergey's Manual of Systematic Bacteriology (Buchanan & Gibbons, 1974; Krieg & Holt, 1984)

3.4 GROWTH STUDIES

Optimal levels of environmental variables namely pH, temperature, NaCl concentration, substrate concentration, carbon sources, nitrogen sources, inoculum concentration and incubation time for maximal growth and enzyme production by P.fluorescens ACMR 171 and ACMR 43, V.costicola ACMR 267 and V.cholerae ACMR 347 were determined for submerged conditions in flask (SmF) and the results obtained for the various studies are presented below:

pH

Optimal pH requirement for maximal production of selected strains was determined by subjecting them to various levels of pH (pH 4-11) in the mineral salts glutamine broth (MSGB). Growth is expressed in terms of optical density at 660 nm and enzyme production as enzyme activity (u/ml) (Fig.1). Results indicate a general linear relationship between growth and enzyme production with reference to pH. Thus maximal growth and maximal enzyme production were observed at same pH on several occasions.

All the strains produced maximal levels of glutaminase at pH 6 (2.114-3.268 u/ml) although they could also produce significant levels at pH ranging from 5 to 8 (1.174-3.268 u/ml). However, optimal pH required for maximal growth varied among the strains. While P.fluorescens ACMR 171 and V.cholerae ACMR 347 required pH 5 (OD 0.958) and pH 7 (OD 0.965) respectively for maximal growth, P.fluorescens ACMR 43 (OD 0.728) and V.costicola ACMR 267 (OD 0.902) grow well at pH 6. In general, all the four strains exhibited significant levels of growth at a wide range of pH varying from pH 5-8 (OD 0.41-1.02). Alkaline pH levels above pH 8 and acidic pH levels below pH 4 did not favour significant growth and enzyme production.

Noticeably the strains (P.fluorescens ACMR 43 and V.costicola ACMR 267) isolated from water exhibited maximal enzyme production along with maximal growth whereas those isolated from sediment showed a slight variation in their requirement of optimal pH for their maximal enzyme production and maximal growth, which was not so marked. In general, among the four strains tested V.cholerae ACMR 347 produced higher levels of glutaminase (3.268 u/ml) followed by P.fluorescens ACMR 171 and V.costicola ACMR 267.

Temperature

Optimal temperature required for maximal growth and glutaminase production by bacteria was analyzed by subjecting the organisms to different incubation temperatures viz., 15, 25, 30, 35, 45 and 55°C. Results presented in Fig.2 indicate that all the strains preferred 35°C for their maximal growth (OD 0.676-0.992) and glutaminase production (2.114-3.268 u/ml). However, they could also record significant levels of growth and enzyme production at other temperatures varying from 25-45°C (OD 0.414-0.992 and 0.938-3.268 u/ml). Incubation at higher temperatures above 45°C and below 25°C did not favour significant growth and enzyme production by all the strains.

Interestingly maximal enzyme production by all the strains was associated with their concurrent maximal growth at 35°C unlike that observed for other parameters. Among the four strains, species of V.cholerae ACMR 347 produced higher levels of glutaminase at 35°C and at 30°C (3.268 u/ml and 3.104 u/ml respectively) followed by P.fluorescens ACMR 43 (2.584 u/ml), V.costicola ACMR 267 (2.348 u/ml) and P.fluorescens ACMR 171 (2.114 u/ml). The same trend was also recorded at 30°C and 25°C. The most interesting observation was that at 15°C both Vibrios (1.174 u/ml for ACMR 267 and 1.292 u/ml for ACMR 347 could record relatively significant levels of enzyme production than Pseudomonas sp., (0.587 u/ml for ACMR 171 and 0.939 u/ml for ACMR 43). Whereas at 45°C, both the Pseudomonas sp. (1.409 u/ml for ACMR 171 and 2.584 u/ml for ACMR 43) could produce relatively higher levels of enzyme than Vibrio sp. (0.938 u/ml for ACMR 267 and 1.409 u/ml for ACMR 347).

Substrate Concentration

The variation in the rate of growth and glutaminase production with reference to the concentration of substrate (0.25-3%) are presented in Fig.3. All the strains recorded significant levels of growth (OD 0.244-1.1) and glutaminase production (0.587-3.168 u/ml) at substrate concentrations

ranging from 0.5-3%. However, the maximal levels of enzyme production by all the strains were effected at 1% substrate concentration except for P.fluorescens ACMR 43 which preferred 0.5% for their maximal enzyme production. While a steady increase in both growth and enzyme production was observed at 0.25-1% substrate concentrations further increase from 1 to 3% resulted in a decrease in the growth and enzyme production of all the strains. Both the Vibrio sp. and P.fluorescens ACMR 43 from marine water exhibited maximal enzyme production along with their maximal growth excepting P.fluorescens ACMR 171.

Interestingly P.fluorescens ACMR 43 recorded comparatively higher levels of enzyme production (2.818 u/ml) along with maximal growth at 0.5% substrate concentration, unlike P.fluorescens ACMR 171 and V.costicola ACMR 267 which recorded relatively only a lesser level of enzyme production at 1% substrate concentration (2.348 u/ml for ACMR 171 and 2.114 u/ml for ACMR 267) which was their optimal substrate concentration level.

In general maximal enzyme production was recorded by V.cholerae ACMR 347 (3.168 u/ml) followed by P.fluorescens ACMR 43 (2.818 u/ml) P.fluorescens ACMR 171 (2.348 u/ml) and

V.costicola ACMR 267 (2.114 u/ml). Interestingly at substrate concentrations ranging from 1-3%, the strains isolated from sediment (P.fluorescens ACMR 171 and V.cholerae ACMR 347) exhibited higher levels of enzyme production.

NaCl Concentration

NaCl concentration required for maximal growth and synthesis of glutaminase by bacteria were tested at various levels of NaCl ranging from 0-10%. Data presented in Fig.4 indicate that all the strains could produce maximum levels of glutaminase in the presence of 3% NaCl except V.cholerae ACMR 347 which did the same in the absence of NaCl. While both Vibrios recorded maximal enzyme production (3.054 u/ml for ACMR 267 and 3.538 u/ml for ACMR 347) along with maximal growth (OD 1.3 for ACMR 267 and 1.084 for ACMR 347), both the strains of P.fluorescens did not record maximal enzyme production (3.459 u/ml for ACMR 171 and 2.584 u/ml for ACMR 43) along with maximal growth (OD 1.1 for ACMR 171 and 0.792 for ACMR 43). However, the variation was insignificant when compared to the level of enzyme produced at their maximal growth point. NaCl concentrations above 3% led to a gradual decline in both growth and enzyme production. While all strains registered an increase in their level of enzyme production along with an increase in NaCl from 0 to 3%, V.cholerae ACMR 347 showed a decrease in enzyme production

along with increase in NaCl concentration from 0 to 7% during the study. Maximal enzyme production was recorded by V.cholerae ACMR 347 (3.538 u/ml) followed by P.fluorescens ACMR 171 (3.459 u/ml), V.costicola ACMR 267 (3.054 u/ml) and P.fluorescens ACMR 43 (2.584 u/ml). In general the enzyme production varied from 0.234-3.459 u/ml for the marine strains (both Pseudomonas sp.) and from 0.117 to 3.538 u/ml for the estuarine strains (both Vibrio sp.) suggesting less differences in their level of enzyme production. Both strains isolated from sediment (ACMR 171 and ACMR 347) recorded maximal enzyme production compared to that of water (ACMR 43 and ACMR 267).

Carbon Sources

Effect of additional carbon sources on maximal growth and enzyme production by glutaminase producing bacteria was determined using different carbon sources incorporated in the mineral media in addition to glutamine. In general, all additional carbon sources tested, recorded significant levels of growth (OD 0.591-1.964) and glutaminase production (1.408-3.838 u/ml). However, among the various carbon sources tested, only glucose enhanced significant levels of both growth (OD 1.24-1.964) and enzyme production (3.054-3.838 u/ml) by all the strains followed by trisodium citrate (OD 0.846-1.326 and 2.114-3.114 u/ml) when compared to glutamine as the only substrate (Table 5). Interestingly

glucose enhanced higher levels of enzyme production by estuarine strains (3.648 u/ml for ACMR 267 and 3.838 u/ml for ACMR 347) than marine strains (3.524 u/ml for ACMR 171 and 3.054 u/ml for ACMR 43).

While glucose resulted in enhanced glutaminase production by all the strains, trisodium citrate favoured enhanced enzyme production in all but P.fluorescens ACMR 171 which responded to maltose for enhanced enzyme production. Na_2CO_3 resulted in a decrease in enzyme production compared to that in glutamine medium for all the strains. Galactose and lactose reduced enzyme levels in three strains (ACMR 171, ACMR 43 and ACMR 347) while playing a neutral role for the fourth strain (ACMR 267). Maltose reduced enzyme production by both Vibrios while enhancing enzyme production by ACMR 171 and ACMR 43 was not influenced by maltose. Starch in fact marginally repressed glutaminase production by P.fluorescens ACMR 43, V.costicola ACMR 267 and V.cholerae ACMR 347 while unaffecting P.fluorescens ACMR 171.

Nitrogen Sources

Effect of nitrogen sources on growth and enzyme production by the selected strains was tested by incorporating various nitrogen sources in growth media in addition to

glutamine. Results presented in Table 6 indicated that all the four strains could register significant levels of growth (OD 0.681-4.024) and glutaminase production (0.704-3.524 u/ml) in the presence of additional nitrogen sources.

While beef extract enhanced maximal enzyme production by both strains of marine Pseudomonas fluorescens (2.348 u/ml for ACMR 171 and 2.818 u/ml for ACMR 43), lysine promoted the same in the estuarine Vibrio sp. (3.288 and 3.524 u/ml respectively for V.costicola ACMR 267 and V.cholerae ACMR 347). While beef extract induced maximal growth in bacteria from water P.fluorescens ACMR 43 (1.846 OD) and V.costicola ACMR 267 (2.982 OD), peptone promoted enhanced growth in the sediment strains P.fluorescens ACMR 171 (2.568 OD) and V.cholerae ACMR 347 (4.024 OD). Except for P.fluorescens ACMR 43 which was induced by beef extract for maximal growth along with maximal enzyme production all the other strains did not exhibit any such simultaneous enhancement of growth and enzyme production for beef extract or any other substrates tested. While beef extract induced the enzyme production by both the marine strains (Pseudomonas), it repressed the enzyme production in both the estuarine Vibrio strains. Lysine did not adversely affect the enzyme production by any strain tested.

Peptone and glutamic acid while inducing the enzyme production in V.costicola ACMR 267, caused the maximal level of repression in all other strains. Enzyme production by V.cholerae ACMR 347 was found to be affected by all of these nitrogen sources except KNO_3 which caused a marginal repression. In general KNO_3 repressed all the strains leading to minimal enzyme production in the media.

Glucose Concentration

Results for the carbon source test indicated that glucose was the only additional nutrient that enhanced significant levels of glutaminase production along with glutamine. Hence in order to find the optimum concentration of glucose that induce maximum level of glutaminase, further experiment was carried out with different concentrations of glucose and the results are presented in Table 7.

In general all the strains registered significant levels of growth (OD 0.452-1.83) and glutaminase production (1.408-3.948 u/ml) at all levels of glucose concentrations tested. However, maximal levels of enzyme production (3.446-3.948 u/ml) by all the strains was recorded at 0.5% glucose concentration except V.costicola ACMR 267 which preferred 1% glucose concentration for its maximal glutaminase

production (3.446 u/ml). While maximal growth of both the P.fluorescens ACMR 171, P.fluorescens ACMR 43 and V.cholerae ACMR 347 occurred at 1% glucose (OD 1.116-1.83), V.costicola ACMR 267 required 2% glucose concentration for its maximal growth (OD 1.225). However, increased levels of glucose concentration above the optimal concentration resulted in a decline in enzyme production by all the strains. Higher titres of enzyme production was recorded by V.cholerae ACMR 347 (3.948 u/ml) followed by P.fluorescens ACMR 171 (3.788 u/ml) P.fluorescens ACMR 43 (3.588 u/ml) and V.costicola ACMR 267 (3.446 u/ml). There was no linear relationship between maximal enzyme production and maximal growth with reference to different glucose concentrations.

While 0.5% glucose enhanced the maximal level of enzyme production in the sediment P.fluorescens ACMR 171 (3.788 u/ml) than in water P.fluorescens ACMR 43, P.fluorescens of water (ACMR 43) showed higher levels of enzyme production than P.fluorescens of sediment (ACMR 171) at glucose concentration of 0.2%, 1% and 2%. Whereas in the case of Vibrios, V.cholerae of sediment (ACMR 347) recorded higher levels of enzyme production than V.costicola of water (ACMR 267) at all glucose concentrations tested.

Inoculum Concentration

Optimal inoculum concentration for maximal growth and enzyme production by all the four strains were determined by inoculating the medium with four different inoculum concentrations of 1, 3, 5, 7% (v/v) and the results are presented in Fig.5. All the strains exhibited significant levels of growth (OD 0.74-1.4) and enzyme production (1.878-3.438 u/ml) at all levels of inoculum tested. However, maximal levels of glutaminase production by both Vibrio sp. and P.fluorescens ACMR 171 was recorded at 3% inoculum concentration while P.fluorescens ACMR 43 required 1% inoculum concentration for maximal enzyme production. Further increase in inoculum concentration resulted in a declining trend in glutaminase production by all the strains although growth demonstrated a linear increase along with increase in inoculum concentration thus recording the maximal growth at 7% inoculum concentration for all the strains.

Maximal enzyme production was registered by V.cholerae ACMR 347 (3.438 u/ml) followed by V.costicola ACMR 267 and P.fluorescens ACMR 43 (2.584 u/ml) and P.fluorescens ACMR 171 (2.348 u/ml). Comparatively V.cholerae ACMR 347 registered a higher level of enzyme

production (2.86 u/ml), at the minimal level of inoculum concentration tested (1%), by other strains.

Incubation Time

Optimal incubation time required for attaining maximal growth and glutaminase production by all the four strains were studied for a total period of 48 hours. From the results presented in Fig.6 it is evident that excluding P.fluorescens ACMR 43 which required 24 hours all the rest could maximally produce the enzyme and grew well by 18 hours itself.

In general, all the strains exhibited significant levels of growth and enzyme production right from 6 hours of incubation onwards till the end of incubation at 48 hours, although the maximum was recorded at 18-24 hours (1.292-2.114 u/ml) for P.fluorescens ACMR 171, 0.704-2.348 u/ml for P.fluorescens ACMR 43, 0.587-2.114 u/ml for V.costicola ACMR 267, 0.587-3.46 u/ml for V.cholerae ACMR 347. Further incubation after 24 hours upto 48 hours did not favour additional growth and enzyme production by all the strains compared to that recorded at 18 hours.

Maximal enzyme production by all the strains occurred at their maximal growth point except P.fluorescens ACMR 43 which rended maximal growth at 30 hours. However, maximal enzyme production was recorded in V.cholerae ACMR 347 (3.46 u/ml) followed by P.fluorescens ACMR 171 , and P.fluorescens ACMR 43 (2.348 u/ml) and V.costicola ACMR 267 (2.114 u/ml).

Interestingly at 6 hours of growth both marine Pseudomonas strains exhibited higher enzyme production (1.292 u/ml for P.fluorescens ACMR 171 and 0.704 u/ml for P.fluorescens ACMR 43) than estuarine Vibrio strains (0.587 u/ml for V.costicola ACMR 267 and 0.587 u/ml for V.cholerae ACMR 347). Whereas at 48 hours of incubation both strains isolated from sediment possessed higher levels of enzyme production (1.878 u/ml for P.fluorescens ACMR 171 and 2.00 u/ml for V.cholerae ACMR 347) than those isolated from water (1.587 u/ml for P.fluorescens ACMR 43 and 1.408 u/ml for V.costicola ACMR 267).

Growth Curve

Growth curves obtained for all the four bacteria grown in both mineral medium and nutrient broth are presented in Fig.7-10. In general, all the strains after

remaining in the lag phase for about 3-5 hours initially, spent about 7 hours in the logarithmic phase and later entered the stationary phase at about 10-12 hours of growth which prevailed till the end of incubation (24 hours), in both the media tested.

Generation time for all the strains except V.cholerae ACMR 347 were found to be longer in mineral media (72 min. for P.fluorescens ACMR 171, 60 min. for P.fluorescens ACMR 43, 84 min. for V.costicola ACMR 267) than in nutrient broth (54 min. for ACMR 171, 48 min. for ACMR 43 and 60 min. for ACMR 267) whereas V.cholerae ACMR 347 possess a longer generation time in nutrient broth (42 min.) than in mineral media (36 min.). Among the four strains longer generation time was exhibited by V.costicola ACMR 267 (84 min) while V.cholerae ACMR 347 possess the shortest generation time (36 min).

3.5 PRODUCTION OF EXTRA AND INTRACELLULAR GLUTAMINASE

Production of glutaminase, both as extracellular and intracellular, during growth in three different media (mineral media supplemented with 1% glutamine, nutrient broth and nutrient broth added with 1% glutamine) at optimal

conditions was observed for both the P.fluorescens strains (ACMR 171 and ACMR 43) and for V.costicola (ACMR 267) and V.cholerae (ACMR 347) (Table 8).

Extracellular glutaminase fractions were in higher titres than intracellular during their growth in all the 3 types of media tested. However, both the fractions were higher in mineral media supplemented with 1% glutamine compared to that in nutrient broth with and without glutamine. A comparative analysis of data indicate that glutaminase production as extracellular fraction is about 2.6-6.8 times greater than intracellular fraction. Further on an average about 2.5 fold production of glutaminase could be achieved in mineral media added with glutamine than in nutrient broth with glutamine. Nutrient broth did not favour intracellular synthesis of glutaminase by all the strains. However, on addition of 1% glutamine meagre levels of intracellular fractions were noticed for all the strains.

Both the P.fluorescens strains produced significant levels of extracellular and intracellular glutaminase when grown in mineral media (0.704-1.878 u/ml) for ACMR 171 and 0.469-2.348 u/ml for ACMR 43. While P.fluorescens ACMR 171 recorded feeble amount of glutaminase in nutrient broth

(0.234 u/ml) P.fluorescens ACMR 43 recorded not even detectable levels of enzyme. Production of intracellular fraction in both cases were not detectable. However, both the strains registered an increased production of both intra and extracellular glutaminase during growth in nutrient broth added with glutamine (0.393 u/ml and 1.408 u/ml for ACMR 171 and 0.234 u/ml and 1.057 u/ml for ACMR 43).

Similarly both strains of V.cholerae ACMR 347 and V.costicola ACMR 267 recorded significant levels of production of intracellular and extracellular fractions of glutaminase in mineral media (0.469 u/ml and 2.114 u/ml for ACMR 171 and 0.413 u/ml and 2.818 u/ml for ACMR 43). While both strains registered feeble levels of extracellular fraction of enzyme when grown in nutrient broth, there was no detectable levels of intracellular fraction in both cases. Eventhough both strains produced extracellular fractions of the enzyme in appreciable levels in nutrient media with 1% glutamine, only meagre amount of intracellular fraction was recorded by V.costicola ACMR 267.

3.6 PURIFICATION AND RECOVERY OF GLUTAMINASE FROM PSEUDOMONAS sp. AND VIBRIO sp.

Glutaminase isolated from Pseudomonas sp. and Vibrio sp. were purified and recovered after $(NH_4)_2SO_4$

precipitation (50-80% saturation) and dialysis followed by ionexchange chromatography. Results obtained for purification steps with reference to total activity, specific activity and yield of the enzyme are presented in Table 9.

In general, total activity of crude extract of all the four bacteria ranged from 281.8-393.7 units whereas the total activity in the final purified preparation ranged from 129.2-140.9 units. Initially the specific activity of the crude extracts of all strains varied from 1.5-2.2 units/mg of protein. However, after purification specific activities of enzyme preparation were observed to be increased significantly (61.7-88.49 u/mg of protein). After purification 40.22-56.00 fold purified glutaminases were obtained with a yield ranging from 35.79-45.84%.

Purified preparation of glutaminase with maximal specific activity was obtained from P.fluorescens ACMR 43 (88.49 u/mg of protein) followed by P.fluorescens ACMR 171 (80.75 u/mg of protein) V.cholerae ACMR 347 (71.77 u/mg of protein) and V.costicola ACMR 267 (61.7 u/mg of protein). Maximal yield of purified glutaminase was recorded for P.fluorescens ACMR 43 (45.84%) followed by P.fluorescens ACMR 171 (42.31%), V.costicola ACMR 267 (39.28%) and

V.cholerae ACMR 347 (35.79%). However, maximal fold of recovery of glutaminase was from V.costicola ACMR 267 (56.09) followed by V.cholerae ACMR 347 (47.84); P.fluorescens ACMR 171 (47.50) and P.fluorescens ACMR 43 (40.22).

Glutaminase with the highest specific activities and yield were obtained from Pseudomonas strains when compared to that of Vibrio strains. Whereas maximal fold of recovery of glutaminase was incurred from Vibrio strains (56.09 for ACMR 267 and 47.84 for ACMR 347) when compared to Pseudomonas strains (47.5 for ACMR 171 and 40.22 for ACMR 43).

Effect of pH on the Activity and Stability of Glutaminase

Effect of pH on the activity and stability of purified glutaminase prepared from the four bacteria was tested by subjecting them to various pH levels ranging from pH 4-10. Data presented in Fig.15 indicate that enzymes of all the strains were maximally active and stable at pH 6 except P.fluorescens ACMR 171 which preferred pH 8 for the same.

In general, glutaminase of all the strains could demonstrate stability and considerable activity over a wide range of pH (pH 4-9). Relatively they were more stable and active in the acidic range of pH (4-7) than in the alkaline range (pH 7-9). Thus except P.fluorescens ACMR 171 all the rest lost their stability at pH 9 and 10.

Maximal activity was recorded by glutaminase of P.fluorescens ACMR 171 (1.527 u/ml) followed by P.fluorescens ACMR 43 (1.409 u/ml) V.cholerae ACMR 347 (1.409 u/ml) and V.costicola ACMR 267 (1.292 u/ml). However, all the strains exhibited 100% stability at their optimal pH except V.cholerae ACMR 347 which retained only 95.05% of its optimal activity at the optimal pH.

While the glutaminase of P.fluorescens ACMR 171 was maximally active and stable at pH 8, those of other strains preferred pH 6 for their maximal activity and stability.

Effect of Temperature on the Activity and Stability of Glutaminase

The effect of temperature on the activity and stability of glutaminase was tested by subjecting them to

various temperatures ranging from 30-70°C. From the results (Fig.16) it is evident that glutaminase of all the strains were active and stable at temperatures varying from 30-60°C with their maximal activity and stability at 40°C. A further increase in temperature to 70°C resulted in a decrease in the activity and stability of the enzymes of all strains except that of P.fluorescens ACMR 171 which lost their activity completely at this temperature.

Maximal activity was recoreded by the glutaminase of P.fluorescens ACMR 171 (1.527 u/ml) followed by P.fluorescens ACMR 43 (1.409 u/ml) V.costicola ACMR 267 (1.409 u/ml) and V.cholerae ACMR 347 (1.292 u/ml). While glutaminase from both Vibrio strains recorded 100% of their maximal activity and stability at their optimal temperature during stability tests, those of P.fluorescens ACMR 171 and P.fluorescens ACMR 43 retained only 92.27% and 91.6% respectively of their maximal activity during stability tests at their optimal temperature. Comparatively glutaminase of sediment bacteria exhibited higher levels of activity at 70°C (0.234 u/ml for P.fluorescens ACMR 171 and 0.234 u/ml) for V.cholerae ACMR 347) than those of bacteria from water (0.117 u/ml) for P.fluorescens ACMR 43 and 0.117 u/ml for V.costicola ACMR 267).

Effect of Substrate Concentration on the Activity of Glutaminase

Effect of substrate concentration on the activity of prepared glutaminase was tested by subjecting them to various levels of substrate concentrations ranging from 0.01-0.1 M. The results presented in Fig.17 suggested that enzymes of all the four strains uniformly reacted sharply to an increase in substrate concentration from 0.01-0.04 M by rapid increase in activity. However, they responded passively by remaining in a steady state towards a further increase in substrate concentration from 0.04 M to 0.1 M.

While glutaminase of bacteria from water preferred 0.06 M substrate concentration for their maximal activity (1.409 u/ml for P.fluorescens ACMR 43, 1.409 u/ml for V.costicola ACMR 267) those of sediment (P.fluorescens ACMR 171, 1.409 u/ml and V.cholerae ACMR 347, 1.527 u/ml) opted 0.04 and 0.08 M respectively for their maximal activity.

Eventhough optimal substrate concentration for maximal activity of glutaminase of each strain varied from 0.04-0.08 M the results indicate that 0.04 M substrate concentration could promote significant level of glutaminase activity in all the cases.

Michaelis - Menten constant of glutaminase from selected strains was calculated using Lineweaver - Burk plot. It varied among the strains from 1.0×10^{-4} M to [illegible] M. Km of glutaminase was 1.0×10^{-4} M for P.fluorescens ACMR 171; 4.16×10^{-5} M for P.fluorescens ACMR 43; 9.54×10^{-5} M for V.costicola ACMR 267; 1.5×10^{-5} M for V.cholerae ACMR 347.

Effect of Nacl Concentration on the Activity of Glutaminase

The effect of NaCl on enzyme activity of the prepared glutaminase of bacteria was tested by subjecting them to various concentrations of NaCl ranging from 0-25%. Results presented in Fig.18 show that NaCl concentration of 0-5% did not influence the enzyme activity of all bacteria tested. Moreover, glutaminase was not influenced drastically by high concentrations of NaCl (from 7-25%) in the medium. Thus except for the enzyme from V.cholerae ACMR 347 whose activity was decreased rather rapidly to 33.28% of optimal activity at 25% NaCl concentration, enzymes of all other strains could retain 49.96-58.33% of optimal activity at 25% NaCl concentration.

Glutaminase isolated from both the strains of Pseudomonas fluorescens were more salt tolerant, retaining more than 50% of their activity even in the presence of

25% NaCl whereas glutaminase from Vibrio sp. lost about 60% of their activity at the same concentration of NaCl.

Among the glutaminase from all strains, the enzyme from P.fluorescens ACMR 171 was unaffected upto 3% NaCl indicating a salt tolerance while enzymes from other strains lost about 8.31% of their activity in the presence of 3% NaCl. Interestingly glutaminase of Pseudomonas fluorescens ACMR 43 isolated from marine water was less affected by the presence of higher concentration of NaCl than those of estuarine environment, ACMR 171. Both Pseudomonas sp. (ACMR 171 and ACMR 43) retained 76.88% and 75.01% respectively of their maximal activity even in the presence of 20% NaCl while glutaminase from Vibrio sp. exhibited only 66.64% (ACMR 267) and 41.6% (ACMR 347) of the maximal activity at 20%. Among the estuarine bacteria V.cholerae ACMR 347 was greatly influenced by the presence of NaCl where a linear decrease in enzyme activity was observed along with an increase in NaCl concentration from 1 to 20%.

Effect of Incubation Time on the Activity of Glutaminase

Optimal reaction time required for the maximal rate of deamidation of glutamine by glutaminase was tested incubating the enzyme with the substrate for different time

intervals. Data presented in Fig.19 indicate that maximum rate of hydrolysis by glutaminase could be effected within 10-15 min. Further incubation did not favour any increase in enzyme activity, and led to a marginal decline.

While glutaminase of sediment bacteria required 15 min. of incubation for their maximal activity (1.409 u/ml for P.fluorescens ACMR 171 and 1.409 u/ml for V.cholerae ACMR 347) those obtained from bacteria isolated from water required only 10 min. to record their maximal activity (1.409 u/ml for P.fluorescens ACMR 43 and V.costicola ACMR 267). However, even within 3-5 min. the enzymes of all bacteria could demonstrate relatively significant levels of activity. There was no marked increase in activity along with further raise in duration after 5 min., when compared to the activity recorded at 5 min. in all the cases (1.057 u/ml).

Substrate Specificity

The substrate specificity of the prepared glutaminase was tested with glutamine, asparagine and a combination of glutamine and asparagine at 0.04 M level. Data obtained (Table 10) suggest glutamine as the preferred substrate for exhibiting maximum activity. Asparagine was

deamidated by glutaminase of all tested strains except that of P.fluorescens ACMR 43 which utilised at very low magnitude. Presence of asparagine along with glutamine also resulted in a reduction in the total enzyme activity except for P.fluorescens ACMR 171 which recorded enhanced activity compared to that of glutamine alone.

Effect of Heavy Metals on the Activity of Glutaminase

Effect of heavy metals on the activity of glutaminase of the four bacteria was tested using various heavy metals viz., Pb, Co, Mn, Hg, Cu, Fe, Ca and Zn at 1 mM level in the reaction mixture.

In general, all of the heavy metals tested influenced glutaminase activity significantly (Table 11). Among the heavy metals tested, Hg effected 100% inhibition of the glutaminase of P.fluorescens ACMR 171 and V.costicola ACMR 267 while allowing glutaminase of P.fluorescens ACMR 43 and V.cholerae ACMR 347 to retain 24.9% and 22% of the optimal activity respectively. Although others did not effect very high level of inhibition, still rendered significant levels of inhibition. Thus the loss of activity of glutaminase varied from 44.5-61.8% for Fe; 33.0-55.7% for Ca; 23.0-55.7% for Mn; 25.03-50.1% for Pb; 23.0-44.5% for

both Co and Cu and 11.2-25.03% for Zn in order of drastic effect on the enzymes of all bacteria tested. Further the results clearly indicated that while Hg was highly inhibitory Zn was lesser inhibitive among the metals tested against glutaminase of all strains. The order of inhibitory efficiency varied for the enzyme of each strain. Thus glutaminase of P.fluorescens ACMR 171 and V.costicola ACMR 267 were inhibited by Fe followed by Pb, Ca, Cu, Co, Mn while glutaminase of P.fluorescens ACMR 43 and V.cholerae ACMR 347 were highly inhibited by Mn followed by Fe, Ca, Cu, Co and Pb.

Presence of heavy metals affected the activity of glutaminase of Vibrio strains more than that of Pseudomonas. Maximum loss of activity due to the presence of heavy metals was in the case of V.costicola ACMR 267 glutaminase, followed by V.cholerae ACMR 347 P.fluorescens ACMR 43 and P.fluorescens ACMR 171 glutaminase.

Effect of other Substances on the Activity of Glutaminase

Effect of inhibitory compounds other than heavy metals on the activity of glutaminase was estimated with glutamic acid, aspartic acid, alpha ketoglutarate, EDTA, phosphate, borate, tris ion, phenolphthalein and bromocresol

purple. Results shown in Table 12 indicate that glutamic acid, aspartic acid and EDTA had not influenced the activity of glutaminase while phosphate and tris ion enhanced the activity of glutaminase. Those compounds which attributed inhibition of glutaminase included alpha keto-glutarate (47.45-75.08% loss of activity), phenolphthalein (41.47-55.7% loss of activity) bromocresol purple (36.97-55.63% loss of activity) in the order of merit. Although borate showed inhibition of glutaminase (11.2-15.94% loss of activity) it was only marginal when compared to others. The presence of these inhibitor compounds greatly influenced the activity of glutaminases from V.costicola ACMR 267 followed by V.cholerae ACMR 347 and P.fluorescens ACMR 171 and P.fluorescens ACMR 43.

Alpha ketoglutarate caused maximum loss of glutaminase activity of P.fluorescens ACMR 43 (75.08%) followed by P.fluorescens ACMR 171 (66.7%), V.cholerae ACMR 347 (66.7%) and V.costicola ACMR 267 (47.45%).

While phenolphthalein caused the maximal inhibition of glutaminase of P.fluorescens ACMR 43 (50.06%) and V.cholerae ACMR 347 (55.7%), bromocresol purple inhibited glutaminase of both the strains of P.fluorescens maximally

(55.63% of ACMR 171 and 55.06% of ACMR 43). Whereas percentage of inhibition recorded by glutamic acid, EDTA, and tris ion were of not high magnitude.

Glutaminase from both Pseudomonas fluorescens strains were not affected by the presence of glutamic acid, aspartic acid and EDTA while glutaminase from both Vibrio strains were influenced by the presence of glutamic acid (26.42% of V.costicola ACMR 267 and 11.27% V.cholerae ACMR 347).

3.7 PRODUCTION OF GLUTAMINASE BY SSF

Glutaminase production through solid state fermentation was studied by using wheat bran as the solid support moistened with mineral media supplemented with 1% glutamine. The process was optimized both for enzyme production and extraction and recovery of the enzyme.

Optimization of Extraction Parameters for Recovery of the Enzyme Produced by SSF

Extraction procedures for maximal recovery of extracellular glutaminase from bacterial wheat bran (BWB) were optimized in terms of the drying temperature of BWB,

extraction media, buffer system, pH of extraction, ratio of bran to buffer, extraction temperature and contact time of bran with buffer.

Drying Temperature

Effect of drying temperature of the bacteria grown on wheat bran (BWB) on maximal enzyme recovery was determined by drying the BWB at different temperatures ranging from 30-50°C for 1 hour. From the results presented in Table 13 it could be noted that drying of BWB at 40°C favoured maximal recovery of glutaminase from all strains (52.86-58.74 u/g) except for V.costicola ACMR 267 where drying at 50°C yielded high titres of glutaminase (52 u/g). Drying at 30°C yielded relatively lesser levels of enzyme units (35.24-46.99 u/g) by all strains when compared to that obtained at 50°C (41.12-52.86 u/g).

Extraction Media

Appropriate extraction medium that enhance maximal yield of enzyme recovery from the BWB was determined by extracting with various types of extraction media including ethanol, distilled water, distilled water added with 1% NaCl, tap water and phosphate buffer of pH 6 or 8 (according to

the optimum pH of the enzyme). Results presented in Table 14 indicate that in general, buffer with respective optimum pH of the enzyme promoted maximum enzyme recovery from all strains (41.12-46.99 u/g) followed by distilled water added with 1% NaCl (29.37-41.12 u/g) tap water (23.49-35.24 u/g) and ethanol (23.49-29.37 u/g) which yielded only less significant level of glutaminase.

Maximal recovery of glutaminase was obtained by extraction with phosphate buffer (pH 6 for ACMR 43, ACMR 267, ACMR 347 and pH 8 for ACMR 171) from both the Pseudomonas fluorescens ACMR 171 and ACMR 43 and V.cholerae ACMR 347 (46.99 u/g each) followed by V.costicola ACMR 267 (41.12 u/g). Among other extraction media used distilled water plus 1% NaCl yielded appreciable levels of enzyme recovery from all strains (35.24 u/g each from both species of Pseudomonas fluorescens and 41.12 u/g from V.cholerae ACMR 347) except for V.costicola ACMR 267 (29.37 u/g) in which case distilled water alone yielded extraction of more enzyme units (35.24 u/g). Tapwater and aqueous ethanol recorded the lowest level of enzyme recovery.

pH of Extraction Media

Since buffer system was identified as the most suitable extraction medium for obtaining maximal enzyme recovery from wheat bran, the effect of pH of buffer system on the recovery was tested by using buffers of various pH ranging from 5-9 for the extraction process. Buffers in the acidic pH range favoured maximum enzyme recovery from BWB for all species except P.fluorescens ACMR 171 where buffer with pH 8 was found to be most suitable (Table 15). In general, buffers with pH in the range of 5-8 yielded significant levels of enzyme units (35.24-52.86 u/g).

Extraction with phosphate buffer having pH 6 and pH 5 recorded maximal enzyme recoveries from P.fluorescens ACMR 43, V.cholerae ACMR 347, V.costicola ACMR 267 whereas, P.fluorescens ACMR 171 preferred buffer with pH 8 and pH 9 for maximal enzyme recoveries.

Different Buffer Systems

Influence of different buffer systems of optimal pH on the extent of enzyme recovery was tested by using acetate buffer of pH 6, phosphate buffer of pH 6 or pH 8 and borate buffer of pH 8. Results presented in Table 16

indicate that extraction with phosphate buffer (pH 6) registered maximal enzyme recovery in both Vibrio sp. (52.86 u/g) while P.fluorescens ACMR 171 yielded higher levels of enzyme units with phosphate buffer of pH 8 (52.86 u/g) than borate buffer (pH 8) (41.12 u/g). P.fluorescens ACMR 43 recorded maximal enzyme extraction in acetate buffer of pH 6 (52.86 u/g) although significant levels could be obtained with phosphate buffer of pH 6 (46.99 u/g). Similar results were also obtained with acetate buffer at appreciable levels of enzyme from both Vibrio sp. (46.99 u/g for ACMR 267 and 41.12 u/g for ACMR 347).

Ratio of Bran to Buffer

Effect of bran to buffer ratio on the enzyme recovery was tested by adding buffer to fermented BWB at different ratios of 1:3, 1:5 and 1:10. Data obtained (Table 17) shows that in general, a ratio of 1:5 is optimum to recover maximal levels of enzyme from BWB of all strains (46.99-52.86 u/g). Further increase in the ratio to 1:10 did not favour appreciable levels of enzyme recovery. 1:3 ratio could also yield significant levels of enzyme recovery (17.62-28.19 u/g).

Effect of Contact Time

Effect of contact time of buffer with fermented BWB was determined by allowing the buffer to be in contact with BWB for various time intervals of 30, 60, 90 and 120 min. Results shown in Table 18 suggest that maximal amount of enzyme could be recovered within 60-90 min. of contact. Further increase in contact time did not favour any significant increase in enzyme recovery.

Glutaminase of BWB of both strains of *Pseudomonas fluorescens* was extracted at maximal levels by a contact time of 60 min. (52.86 u/g for ACMR 171, 46.99 u/g for ACMR 43) while both BWB of *Vibrio* sp. required a longer contact time of 90 min. (52.86 u/g for ACMR 267 and 46.99 u/g for ACMR 347). In general, the results indicate that contact time of 90 min. is necessary to obtain significant levels of glutaminase from BWB of all the strains.

Effect of Contact Temperature

Effect of contact temperature on enzyme recovery was determined by keeping BWB and buffer to be in contact at different temperatures of 25, 35 and 45°C and the data

obtained are presented in Fig.19. In general, enzyme recovery from all strains was maximal at 35°C except for a slight increase recorded at 25°C for P.fluorescens ACMR 43 and V.cholerae ACMR 347. Extraction at higher temperatures did not favour enzyme recovery.

Maximal enzyme recovery was recorded by P.fluorescens ACMR 43 (41.12 u/g) and V.cholerae ACMR 347 (41.12 u/g) at 25°C followed by P.fluorescens ACMR 171 (35.24 u/g) and V.costicola ACMR 267 (33.24 u/g) at 35°C. Both the strains from the sediment registered an increased yield of glutaminase at a contact temperature of 45°C (35.24 u/g) than those from water (29.37 u/g). It was observed that glutaminase from all the strains could be recovered in significant levels at contact temperatures ranging from 25-35°C (29.37-41.12 u/g).

Effect of Operational Parameters on Enzyme Production by SSF

Effect of operational parameters viz., particle size of wheat bran, moisture content, pH, temperature, substrate concentration, NaCl concentration, inoculum concentration and incubation time on enzyme production by SSF was determined and the results are presented below.

Effect of Particle Size of Wheat Bran

Effect of particle size of wheat bran on enzyme production during SSF was estimated by using wheat bran of different particle size. Data obtained (Table 20) indicate that among the four strains presented both the strains of Pseudomonas and V.cholerae ACMR 347 preferred wheat bran of 1.20 mm size for maximal enzyme production (41.12, 46.99 u/g) and particles varying from 1.41-2.06 mm in size were required by V.costicola ACMR 267 for maximal enzyme production.

Effect of Moisture Content

Effect of moisture content of wheat bran medium on the enzyme production was studied by adjusting the moisture content to various levels ranging from 20-70% (w/w). Results presented in Table 21 indicate that a moisture content of 40-70% is required for significant level of enzyme production by all strains (41.12-52.86 u/g) with an optimum between 50-60% (P.fluorescens ACMR 171, 52.86%; P.fluorescens ACMR 43, 46.99%; V.costicola ACMR 267, 46.99% and V.cholerae ACMR 347, 41.12%).

Results further show that there is a linear relationship between moisture content and enzyme production

so that the level of enzyme production by all the four strains increased significantly along with the raise in moisture level upto 60% where all recorded their maximal enzyme production. Except P.fluorescens ACMR 43, which did not show any change in the enzyme level, all others responded to 70% moisture content level by a sudden decline in enzyme production, when compared to that of 60% moisture content. Among the four, V.costicola ACMR 267 could record relatively higher levels of enzyme production than others at 40% moisture content itself, which in fact remained unchanged at 50-60% of moisture level. An overall assessment of the results indicate that 50% of moisture content would be enough to achieve maximal enzyme production by SSF.

Effect of pH

Effect of pH on the enzyme production by all strains in SSF was tested at various pH levels (pH 4-10) (Fig.20). Significant levels of glutaminase production by all strains were observed at a wide range of pH from 4-10. However, maximal enzyme production was recorded at pH 6 (46.99-52.86 u/g) except for P.fluorescens ACMR 171 which preferred pH 5 for the same. Relatively higher levels of enzyme production by all strains could be recorded at pH 5-7. Further there were no marked differences in the

levels of enzyme produced at their optimal pH for all the four strains. Both strains of Pseudomonas fluorescens and P.costicola ACMR 267 recorded 52.87 u/g each and V.cholerae ACMR 347 which recorded only 46.99 u/g. Alkaline pH above pH 8 and acidic pH below 5 did not enhance the enzyme production by all the strains.

Effect of Temperature

Effect of incubation temperature on enzyme production in the WB medium was tested at different temperature ranging from 25-55°C. Results presented in Fig.21 indicate that all strains could produce glutaminase at appreciable levels at a wide range of temperature varying from 15 to 45°C (11.72-46.99 u/g), with a maximum at 35°C. Maximal production of glutaminase was recorded by P.fluorescens ACMR 171 (46.99 u/g) and V.costicola ACMR 267 (46.95 u/g) followed by P.fluorescens ACMR 43 (41.12 u/g) and V.cholerae ACMR 347, (41.12 u/g).

Comparatively all could produce significant levels of enzyme at 15-25°C than at higher temperatures about 35°C where, they recorded a decline in the levels of glutaminase. Relatively the level of enzyme production at 45°C (23.45-29.37 u/g) was in appreciable level than at 55°C (5.85 u/g).

The data obtained in this study suggest that 35°C followed by 25, 45 and 15°C could favour maximal enzyme production in SSF.

Effect of Substrate Concentration

Effect of substrate concentration on enzyme production was determined at different substrate concentrations (0-3%). Among them 1% substrate concentration enhanced glutaminase production by all strains (41.12-46.99 u/g) except P.fluorescens ACMR 171 which required only 0.5% substrate concentration to produce maximal levels of glutaminase (58.74 u/g) (Fig.22). Eventhough all the strains could record glutaminase production, (1-6 u/g) on wheat bran not added with glutamine, addition of 0.25% glutamine effected a rapid increase in glutaminase production (17.62-23.49 u/g). Maximal enzyme production was recorded by P.fluorescens ACMR 171 (58.74 u/g) followed by V.costicola ACMR 267 (46.99 u/g), P.fluorescens ACMR 43 (41.12 u/g) and V.cholerae ACMR 347 (41.12 u/g).

In general, at lower substrate concentrations from 0-0.5% bacteria from sediment recorded higher levels of enzyme than those from water. Whereas at higher concentrations of 2% and 3% bacteria from water could produce comparatively higher level of enzyme production.

Effect of NaCl Concentration

Effect of NaCl concentration on enzyme production by SSF was determined by adjusting the NaCl concentration of mineral medium to 0-7%. Data presented in Fig.23 suggest that NaCl concentrations upto 3% enhanced higher titers of glutaminase (29.37-58.74 u/g) in all the strains. However, an increase in NaCl concentration above 3% resulted in a linear decrease in the enzyme production by all the strains.

Optimum concentration of NaCl required for the maximal enzyme production varied for the strains. Both the P.fluorescens ACMR 171 and V.costicola ACMR 267 required 3% NaCl for their maximal enzyme production (52.86 and 46.99 u/g respectively). Whereas P.fluorescens ACMR 43 preferred 1% NaCl concentration for the same (41.12 u/g). On the other hand V.cholerae ACMR 347 produced higher enzyme production in the absence of NaCl (58.74 u/g).

With respect to the relationship between NaCl concentration and enzyme production it is evident from the figure that the response to NaCl varied among the strains. Thus among the four, V.cholerae ACMR 347 recorded a decline in enzyme production along with raise in NaCl from 0% to 7%.

Whereas both P.fluorescens and V.costicola ACMR 267 showed increased enzyme production along with increase in NaCl upto 1% and 3% respectively and later recorded a decline at higher NaCl concentration.

Quantitatively maximal enzyme production was recorded by both the sediment bacteria V.cholerae ACMR 267 (58.74 u/g) and P.fluorescens ACMR 171 (52.86 u/g) followed by bacteria from water, V.costicola ACMR 267 (46.99 u/g) and P.fluorescens ACMR 43 (41.12 u/g).

Effect of Carbon Sources

Effect of additional carbon sources on enzyme production by SSF was determined by incorporating various carbon sources other than glutamine to wheat bran medium. Results presented in Table 22 indicate that among the various carbon sources tested only maltose enhanced glutaminase production (46.99-64.77 u/g). Whereas all others did not promote enzyme production in all the strains except P.fluorescens ACMR 171 which showed enhancement of enzyme production in response to glucose alone. Maximal enzyme production was recorded by V.costicola ACMR 267 (64.67 u/g using maltose) followed by P.fluorescens ACMR 171 (58.74 u/g using glucose) and P.fluorescens ACMR 43 and V.cholerae ACMR 347 (46.99 u/g each using maltose).

In general, maltose was found to be the only carbon source that favoured maximal glutaminase production by all strains.

Effect of Nitrogen Sources

Influence of additional nitrogen source besides glutamine on enzyme production in SSF was studied by the addition of various nitrogen sources in the medium. It could be seen from the Table 23 that all nitrogen sources tested either remained non-influential or resulted in a reduction in the level of enzyme production. Thus beef extract, yeast extract, peptone, KNO_3 and $NaNO_3$ led to minimal enzyme production by all strains (17.62-41.12 u/g for P.fluorescens ACMR 171; 11.72-35.24 u/g for P.fluorescens ACMR 43, 17.62-35.24 u/g for V.costicola ACMR 267, 17.62-29.37 u/g for V.cholerae ACMR 347). Except in V.cholerae ACMR 347, all others responded by a reduction in enzyme production to glutamic acid. Whereas lysine, while marginally enhancing enzyme production by P.fluorescens ACMR 43 (46.99 u/g), did not influence others.

Effect of Inoculum Concentration

Data presented in Fig.24 indicate that 3% inoculum promoted significant level of enzyme by all the strains

(41.12-52.86 u/g) and a further raise in inoculum concentration resulted in a decline in the level of enzyme production. However, the quantum of enzyme produced at 1% and 5 to 7% were at appreciable levels. Excepting P.fluorescens ACMR 43 (41.12 u/g) all the other three recorded maximal levels of enzyme at similar levels (52.86 u/g).

Effect of Incubation Time

Effect of incubation period on the enzyme production by SSF was determined by incubating the BWB for various periods at their optimal conditions. Maximal enzyme production was observed at 24 hours of incubation by all the strains (35.24-52.86 u/g) except P.fluorescens ACMR 171 which produced maximal enzyme by 18 hours itself (52.86 u/g) (Fig.25). However, the enzyme production recorded during the period from 6 hours to 48 hours was at appreciable level, for all the strains.

Maximal enzyme production was recorded by both the strains of Pseudomonas fluorescens and V.costicola ACMR 267 (52.86 u/g each) followed by V.cholerae ACMR 347 (46.99 u/g).

3.8 COMPARATIVE ACCOUNT OF ENZYME PRODUCTION BY BACTERIA IN SSF AND SmF

In general, all the four strains recorded significantly higher levels of enzyme production in SSF than

in SmF (Tables 24a-h). All the four strains showed marginal differences in their optimal requirements for maximal enzyme production with reference to the fermentation process ie., solid state or submerged fermentation.

P.fluorescens ACMR 171 preferred pH 5 for maximal enzyme production in SSF (52.86 u/g) while it could do the same at pH 6 in SmF (2.114 u/ml). All the other strains produced their maximal level of enzyme production at the same optimum pH 6 in both SSF and SmF process. Interestingly all the four strains recorded maximal level of enzyme production at 35°C in both SSF and SmF. While 1% concentration of glutamine was preferred by P.fluorescens ACMR 171 in SmF (2.348 u/ml) it required only 0.5% level in SSF (58.74 u/g). In contrast P.fluorescens ACMR 43 required only lower level (0.5%) of glutamine concentrations in SmF (2.818 u/ml) than in SSF (1%, 41.12 u/g). P.fluorescens ACMR 43 registered maximal level of enzyme production at 3% NaCl concentration (3.054 u/ml) and at 1% inoculum level (2.585 u/ml) in SmF while in SSF it required 1% NaCl concentration (41.12 u/g) and 3% inoculum level (41.12 u/g). Both P.fluorescens produced maximal level of enzyme production within 18 hours in SSF and SmF. While both Vibrio strains produced maximal enzymes within 18 hours in SmF (2.114 u/ml for ACMR 267 and

3.46 u/ml for ACMR 347) they required 24 hours in SSF for the same (52.86 u/g, for ACMR 267 and 46.99 u/g for ACMR 347) P.fluorescens (ACMR 171) preferred glucose as an additional carbon source for enhanced glutaminase production in both SSF and SmF, (3.524 u/ml in SmF and 58.74 u/g in SSF) while all the others opted glucose in SmF (3.054 u/ml for ACMR 43, 3.648 u/ml for ACMR 267 and 3.838 u/ml for ACMR 347) and maltose in SSF (46.99 u/g for ACMR 43, 70.48 u/g for ACMR 267 and 46.99 u/g for ACMR 347). In a similar fashion while beef extract enhanced enzyme production of both P.fluorescens strains in SmF (2.348 u/ml for ACMR 171, 2.818 u/ml for ACMR 43), lysine did the same in SSF (46.99 u/g for ACMR 171, 46.99 u/g for ACMR 43, 41.12 u/g for ACMR 267) whereas lysine induced Vibrios which produced maximal enzyme units in both SmF and SSF.

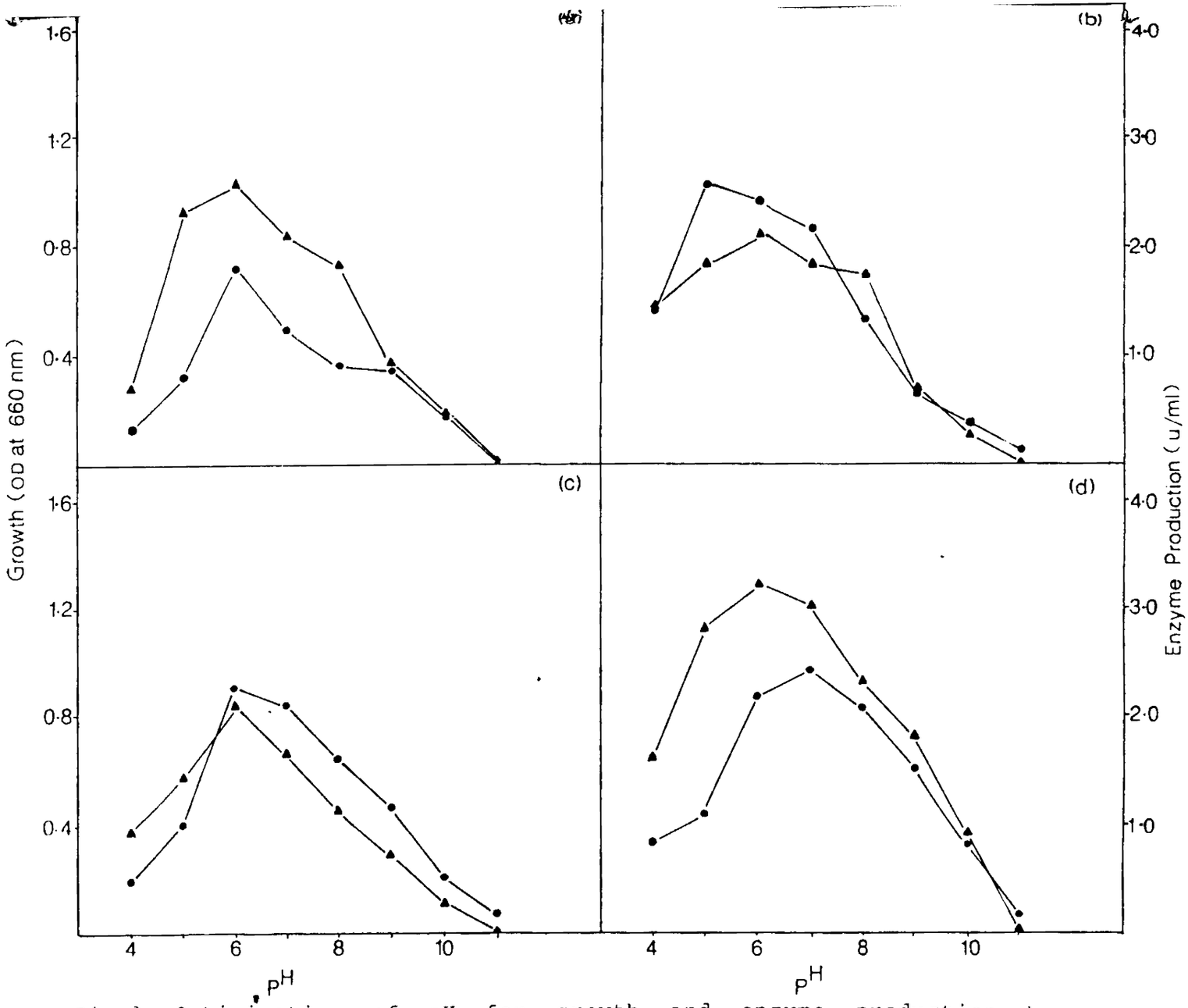


Fig.1: Optimization of pH for growth and enzyme production by Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43
(b) P.fluorescens ACMR 171
(c) V.costicola ACMR 267
(d) V.cholerae ACMR 347

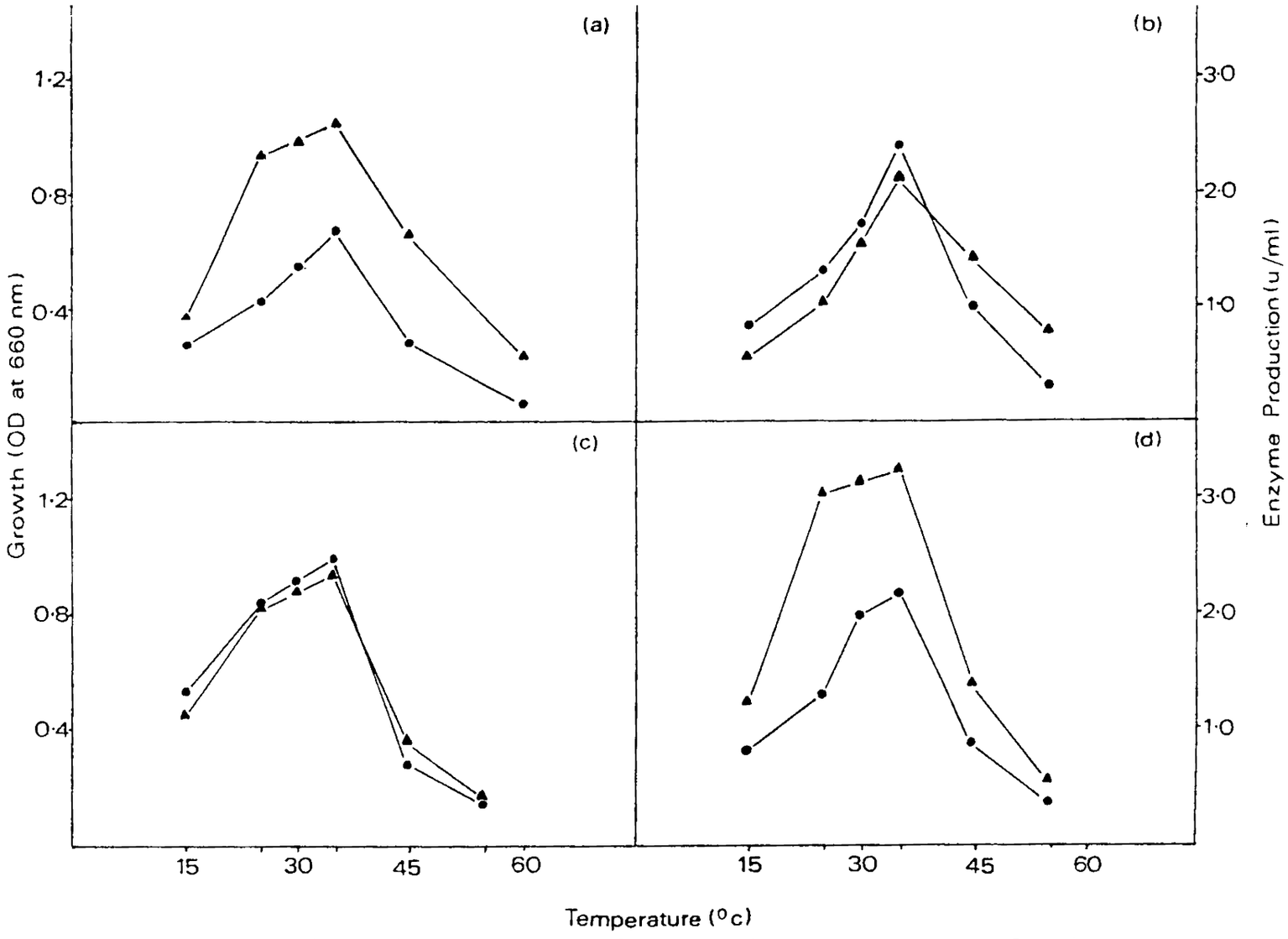


Fig.2: Optimization of temperature for growth and enzyme production by Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43

(b) P.fluorescens ACMR 171

(c) V.costicola ACMR 267

(d) V.cholerae ACMR 347

●—● Growth

▲—▲ Enzyme production

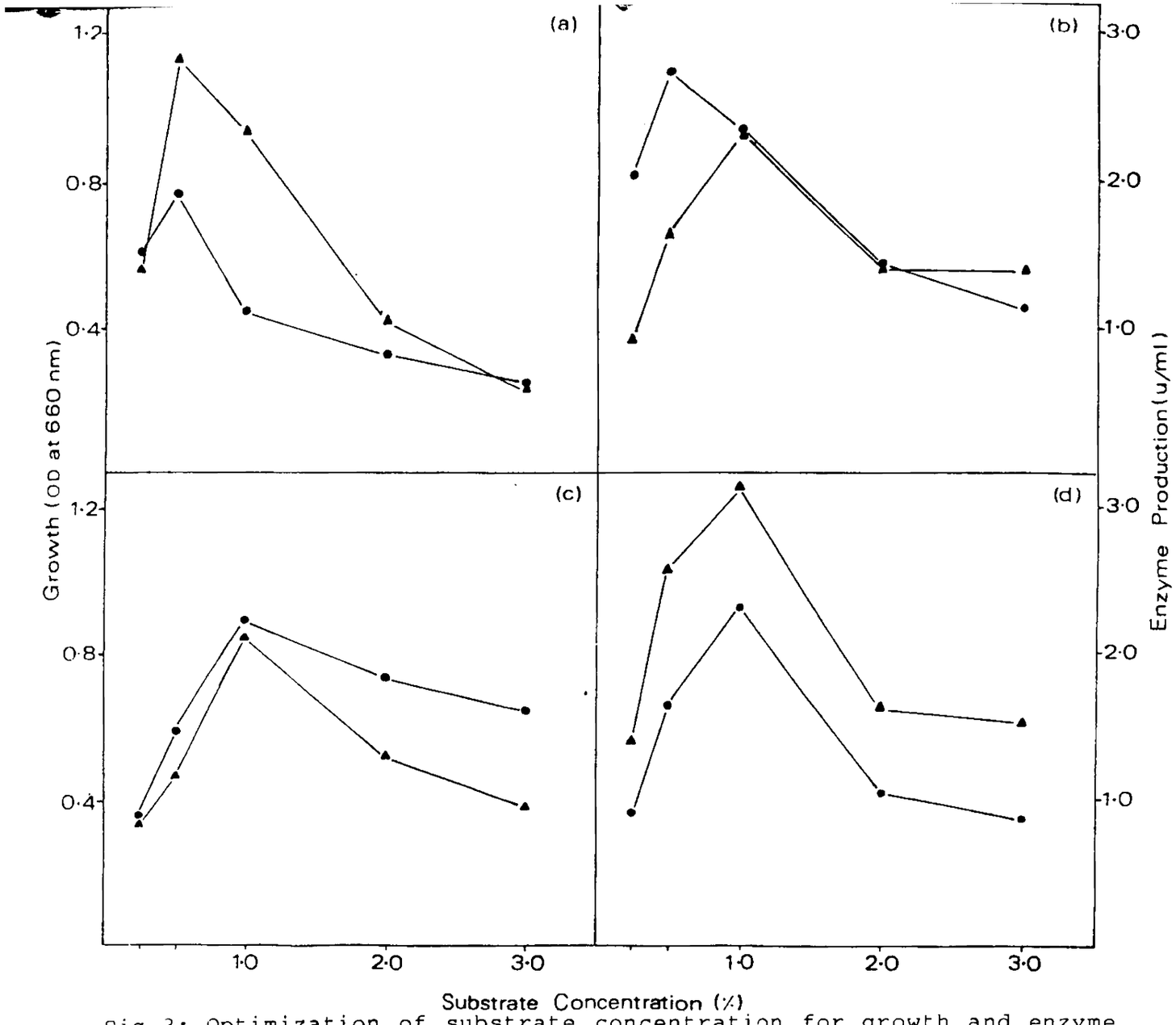


Fig.3: Optimization of substrate concentration for growth and enzyme production by Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43

(b) P.fluorescens ACMR 171

(c) V.costicola ACMR 267

(d) V.cholerae ACMR 347

●—● Growth

▲—▲ Enzyme production

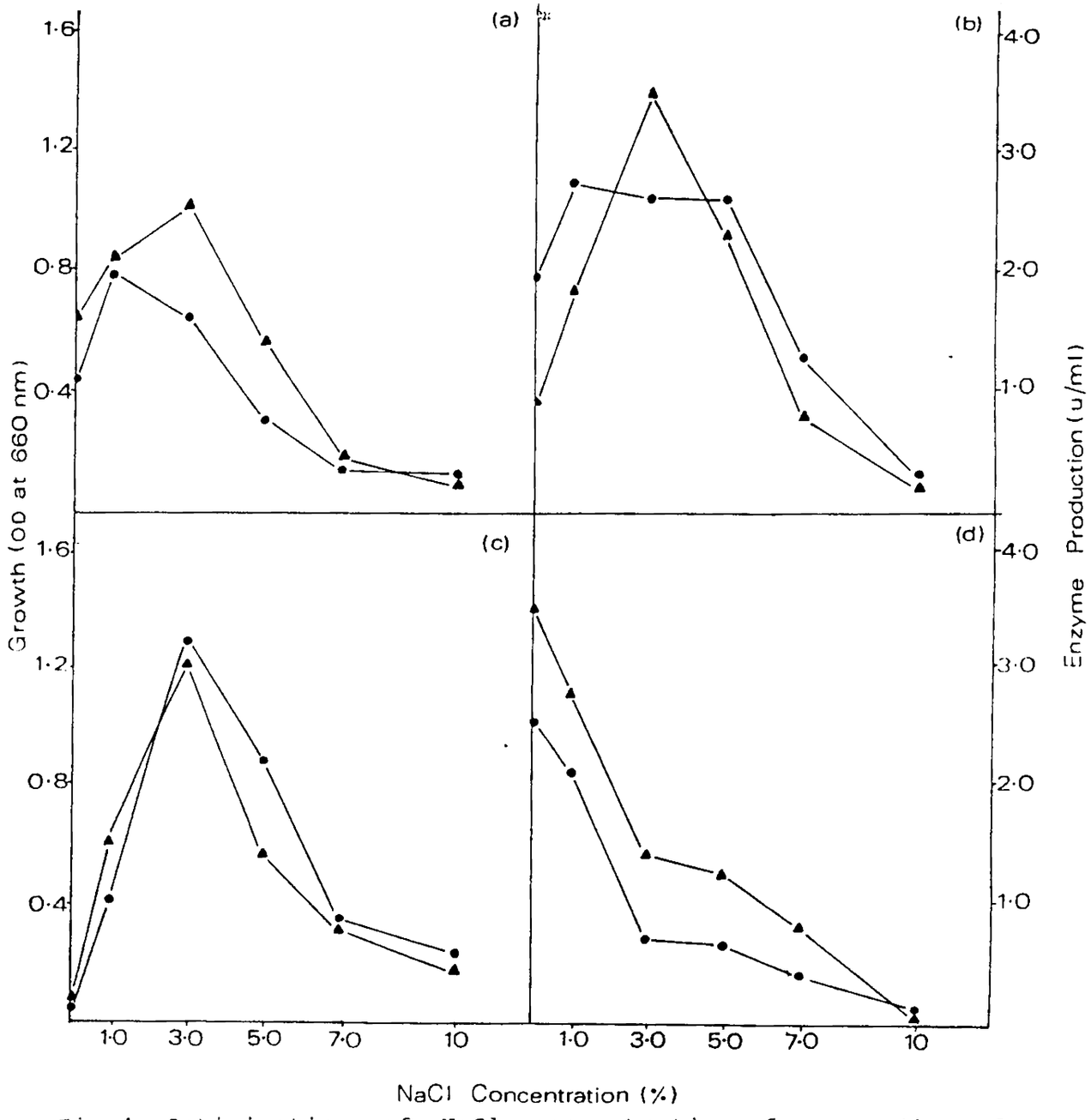


Fig.4: Optimization of NaCl concentration for growth and enzyme production by Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43
(b) P.fluorescens ACMR 171
(c) V.costicola ACMR 267
(d) V.cholerae ACMR 347

●—● Growth
▲—▲ Enzyme prod.

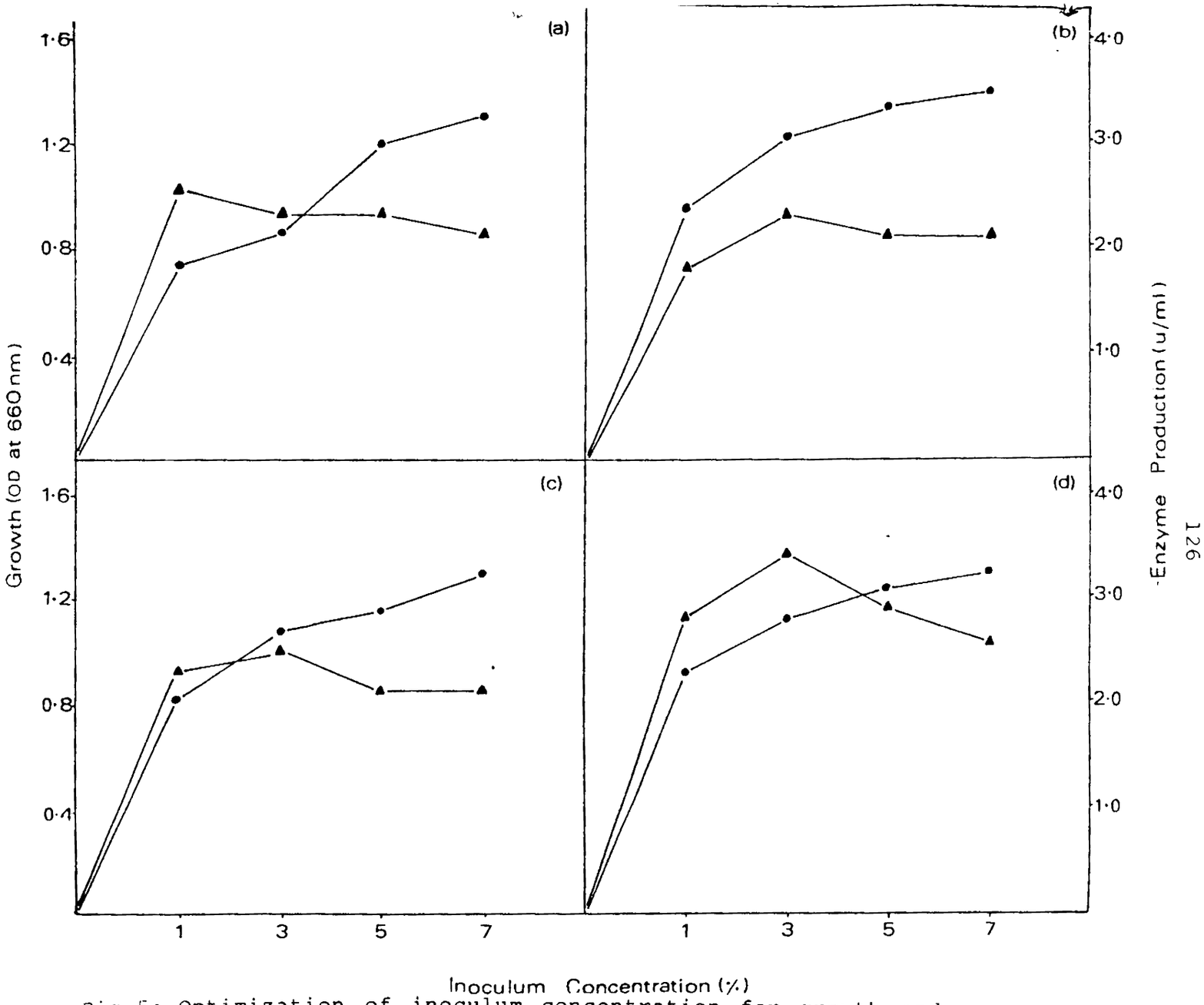


Fig.5: Optimization of inoculum concentration for growth and enzyme production by Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43
(b) P.fluorescens ACMR 171
(c) V.costicola ACMR 267
(d) V.cholerae ACMR 347

●—● Growth
▲—▲ Enzyme produ...

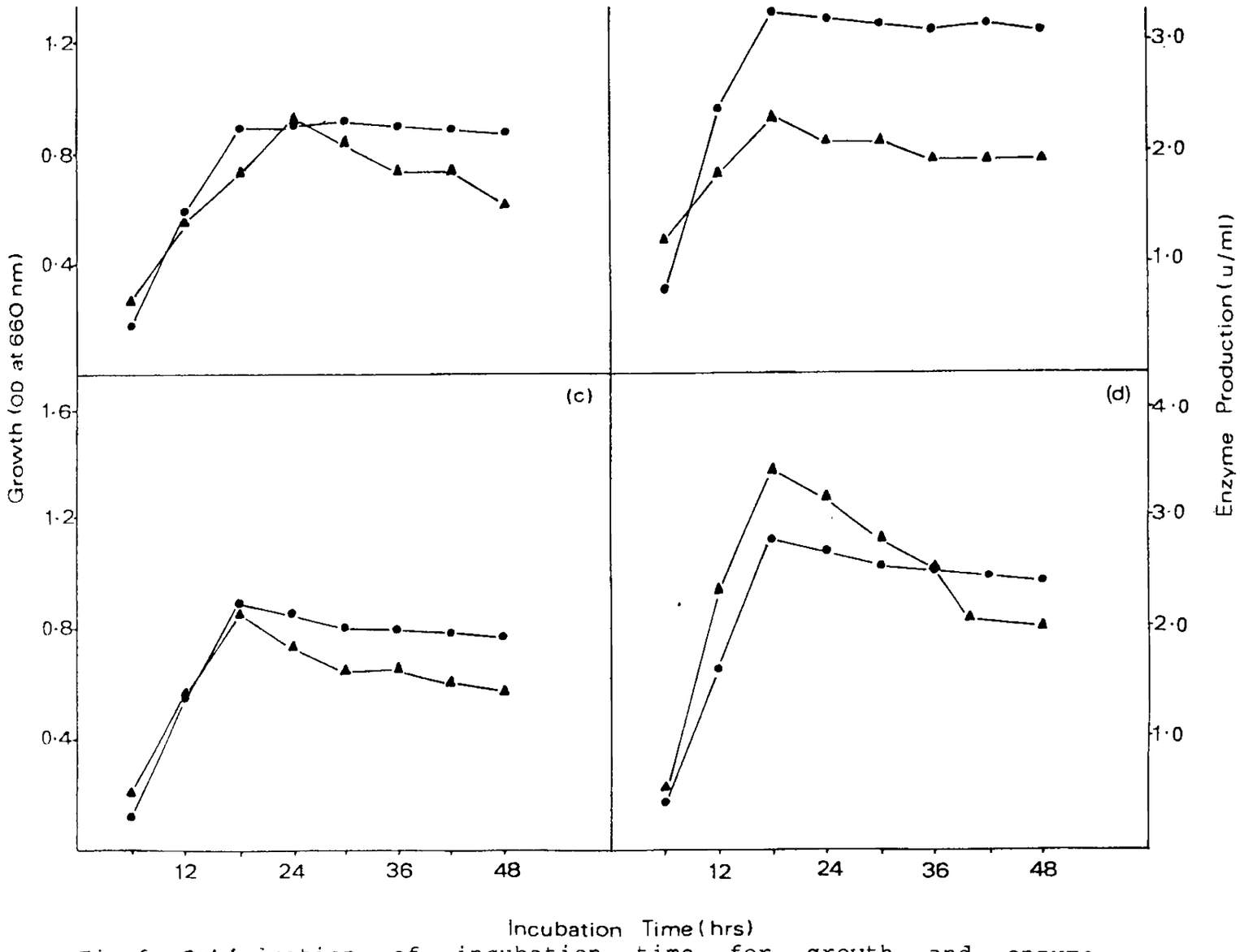


Fig.6: Optimization of incubation time for growth and enzyme production by Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43
(b) P.fluorescens ACMR 171
(c) V.costicola ACMR 267
(d) V.cholerae ACMR 347

●—● Growth
▲—▲ Enzyme production

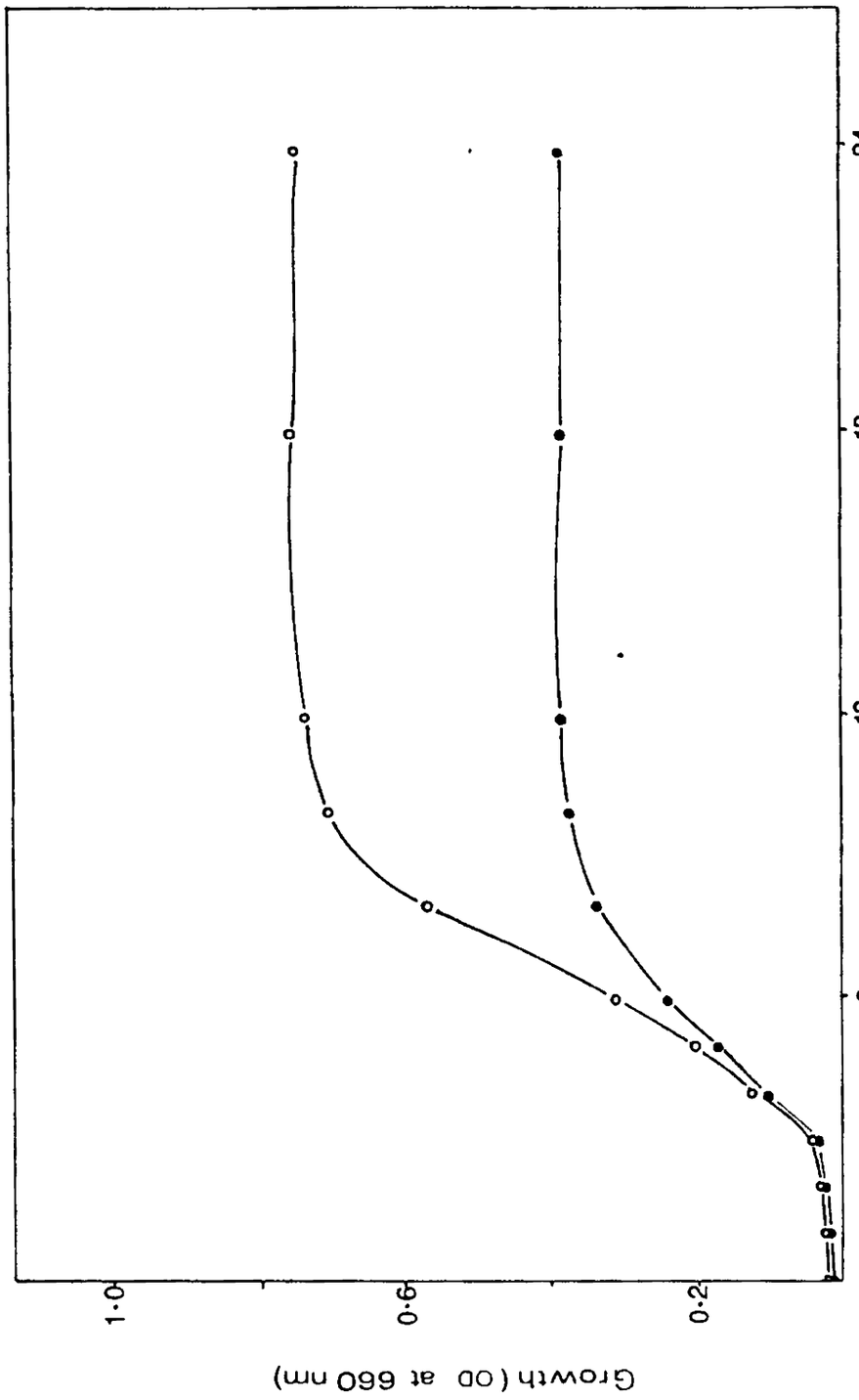


Fig.7: Growth curve of Pseudomonas fluorescens ACMR 43
o—o Nutrient broth ●—● Mineral media

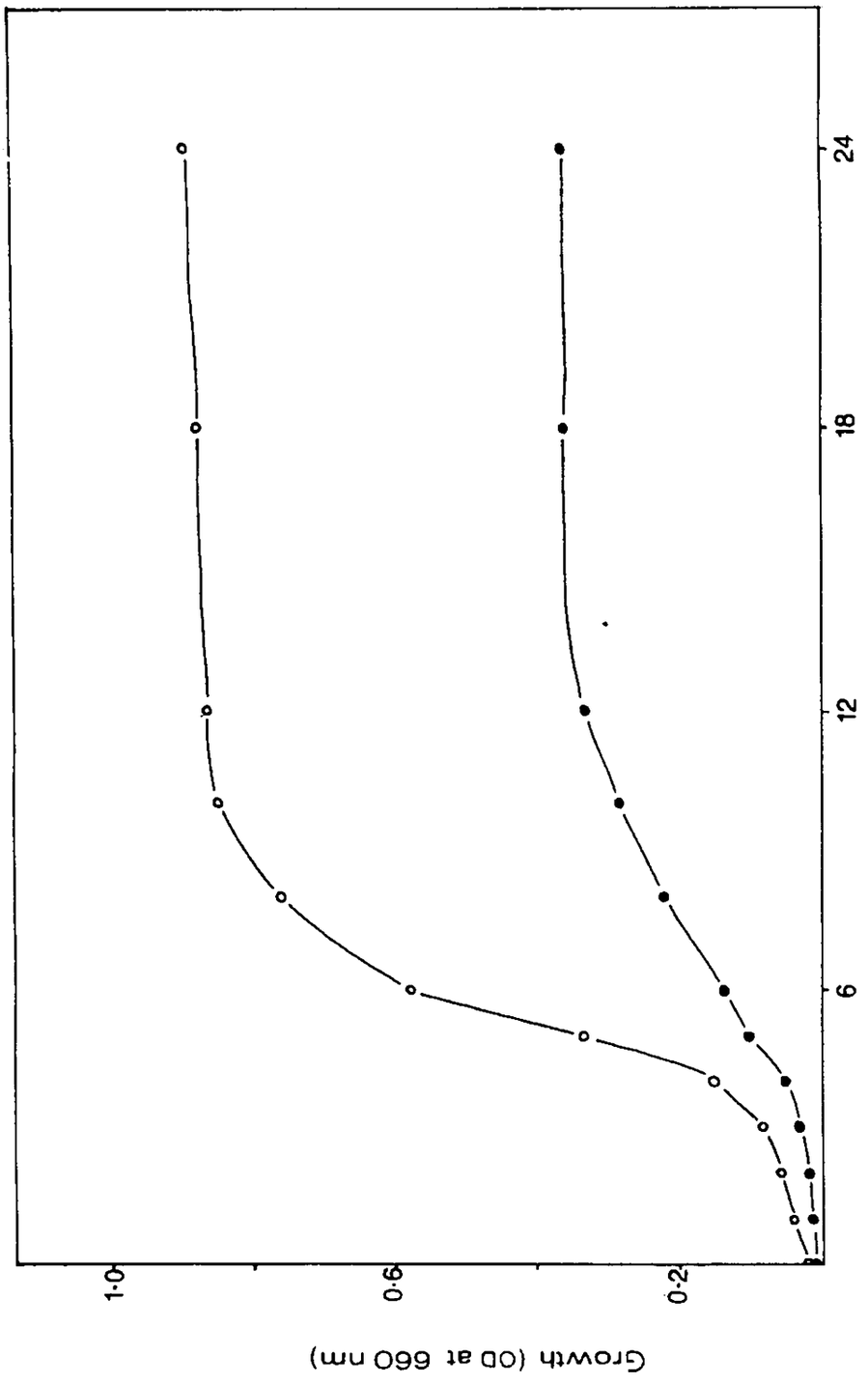


Fig.8: Growth curve of Pseudomonas fluorescens ACMR 171

o——o Nutrient broth ●——● Mineral media

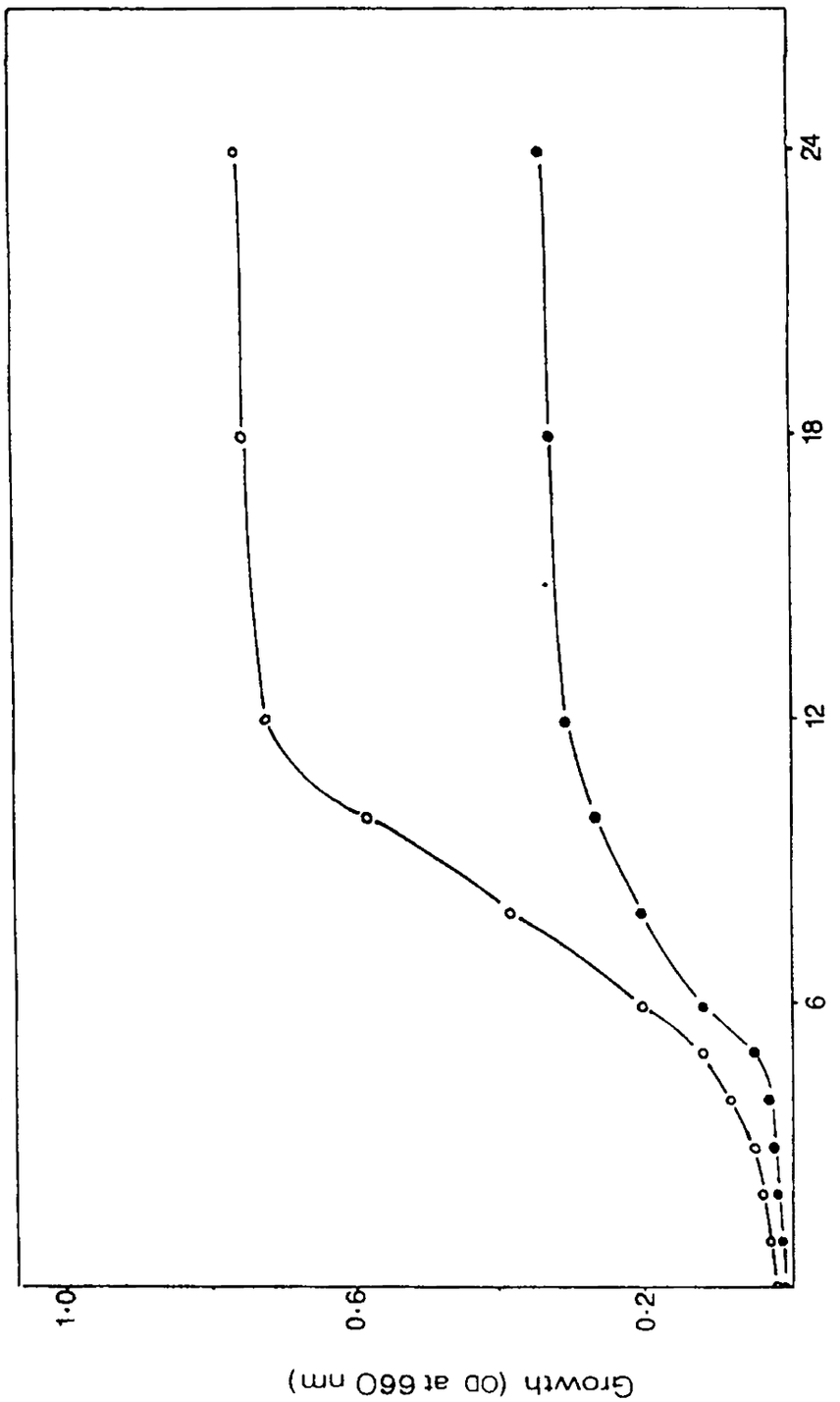


Fig.9: Growth curve of Vibrio costicola ACMR 267

o—o Nutrient broth ●—● Mineral media

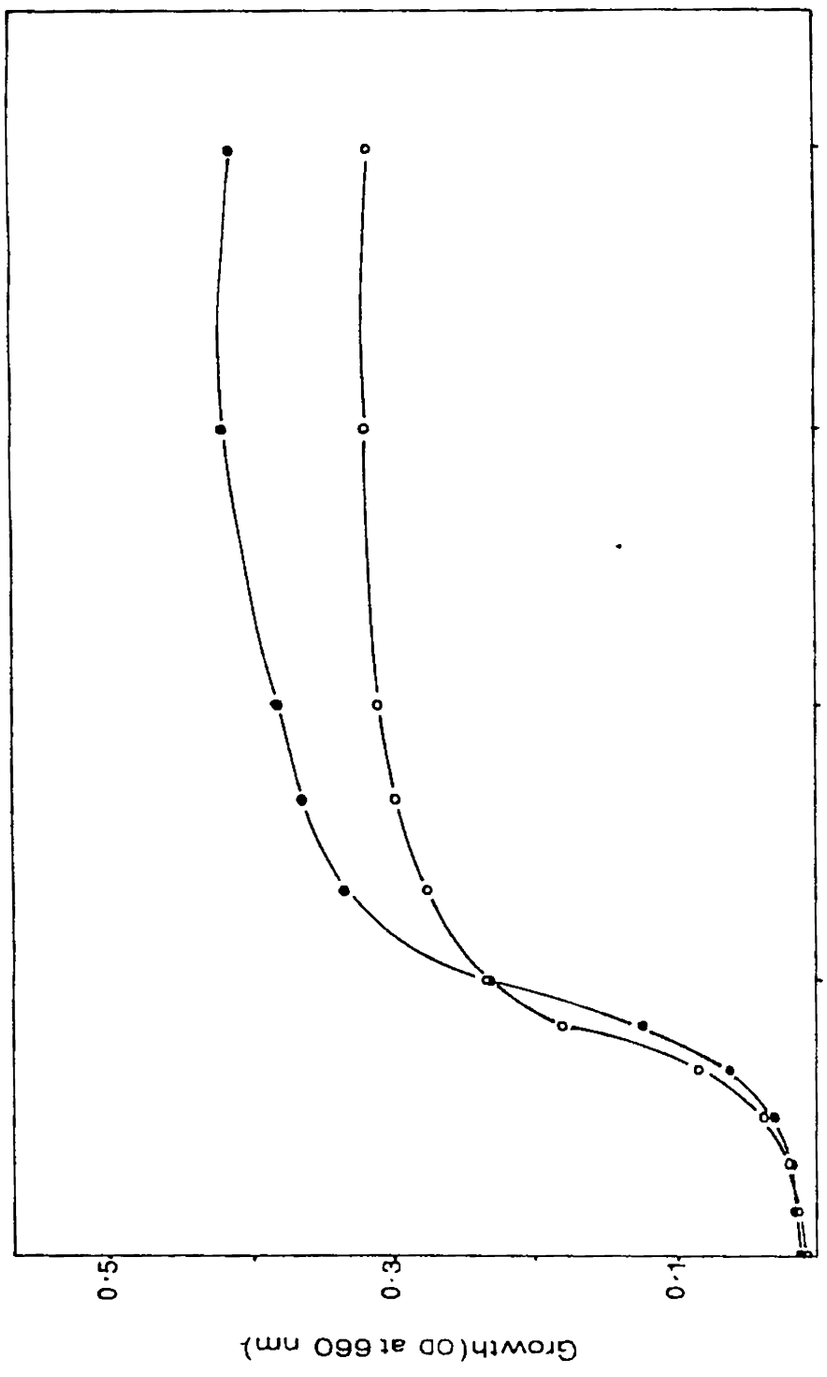


Fig.10: Growth curve of Vibrio cholerae ACMR 347

o—o Nutrient broth ●—● Mineral media

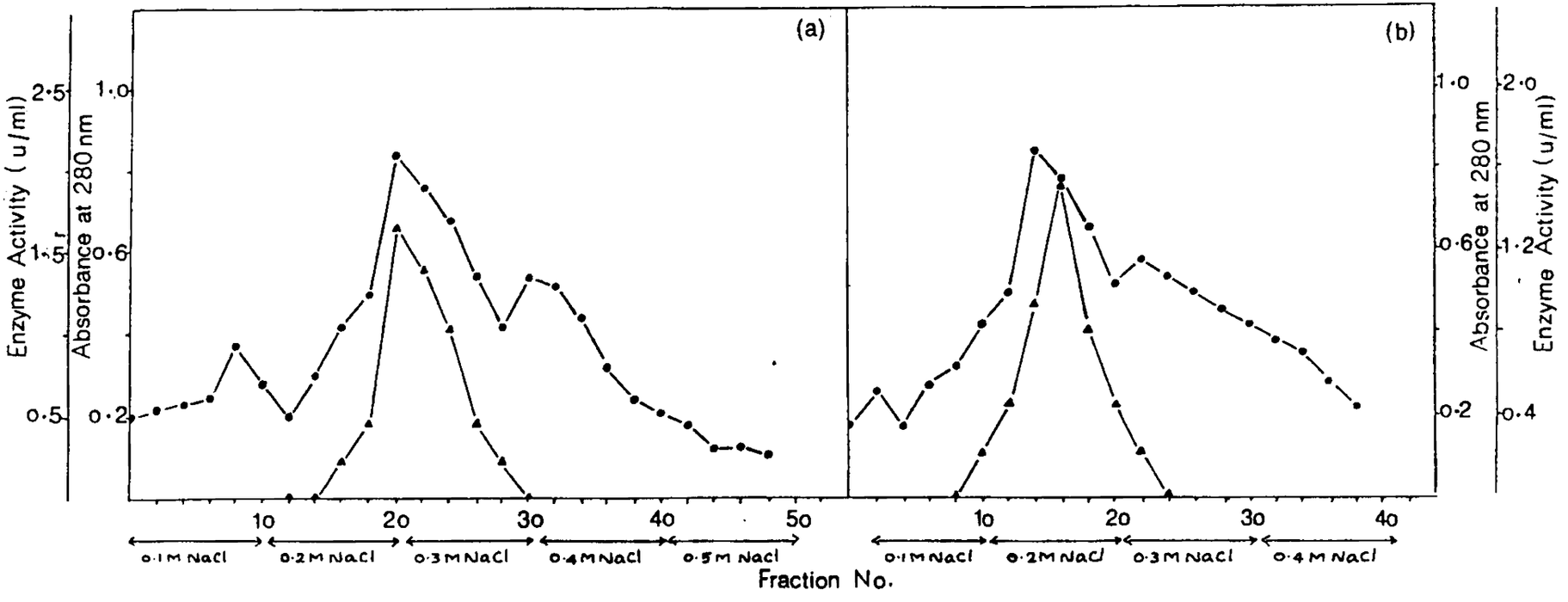


Fig.11: Elution profile of glutaminase from Pseudomonas fluorescens ACMR 43 by column chromatography on

(a) Seralite SRA-400 (b) Seralite SRA-120

●—● Absorbance at 280 nm ▲—▲ Enzyme activity

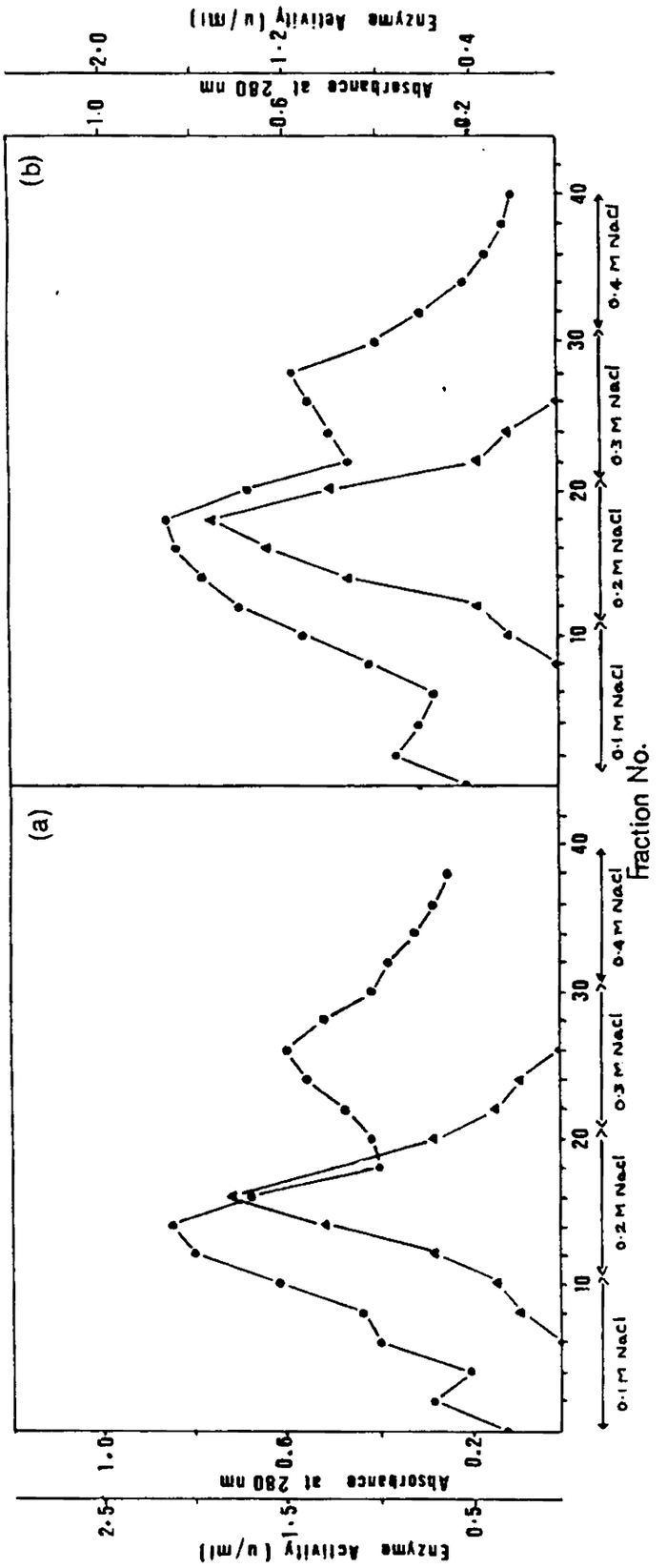


Fig.12: Elution profile of glutaminase from Pseudomonas fluorescens ACMR 171 by column chromatography on

(a) Seralite SRA-400 (b) Seralite SRA-120

●—● Absorbance at 280 nm ▲—▲ Enzyme activity

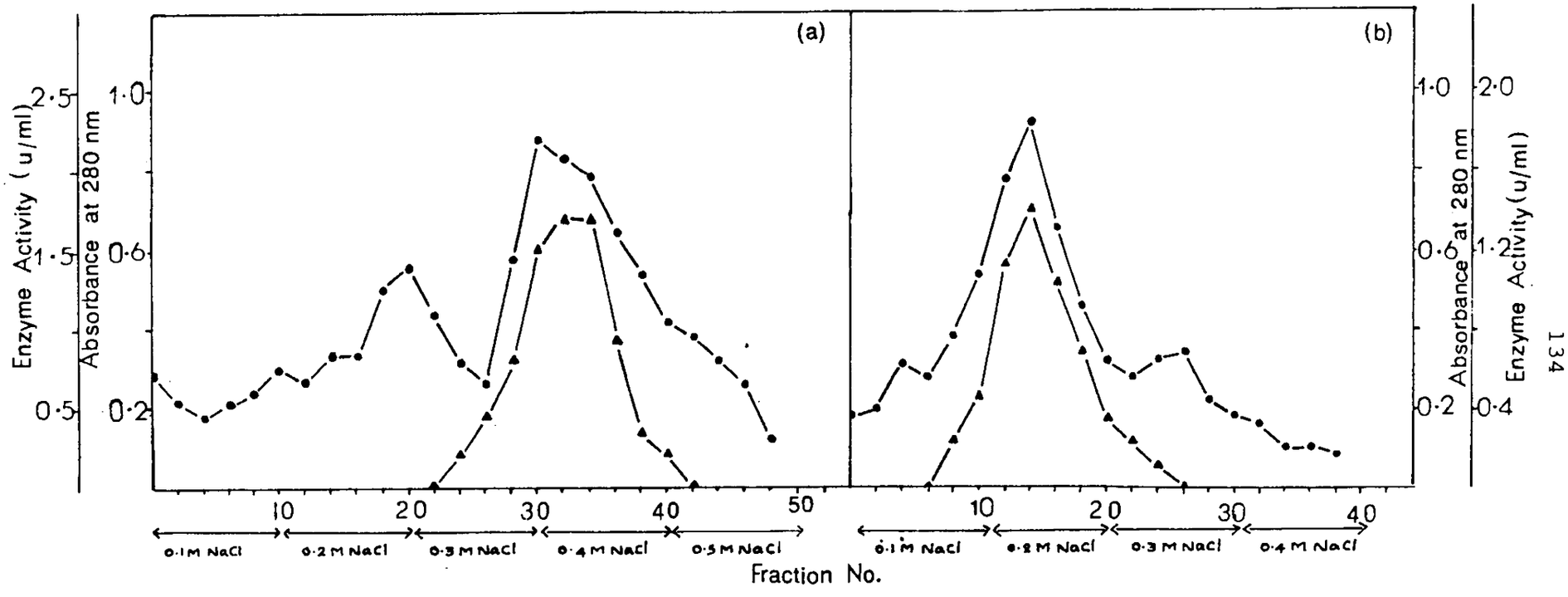


Fig.13: Elution profile of glutaminase from Vibrio costicola ACMR 267 by column chromatography on

(a) Seralite SRA-400 (b) Seralite SRA-120

●—● Absorbance at 280 nm ▲—▲ Enzyme activity

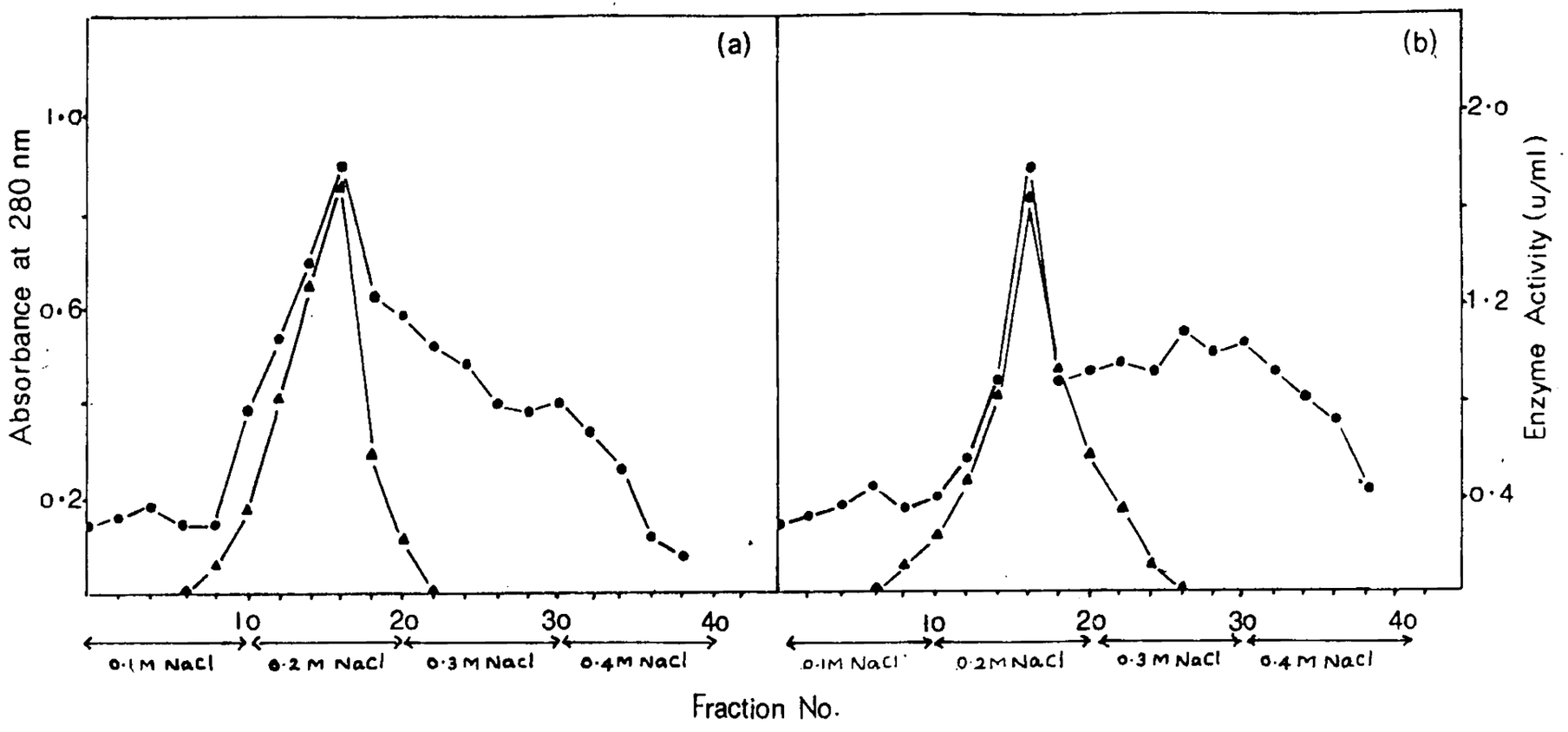


Fig.14: Elution profile of glutaminase from Vibrio cholerae ACMR 347 by column chromatography on

(a) Seralite SRA-400 (b) Seralite SRA-120

●—● Absorbance at 280 nm ▲—▲ Enzyme activity

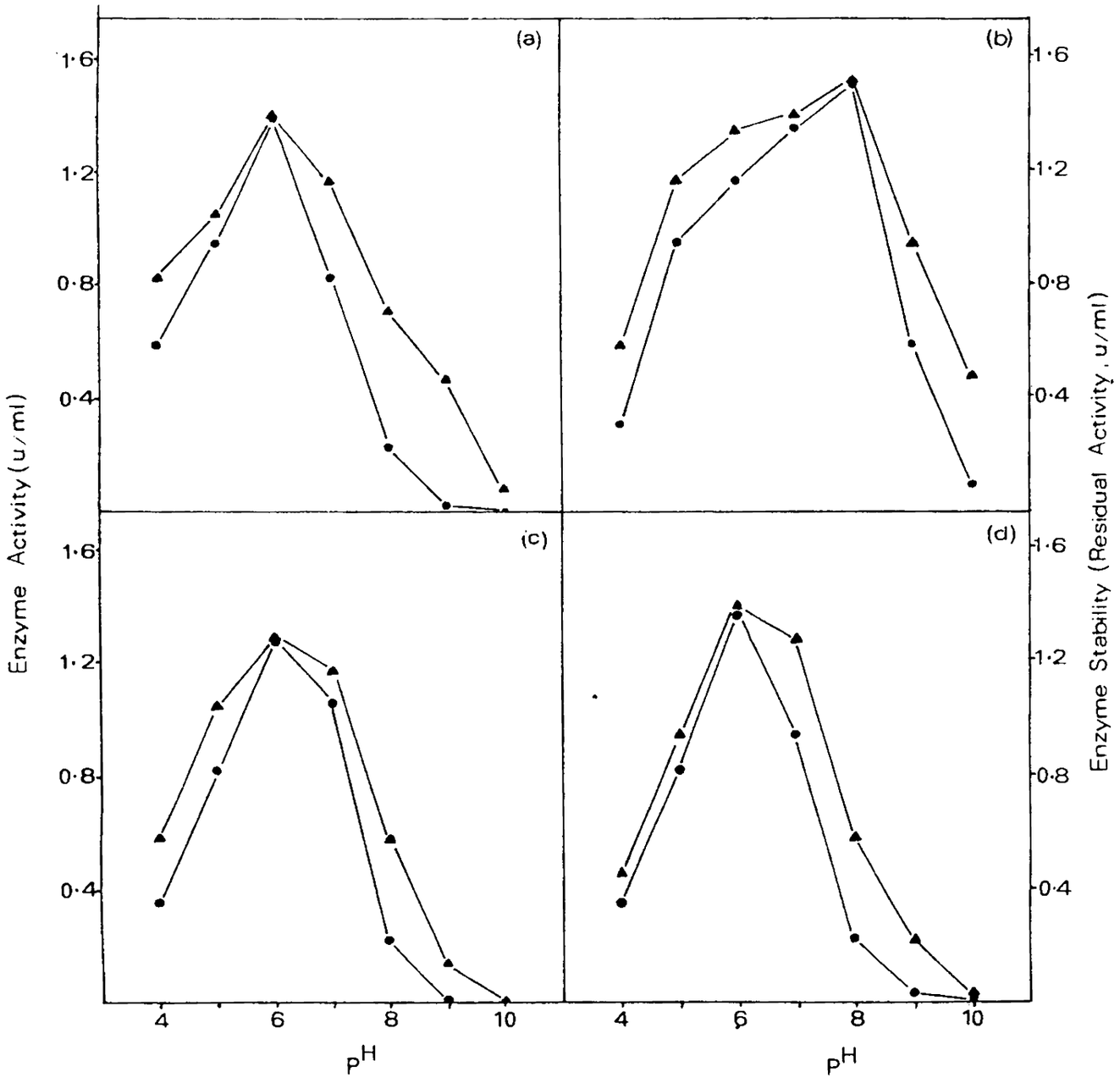


Fig.15: Effect of pH on activity and stability of glutaminase from Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43
(b) P.fluorescens ACMR 171
(c) V.costicola ACMR 267
(d) V.cholerae ACMR 347
▲—▲ Enzyme activity
●—● Enzyme stability

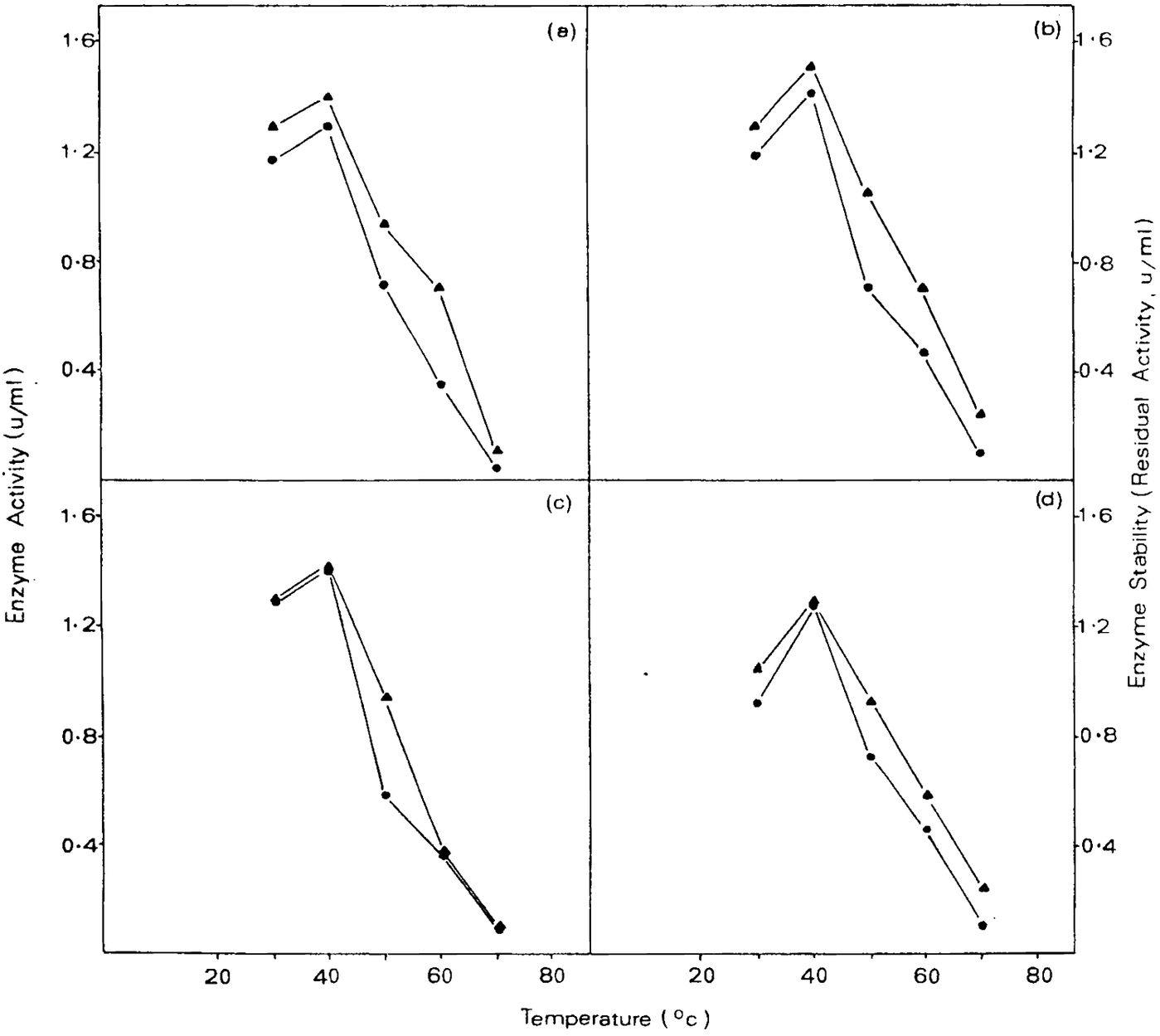


Fig.16: Effect of temperature on activity and stability of glutaminase from Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43
(b) P.fluorescens ACMR 171
(c) V.costicola ACMR 267
(d) V.cholerae ACMR 347

▲—▲ Enzyme activity
●—● Enzyme stability

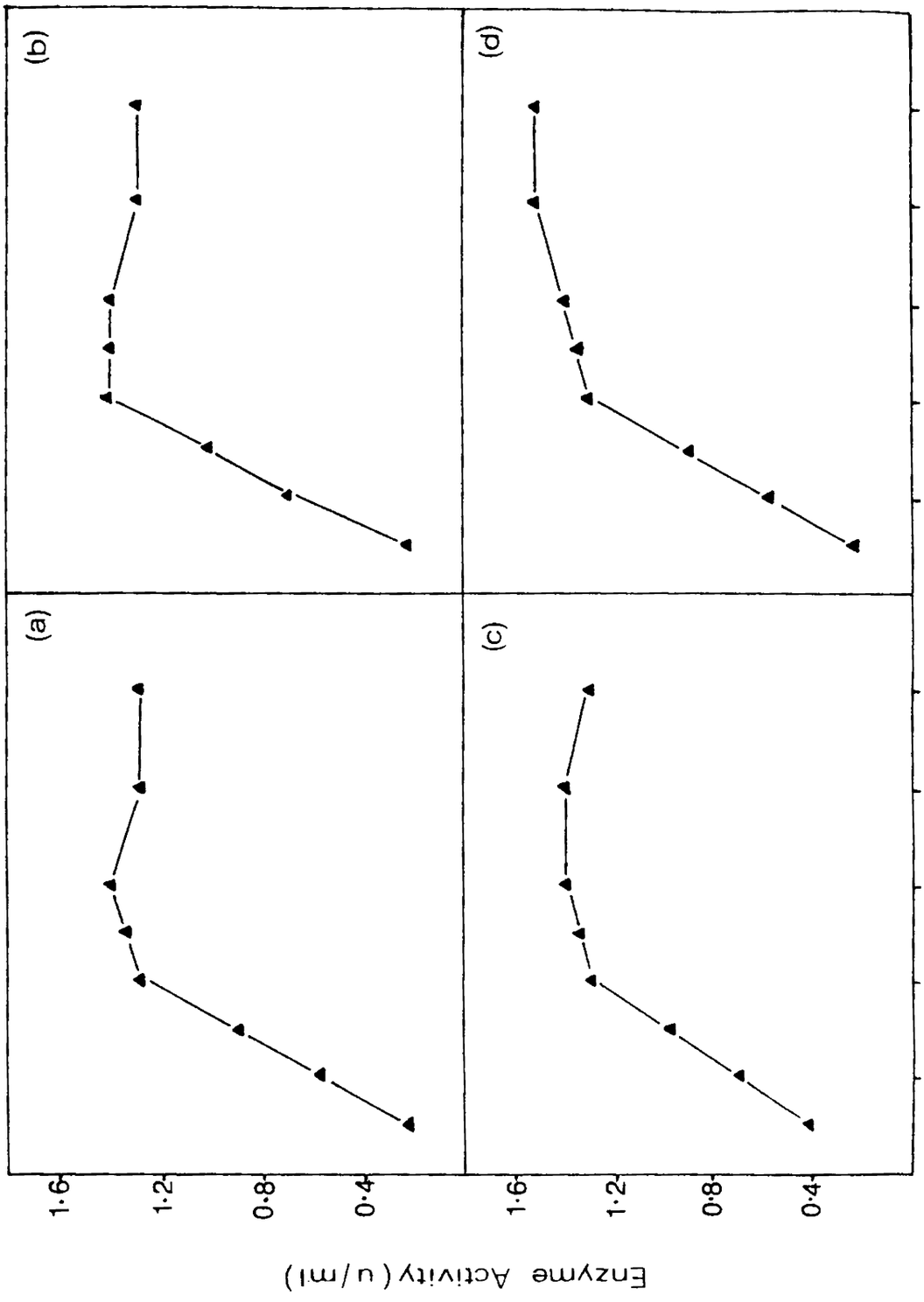


Fig.17: Effect of substrate concentration on activity of glutaminase from Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43 (c) V.costicola ACMR 267

(b) P.fluorescens ACMR 171 (d) V.cholerae ACMR 347

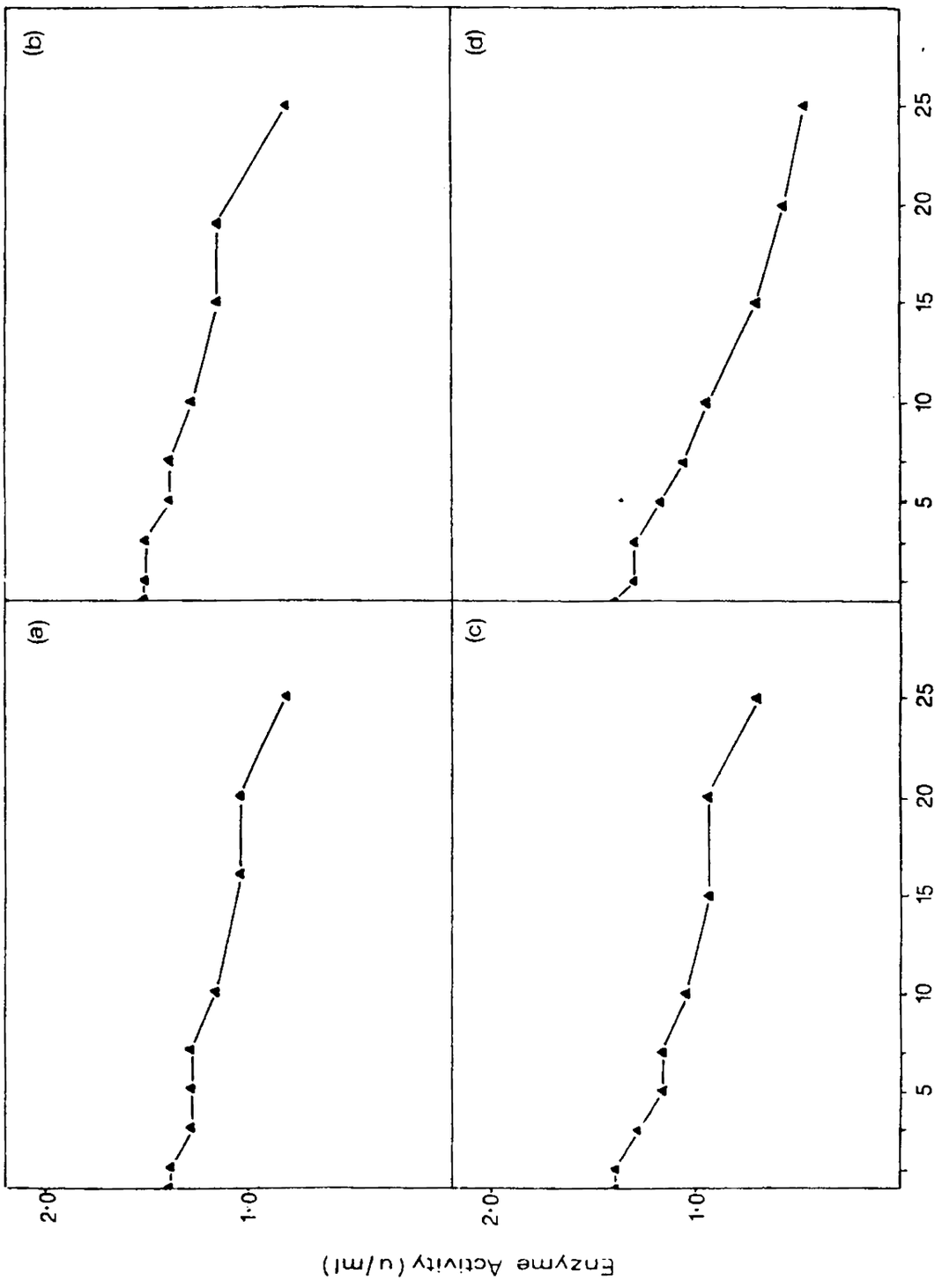


Fig.18: Effect of NaCl concentration on activity of glutaminase from Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43
(b) P.fluorescens ACMR 171
(c) V.costicola ACMR 267
(d) V.cholerae ACMR 347

▲—▲ Enzyme activity

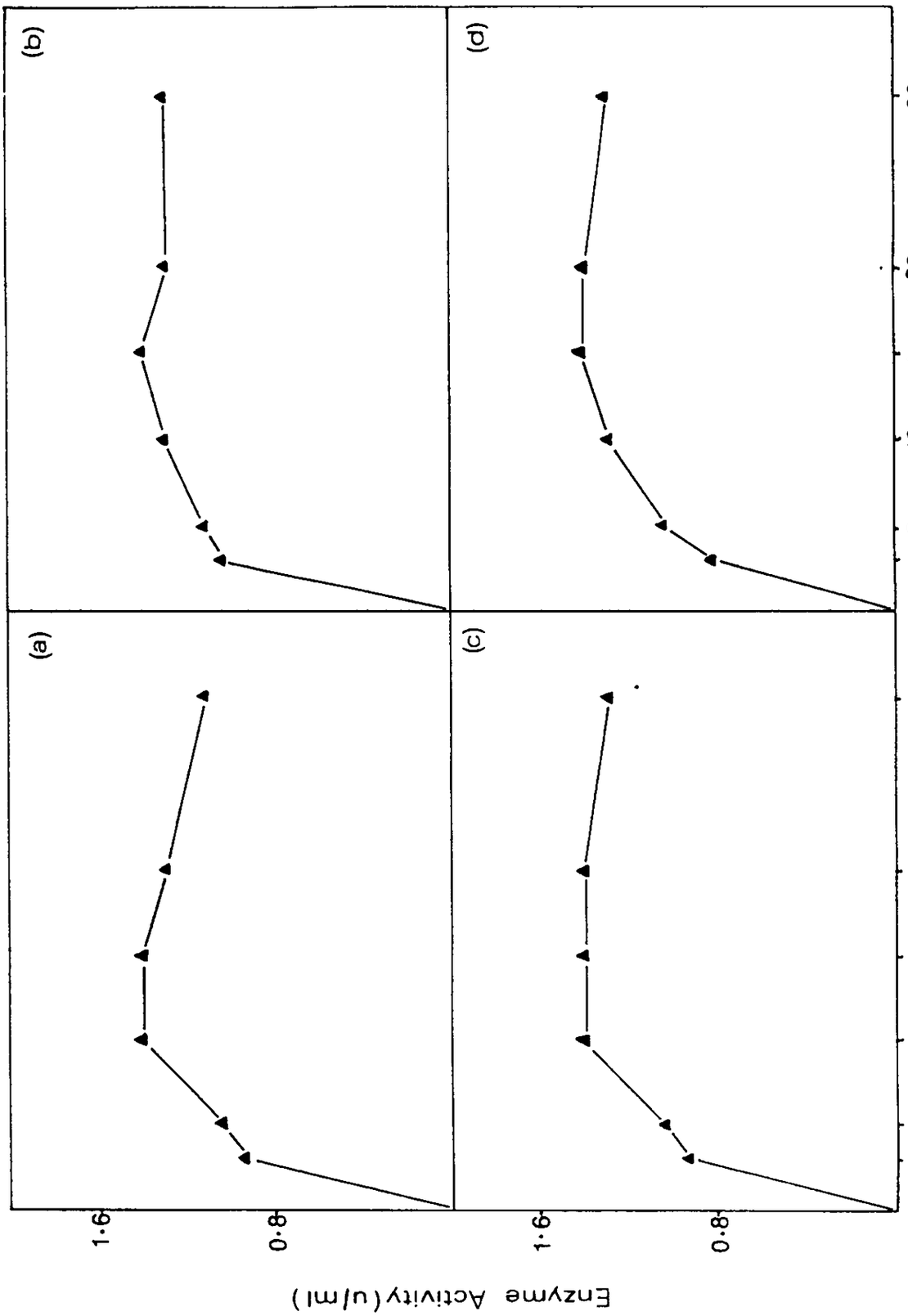


Fig.19: Effect of incubation time on activity of glutaminase from Pseudomonas sp. and Vibrio sp.

(a) P.fluorescens ACMR 43
(b) P.fluorescens ACMR 171
(c) V.costicola ACMR 267
(d) V.cholerae ACMR 347

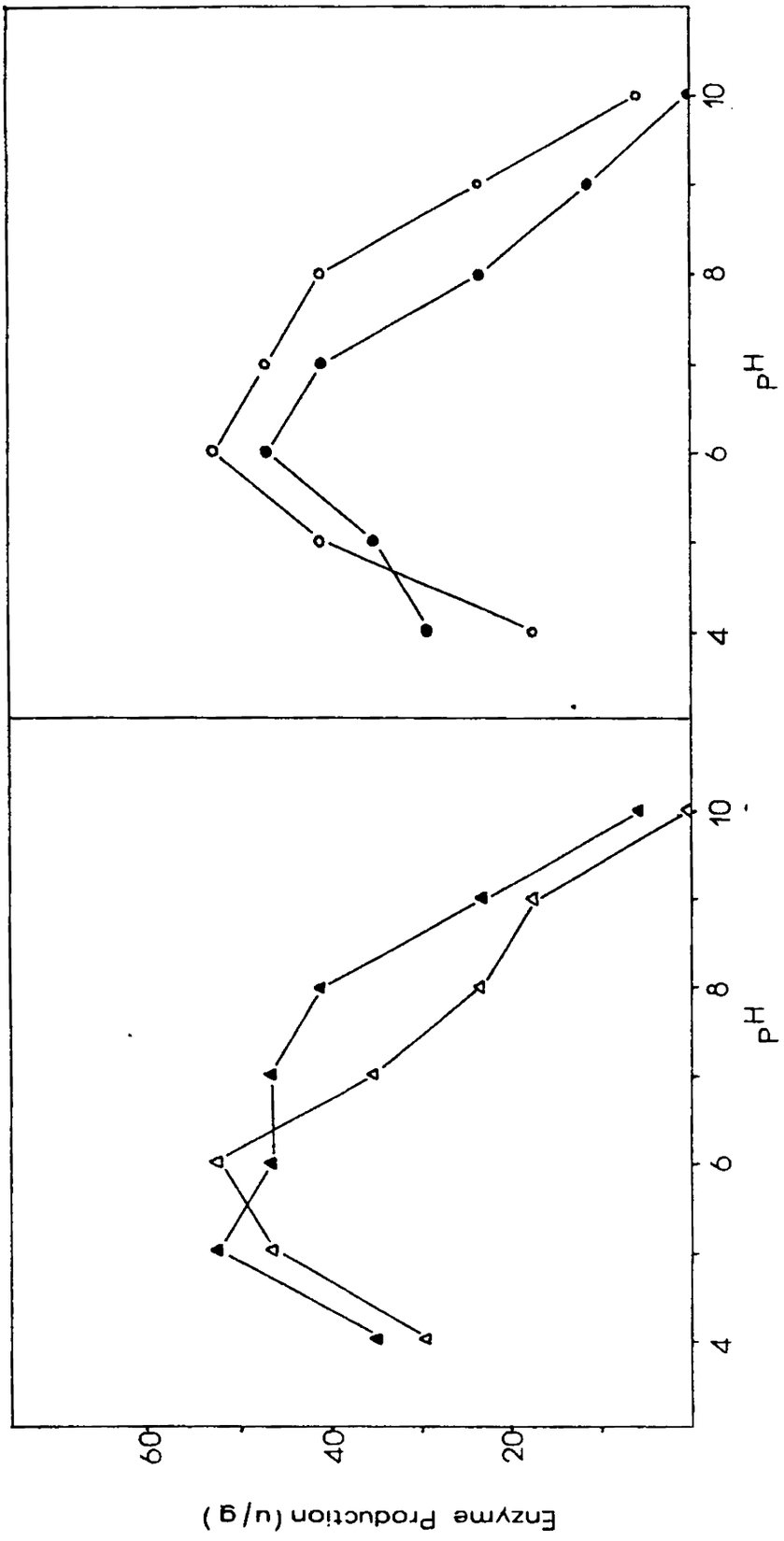


Fig.20: Effect of pH on enzyme production by Pseudomonas sp. and Vibrio sp. in Solid State Fermentation

△—△ P.fluorescens ACMR 43
o—o V.costicola ACMR 267
▲—▲ P.fluorescens ACMR 171
●—● V.cholerae ACMR 347

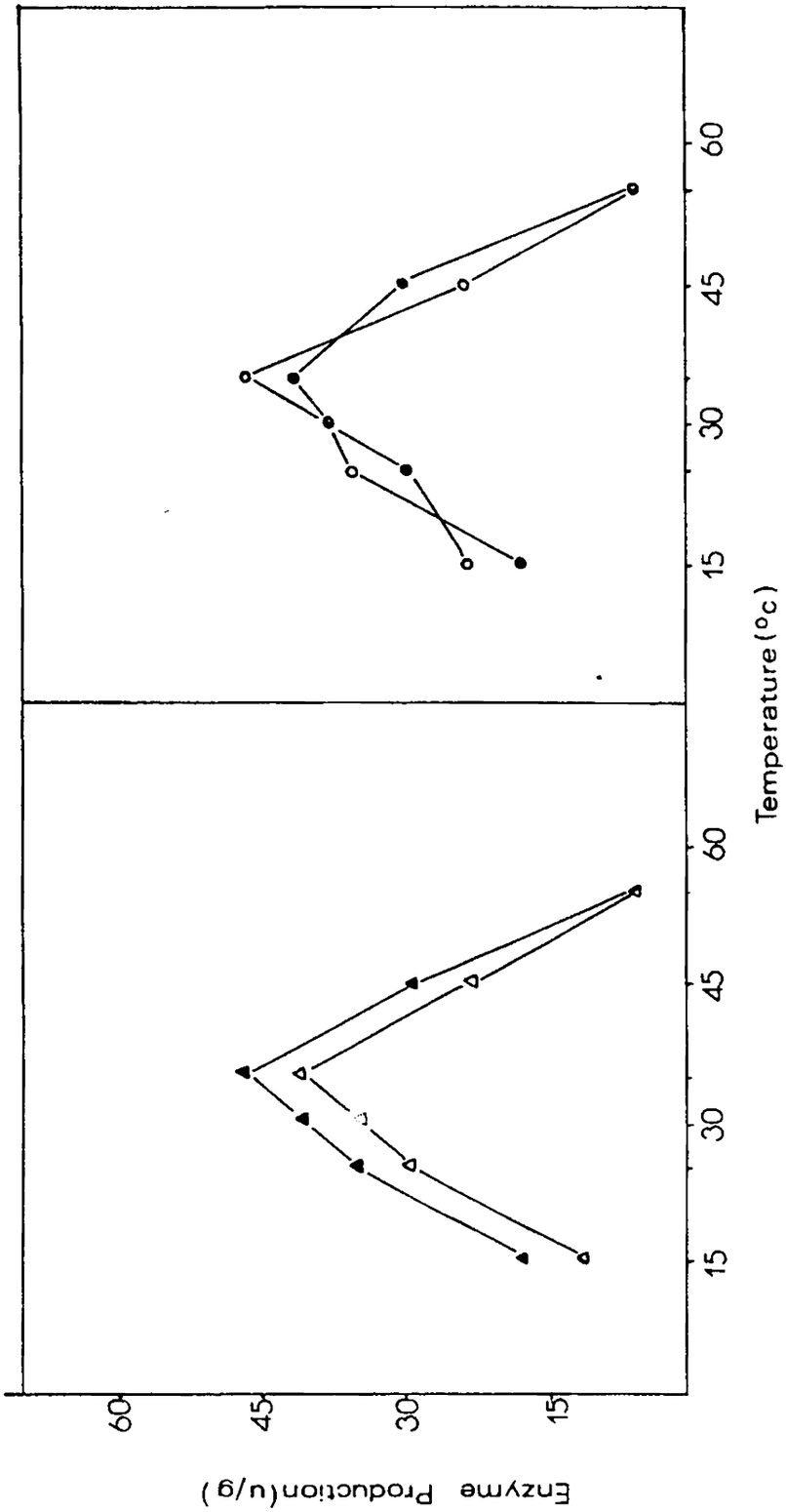


Fig.21: Effect of temperature on enzyme production by Pseudomonas sp. and Vibrio sp. in Solid State Fermentation

▲—▲ P.fluorescens ACMR 43 o—o V.costicola ACMR 267

▲—▲ P.fluorescens ACMR 171 ●—● V.cholerae ACMR 347

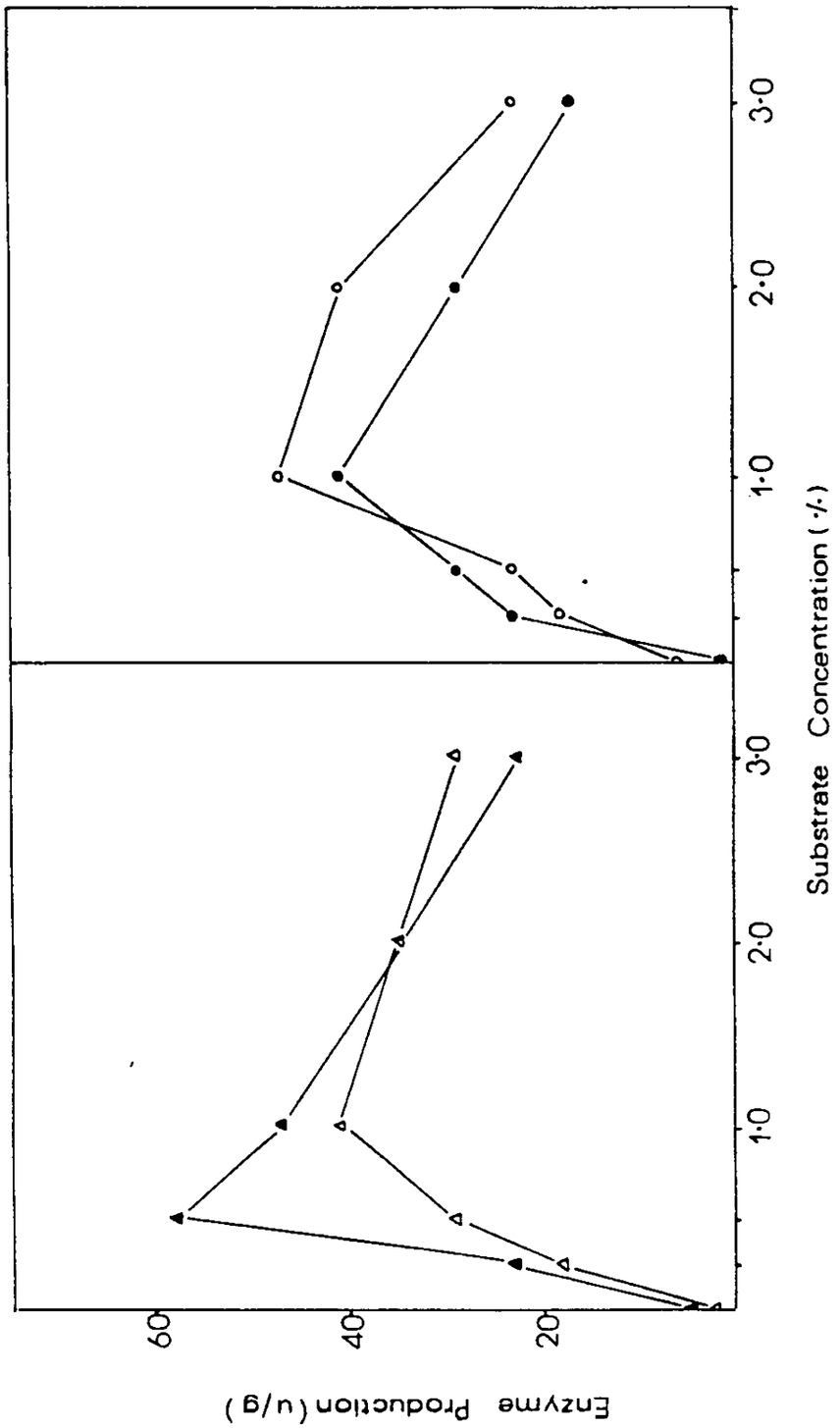


Fig.22: Effect of substrate concentration on enzyme production by Pseudomonas sp. and Vibrio sp. in Solid State Fermentation

△—△ P.fluorescens ACMR 43
▲—▲ P.fluorescens ACMR 171
○—○ V.costicola ACMR 267
●—● V.cholerae ACMR 347

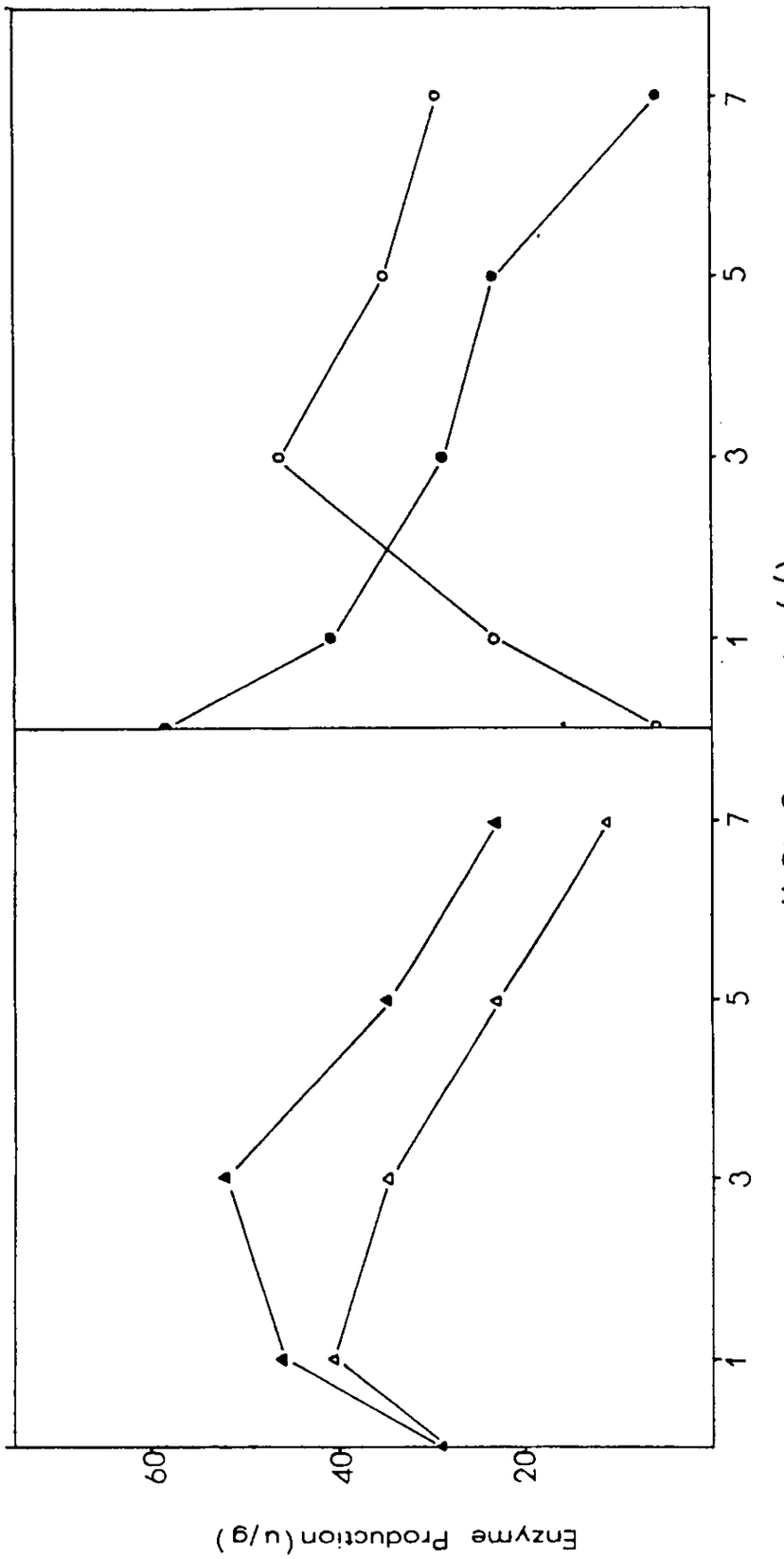


Fig.23: Effect of NaCl concentration on enzyme production by Pseudomonas sp. and Vibrio sp. in Solid State Fermentation

Δ—Δ P.fluorescens ACMR 43
▲—▲ P.fluorescens ACMR 171
o—o V.costicola ACMR 267
●—● V.cholerae ACMR 347

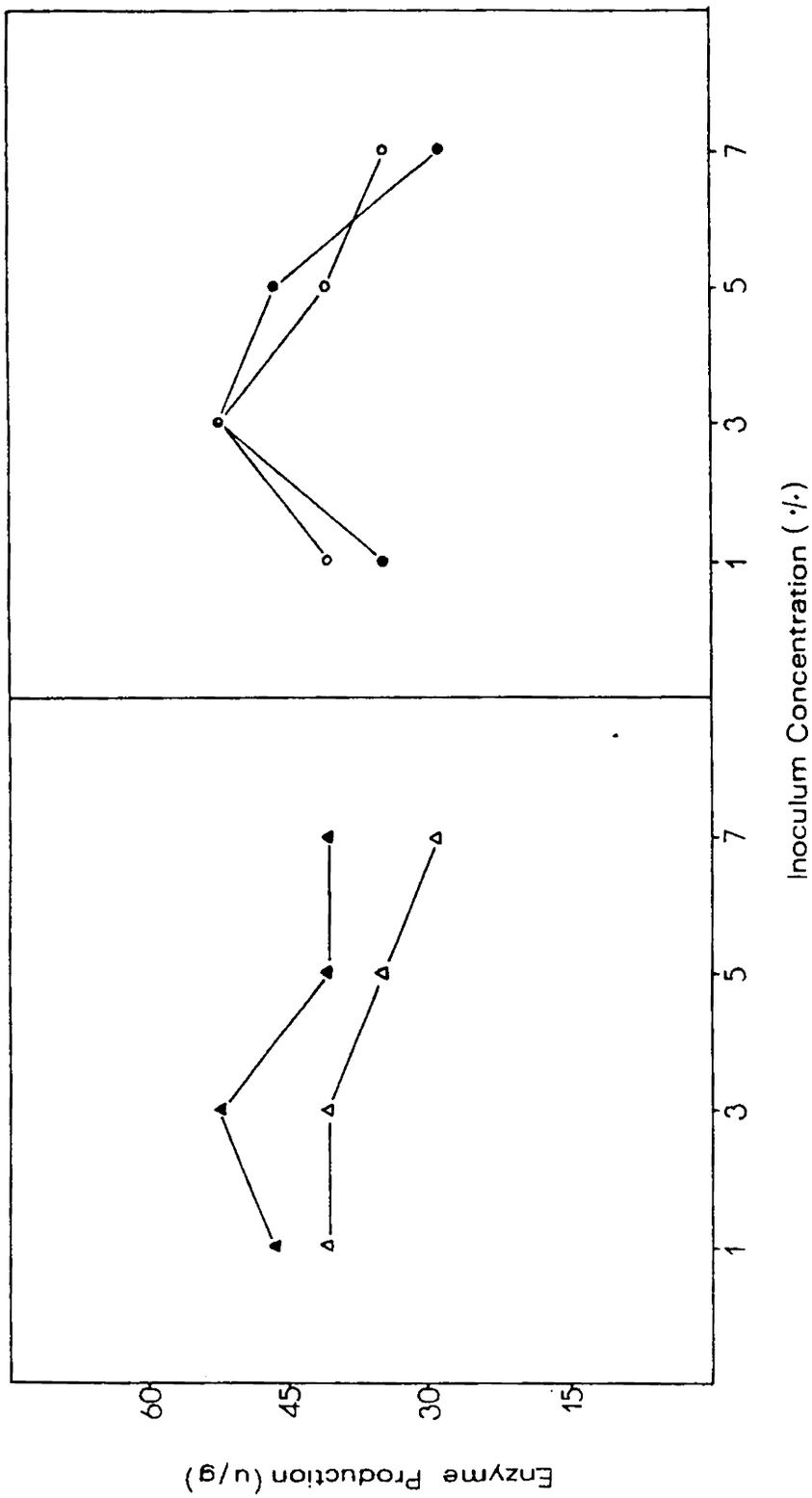


Fig.24: Effect of inoculum concentration on enzyme production by Pseudomonas sp. and Vibrio sp. in Solid State Fermentation

Δ—Δ P.fluorescens ACMR 43
▲—▲ P.fluorescens ACMR 171
o—o V.costicola ACMR 267
●—● V.cholerae ACMR 347

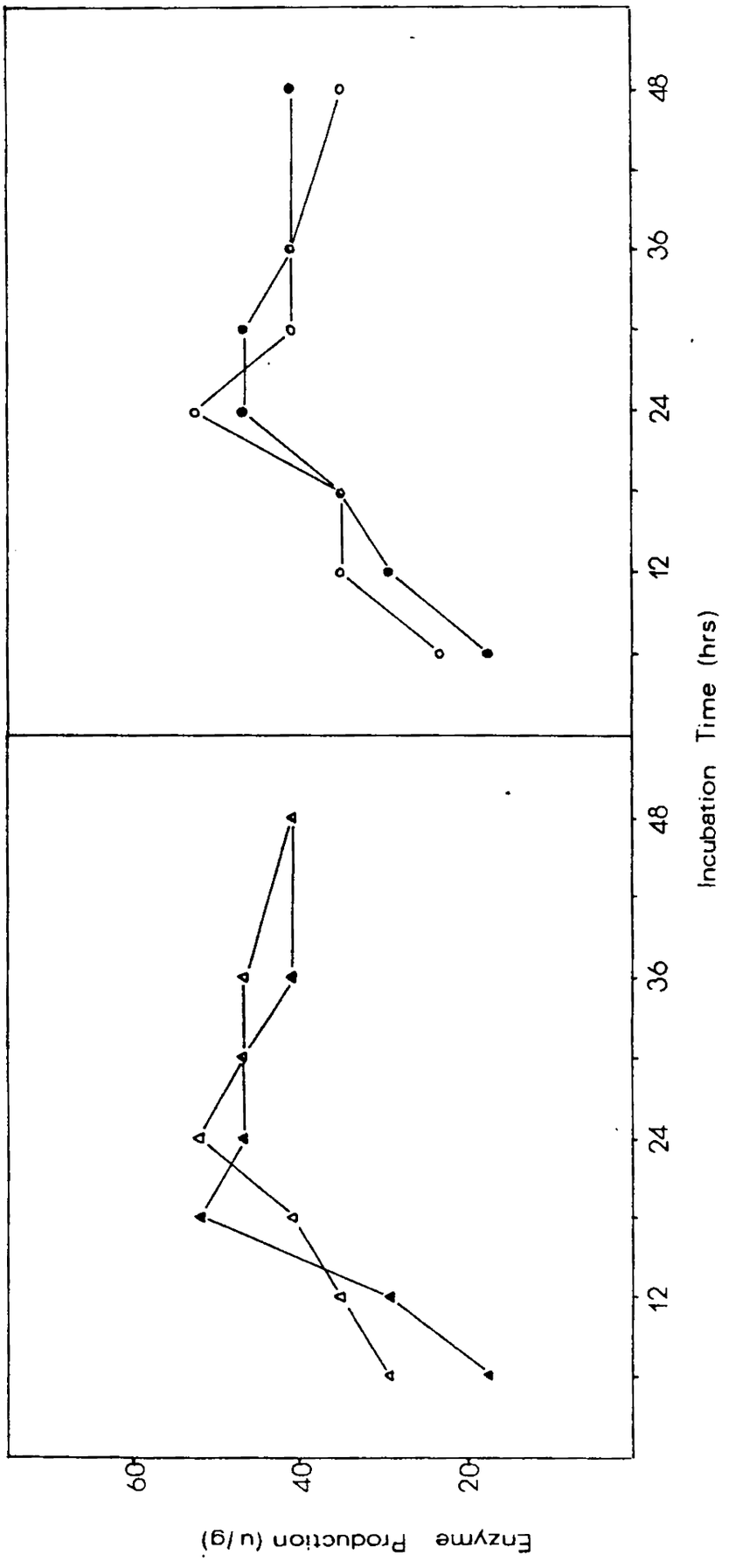


Fig.25: Effect of incubation time on enzyme production by Pseudomonas sp. and Vibrio sp. in Solid State Fermentation

Δ—Δ P.fluorescens ACMR 43
▲—▲ P.fluorescens ACMR 171
o—o V.costicola ACMR 267
●—● V.cholerae ACMR 347

Table 1: Composition of wheat bran

Constituents	Percentage
Moisture	8.80
Total nitrogen	2.33
Fat	4.10
Fibres	10.80
Pentosan	25.10
Ash	6.38
Total sugar	5.40
P_2O_5	3.15
Carbohydrate content	
Starch	14.1
Sugar	7.60
Cellulose	35.20
Hemicellulose	43.10

Source: CFTRI, Mysore

Table 2: Quantitative distribution of L-glutaminase producing heterotrophic bacterial population in various environments of Cochin

(Expressed as No. ml^{-1} or No. g^{-1})

Period	Environment			
	Marine		Estuarine	
	Water	Sediment	Water	Sediment
July 1988	$3.5x10^7$	$1.5x10^7$	$5.0x10^6$	$1.5x10^7$
August 1988	$1.5x10^7$	$3.5x10^7$	$1.7x10^6$	$2.5x10^6$
September 1988	$1.0x10^7$	$1.0x10^5$	$1.8x10^7$	$2.7x10^6$
October 1988	$3.0x10^7$	$3.5x10^7$	$3.0x10^7$	$4.5x10^7$
November 1988	$5.5x10^7$	$4.5x10^7$	$5.6x10^6$	$6.2x10^6$
December 1988	$4.25x10^7$	$4.1x10^7$	$4.8x10^6$	$1.3x10^7$

Table 3: Generic distribution of L-glutaminase producing bacteria in various environments of Cochin

(Expressed as percentage)

Organisms	Environment			
	Marine		Estuarine	
	Water	Sediment	Water	Sediment
Aeromonas	1.0	0.00	5.0	3.33
Vibrio	9.0	12.90	10.0	6.67
Pseudomonas	42.0	41.94	5.0	30.00
Alcaligenes	22.0	17.20	25.0	10.00
Acinetobacter	12.0	11.83	35.0	0.00
Bacillus	10.0	11.83	20.0	33.33
Planococci	4.0	4.30	0.0	16.66
Unidentified	1.0	0.00	0.0	2.20
Grams -ve	89.0	83.87	80.0	63.33
Gram +ve	11.0	16.13	20.0	36.67

Table 4: Characteristic properties of selected strains

(a) Pseudomonas sp.

Properties studied	P.fluorescens ACMR 171	P.fluorescens ACMR 43
Source	Marine sediment	Marine water
(1)	(2)	(3)
Colony morphology		
Shape	Circular	Circular
Colour	Lemon yellow	White
Surface	Umbonate	Convex
Consistency	Opaque	Opaque
Cell morphology	Small thin rods	Long thin rods
Gram reaction	-	-
Motility	+	+
Pycocyanin production	-	-
Chlororaphin production	-	-
Non-fluorescent pigment		
Green	-	-
Orange	-	-
Blue	-	-
Biochemical characters		
Kovac's oxidase	+	+
Levan formation from sucrose	+	+
Gelatin liquifaction	+	+

(Contd....)

(1)	(2)	(3)
Starch hydrolysis	-	-
Lipase production	-	-
Growth at 4°C	+	+
Growth at 41°C	+	+
Denitrification	+	-
Utilization of		
Citrate	+	+
Lactate	+	+
∝-Ketoglutarate	+	+
Glycerol	+	+
Maltose	-	-
Lactose	-	-
Ribose	+	+
Arginine	+	+
Sucrose	+	+
Galactose	+	+
Fructose	+	+
Trehalose	+	+
Propionate	+	+
Butyrate	+	+
Ethanol	+	-
Glycine	-	-

(Contd....)

(1)	(2)	(3)
L-leucine	+	+
L-serine	+	+
L-valine	+	+
L-lysine	+	+
L-ornithine	+	+
L-histidine	+	+
L-tryptophan	+	+
L-glutamate	+	+
L-alanine	+	+
L-aspartate	+	+
L-proline	+	+
Fermentation of		
Glucose	Oxidative	Oxidative
Mannitol	+	+
Xylose	+	+
L-arabinose	+	+

(b) Vibrio sp.

Properties studied	V.costicola ACMR 267	V.cholerae ACMR 347
Source	Estuarine water	Estuarine sediment
(1)	(2)	(3)
Colony morphology		
Shape	Irregular	Circular
Colour	Yellow	White
Surface	Convex	Convex
Consistency	Opaque	Translucent
Cell morphology	Small thin rods	Small thin rods
Gram reaction	-	-
Motility	+	+
Diffusible pigment	-	-
Luminiscence	-	-
Biochemical characters		
Kovac's oxidase	+	+
Indole reaction	-	+
Methyl-red reaction	-	+
Voges Proskauer reaction	+	+
Citrate utilization	-	+
Reduction of NO_3 to NO_2	-	+
Gas from glucose	-	-

(Contd...)

(1)	(2)	(3)
Growth in		
0% NaCl	-	+
10% NaCl	+	-
at 4°C	-	-
at 41°C	+	+
Production of		
Amylase	-	+
Gelatinase	-	+
Lipase	-	+
Caesinase	-	+
Fermentation of		
Glucose	Fermentative	Fermentative
Arabinose	-	-
Mannitol	+	+
Xylose	-	-
Utilization of		
Mannose	-	+
Galactose	-	+
Sucrose	+	+
Trehalose	+	+
Lactose	-	-

(Contd...)

(1)	(2)	(3)
Propionate	+	+
Ribose	+	+
Lactate	+	+
∝-Ketoglutarate	-	+
Ethanol	-	-
L-alanine	+	-
L-serine	+	+
L-leucine	-	-
L-glutamate	-	+
L-histidine	-	-
L-proline	-	+

Table 5: Optimization of requirement of additional carbon sources for growth and enzyme production by Pseudomonas sp. and Vibrio sp. in SmF

Organisms	Parameters analyzed	Glutamine	Additional carbon sources						
			Glucose	Galactose	Starch	Maltose	Lactose	Na_2CO_3	Trisodium citrate
Pseudomonas fluorescens ACMR 43	Growth*	0.7	1.248	0.728	0.792	0.926	0.621	0.591	0.916
	Enzyme production**	2.584	3.054	1.644	2.348	2.584	1.878	2.114	2.668
Pseudomonas fluorescens ACMR 171	Growth	0.9	1.964	1.825	0.992	1.582	1.168	0.946	1.212
	Enzyme production	2.114	3.524	1.878	2.114	2.584	1.644	1.878	2.114
Vibrio costicola ACMR 267	Growth	0.7	1.368	1.036	1.106	0.752	0.852	0.810	0.846
	Enzyme production	2.114	3.648	2.114	1.878	1.878	2.114	1.878	2.348
Vibrio cholerae ACMR 347	Growth	0.9	1.24	1.124	1.268	0.884	1.554	0.924	1.326
	Enzyme production	3.09	3.838	1.644	1.878	1.408	1.878	1.878	3.114

* Growth measured as OD at 660 nm

** Enzyme production expressed as u/ml

Table 6: Optimization of requirement of additional nitrogen sources for growth and enzyme production by Pseudomonas sp. and Vibrio sp. in SmF

Organisms	Parameters analyzed	Glutamine	Additional nitrogen sources						
			Beef extract	Yeast extract	Peptone	Glutamic acid	Lysine	KNO_3	$NaNO_3$
Pseudomonas fluorescens ACMR 43	Growth*	0.7	1.846	1.584	1.642	0.716	0.729	0.681	0.816
	Enzyme production**	2.584	2.818	2.348	1.408	1.644	2.584	2.114	2.114
Pseudomonas fluorescens ACMR 171	Growth	0.9	2.066	2.138	2.568	1.562	1.482	1.064	1.286
	Enzyme production	2.114	2.348	2.114	0.938	1.644	2.114	1.644	1.878
Vibrio costicola ACMR 267	Growth	0.9	2.982	2.342	2.660	0.852	0.718	0.810	0.926
	Enzyme production	2.114	1.878	2.114	3.054	2.818	3.288	1.878	2.114
Vibrio cholerae ACMR 347	Growth	0.9	3.294	3.854	4.024	1.246	0.884	0.719	0.792
	Enzyme production	3.09	1.408	0.704	1.408	3.054	3.524	1.644	1.878

* Growth measured as OD at 660 nm

** Enzyme production expressed as u/ml

Table 7: Optimization of glucose concentration for growth and enzyme production by *Pseudomonas* sp. and *Vibrio* sp. in SmF

Organisms	Parameters analyzed	Percentage of glucose concentration				
		0.2	0.5	1	2	3
Pseudomonas fluorescens ACMR 43	Growth*	0.452	0.791	1.328	0.878	0.615
	Enzyme production**	2.818	3.588	3.288	2.114	1.408
Pseudomonas florescens ACMR 171	Growth	0.850	1.360	1.830	1.768	1,568
	Enzyme production	2.348	3.788	3.050	1.878	1.408
Vibrio costicola ACMR 267	Growth	0.534	0.926	1.117	1.225	1.124
	Enzyme production	2.348	2.750	3.446	2.114	1.878
Vibrio cholerae ACMR 347	Growth	0.618	0.860	1.116	1.046	0.992
	Enzyme production	3.168	3.948	3.564	2.334	1.527

* Growth measured as OD at 660 nm

** Enzyme production expressed as u/ml

Table 8: Production of extracellular and intracellular glutaminase by Pseudomonas sp. Vibrio sp.

Media	Pseudomonas fluorescens ACMR 43		Pseudomonas fluorescens ACMR 171		Vibrio costicola ACMR 267		Vibrio cholerae ACMR 347	
	Extra-cellular glutaminase (u/ml)	Intra-cellular glutaminase (u/ml)	Extra-cellular glutaminase (u/ml)	Intra-cellular glutaminase (u/ml)	Extra-cellular glutaminase (u/ml)	Intra-cellular glutaminase (u/ml)	Extra-cellular glutaminase (u/ml	Intra-cellular glutaminase (u/ml)
Mineral media + 1 % glutamine	2.348	0.469	1.878	0.704	2.114	0.469	2.818	0.413
Nutrient broth + 1% glutamine	1.057	0.234	1.408	0.393	1.174	0.117	0.587	ND
Nutrient broth	ND	ND	0.234	ND	0.469	ND	0.359	ND

ND : Not Detectable

Table 9: Purification of glutaminase from Pseudomonas sp. and Vibrio sp.

Organisms	Purification steps		Total activity	Total protein	Specific activity u/mg of protein	Yield (%)	Fold (x)
Pseudomonas fluorescens ACMN 43	(i)	Crude extract	281.8	128.0	2.20	100.00	--
	(ii)	$(NH_4)_2SO_4$ fractionation	164.4	15.0	10.96	58.33	4.98
	(iii)	Chromatography upon Seralite SRA-400	140.91	3.0	46.97	50.00	21.35
	(iv)	Chromatography upon-Seralite SRA-120	129.2	1.46	88.49	45.84	40.22
Pseudomonas fluorescens ACMN 171	(i)	Crude extract	305.4	179.6	1.7	100	--
	(ii)	$(NH_4)_2SO_4$ fractionation	246.7	16.72	14.75	80.78	8.67
	(iii)	Chromatography upon Seralite SRA-400	176.2	3.52	50.05	57.69	29.44
	(iv)	Chromatography upon Seralite SRA-120	129.2	1.6	80.75	42.31	47.5

(Contd....)

Organisms	Purification steps	Total activity	Total protein	Specific activity u/mg of protein	Yield %	Fold (x)
Vibrio costicola ACMR 267	(i) Crude extract	328.9	299.0	1.1	100.0	--
	(ii) $(NH_4)_2SO_4$ fractionation	211.4	11.02	19.18	64.27	17.44
	(iii) Chromatography upon Seralite SRA-400	152.7	3.99	38.18	46.42	34.71
	(iv) Chromatography upon Seralite SRA-120	129.2	2.09	61.7	39.28	56.09
Vibrio cholerae ACMR 347	(i) Crude extract	393.7	262.4	1.5	100.0	--
	(ii) $(NH_4)_2SO_4$ fractionation	258.4	14.31	18.02	65.63	12.01
	(iii) Chromatography upon Seralite SRA-400	164.4	3.36	49.0	41.76	32.67
	(iv) Chromatography upon Seralite SRA-120	140.9	1.96	71.77	35.79	47.84

Table 10: Substrate specificity of glutaminase from Pseudomonas sp. and Vibrio sp.

Organisms	Parameter analyzed	L-glutamine	L-asparagine	L-glutamine plus L-asparagine
Pseudomonas fluorescens ACMR 43	Enzyme activity (u/ml)	0.939	Nil	0.822
Pseudomonas fluorescens ACMR 171	Enzyme activity (u/ml)	1.057	0.234	1.174
Vibrio costicola ACMR 267	Enzyme activity (u/ml)	1.117	0.359	1.057
Vibrio cholerae ACMR 347	Enzyme activity (u/ml)	1.057	0.359	0.939

Table 11: Effect of heavy metals on the activity of glutaminase from *Pseudomonas* sp. and *Vibrio* sp.

Organisms	Parameters analyzed	Pb	Co	Mn	Hg	Cu	Fe	Ca	Zn
Pseudomonas fluorescens ACMR 43	Enzyme activity u/ml	0.704	0.587	0.469	0.234	0.587	0.469	0.587	0.704
	Loss of activity %	25.030	37.500	50.500	75.100	37.500	50.500	37.500	25.030
Pseudomonas fluorescens ACMR 171	Enzyme activity u/ml	0.587	0.822	0.822	--	0.704	0.469	0.704	0.939
	Loss of activity %	44.500	22.300	22.300	100.000	33.400	55.700	33.400	11.200
Vibrio costicola ACMR 267	Enzyme activity u/ml	0.469	0.587	0.587	--	0.704	0.359	0.469	0.704
	Loss of activity %	50.500	37.500	37.500	100.000	25.030	61.800	50.500	25.100
Vibrio cholerae ACMR 347	Enzyme activity u/ml	0.704	0.587	0.469	0.234	0.469	0.587	0.469	0.822
	Loss of activity %	33.400	44.500	55.700	78.000	55.700	44.500	55.700	22.300

0.939 u/ml was taken as 100% activity for *P.fluorescens* ACMR 43
1.057 u/ml was taken as 100% activity for *P.fluorescens* ACMR 171
0.939 u/ml was taken as 100% activity for *V.costicola* ACMR 267
1.057 u/ml was taken as 100% activity for *V.cholerae* ACMR 347

Table 12: Effect of other substrates on the activity of glutaminase from Pseudomonas sp. and Vibrio sp.

Organisms	Parameters analyzed	Glutamic acid	Aspartic acid	EDTA	Phos-phate	Borate	Tris ion	Phenol-phtha-lein	Bromo-cresol purple	∝-keto-gluta-rate
Pseudomonas fluorescens ACMR 43	Enzyme activity u/ml	0.939	0.939	0.939	0.939	0.822	1.057	0.469	0.469	0.234
	Loss of activity %	0	0	0	0	12.500	0	50.060	50.060	75.080
Pseudomonas fluorescens ACMR 171	Enzyme activity u/ml	1.057	1.057	1.057	1.292	0.939	1.057	0.587	0.469	0.352
	Loss of activity %	0	0	0	0	11.160	0	44.470	55.630	66.700
Vibrio costicola ACMR 267	Enzyme activity u/ml	0.822	1.117	1.057	1.527	0.939	1.057	0.587	0.704	0.587
	Loss of activity %	26.420	0	5.400	0	15.940	5.400	47.450	36.970	47.450
Vibrio cholerae ACMR 347	Enzyme activity u/ml	0.939	1.057	1.057	1.117	0.939	1.409	0.469	0.587	0.352
	Loss of activity %	11.160	0	0	0	11.160	0	55.630	44.470	66.700

0.939 u/ml was taken as 100% activity for P.fluorescens ACMR 43
1.057 u/ml was taken as 100% activity for P.fluorescens ACMR 171
1.117 u/ml was taken as 100% activity for V.costicola ACMR 267
1.057 u/ml was taken as 100% activity for V.cholerae ACMR 347

Table 13: Optimization of drying temperature for recovery of glutaminase from SSF

(Enzyme recovery expressed as u/g)

Organisms	Temperature (°C)		
	30	40	50
Pseudomonas fluorescens ACMR 43	41.12	58.74	46.99
Pseudomonas fluorescens ACMR 171	46.99	52.86	41.12
Vibrio costicola ACMR 267	35.24	46.99	52.86
Vibrio cholerae ACMR 347	41.12	52.86	46.99

Table 14: Optimization of extraction medium for recovery of glutaminase from SSF

(Enzyme recovery expressed as u/g)

Organisms	Extraction Media				
	Phosphate buffer (0.2 M) pH 6 or 8	Distilled water	Distilled water plus 1% NaCl	Tap water	Aqueous ethanol
Pseudomonas fluorescens ACMR 43	46.99	35.24	35.24	23.49	29.37
Pseudomonas fluorescens ACMR 171	46.99	29.37	35.24	23.49	23.49
Vibrio costicola ACMR 267	41.12	35.24	29.37	23.49	29.37
Vibrio cholerae ACMR 347	46.99	35.24	41.12	35.24	23.49

Table 15: Optimization of pH of extraction for recovery of glutaminase from SSF

(Enzyme recovery expressed as u/g)

Organisms	pH of Extraction				
	5	6	7	8	9
Pseudomonas fluorescens ACMR 43	46.99	52.86	41.12	41.12	29.37
Pseudomonas fluorescens ACMR 171	35.24	35.24	41.12	46.99	41.12
Vibrio costicola ACMR 267	41.12	46.99	46.99	29.37	23.49
Vibrio cholerae ACMR 347	46.99	52.86	35.24	23.49	17.62

Table 16: Optimization of buffer systems for recovery of glutaminase from SSF

(Enzyme recovery expressed as u/g)

Organisms	Buffer Systems			
	Acetate buffer (0.2 M) pH 6	Phosphate buffer (0.2 M) pH 6	Phosphate buffer (0.2 M) (pH 8)	Borate buffer (0.2 M) pH 8
Pseudomonas fluorescens ACMR 43	52.86	46.99	ND	ND
Pseudomonas fluorescens ACMR 171	ND	ND	52.86	41.12
Vibrio costicola ACMR 267	46.99	52.86	ND	ND
Vibrio cholerae ACMR 347	41.12	46.99	ND	ND

Table 17: Optimization of ratio of bran to buffer for recovery of glutaminase from SSF

(Enzyme recovery expressed as u/g)

Organisms	Ratio of bran to buffer		
	1:3	1:5	1:10
Pseudomonas fluorescens ACMR 43	17.62	46.99	35.24
Pseudomonas fluorescens ACMR 171	28.19	52.86	46.99
Vibrio costicola ACMR 267	21.14	46.99	23.44
Vibrio cholerae ACMR 347	24.66	46.99	35.24

Table 18: Effect of contact time on recovery of glutaminase from SSF

(Enzyme recovery expressed as u/g)

Organisms	Contact Time (min.)			
	30	60	90	120
Pseudomonas fluorescens ACMR 43	41.12	46.99	41.12	35.24
Pseudomonas fluorescens ACMR 171	29.37	52.86	46.99	46.99
Vibrio costicola ACMR 267	29.37	35.24	52.86	46.99
Vibrio cholerae ACMR 347	23.49	35.24	46.99	46.99

Table 19: Effect of contact temperature on recovery of glutaminase from SSF

(Enzyme recovery expressed as u/g)

Organisms	Contact Temperature (°C)		
	25	35	45
Pseudomonas fluorescens ACMR 43	41.12	35.24	29.37
Pseudomonas fluorescens ACMR 171	29.37	35.24	35.24
Vibrio costicola ACMR 267	35.24	35.24	29.37
Vibrio cholerae ACMR 347	41.12	35.24	35.24

Table 20: Effect of particle size on glutaminase production by Pseudomonas sp. and Vibrio sp. in SSF

(Enzyme recovery expressed as u/g)

Organisms	Size of Particles		
	> 2.41 mm	1.41-2.06 mm	< 1.20 mm
Pseudomonas fluorescens ACMR 43	35.24	35.24	46.99
Pseudomonas fluorescens ACMR 171	29.37	35.24	46.99
Vibrio costicola ACMR 267	23.49	46.99	29.37
Vibrio cholerae ACMR 347	29.37	41.12	41.12

Table 21: Effect of moisture content for glutaminase production by Pseudomonas sp. and Vibrio sp. in SSF

(Enzyme recovery expressed as u/g)

	Moisture content (%)					
	20	30	40	50	60	70
Pseudomonas fluorescens ACMR 43	17.62	35.24	41.12	46.99	46.99	46.99
Pseudomonas fluorescens ACMR 171	23.49	29.37	41.12	46.99	52.86	46.99
Vibrio costicola ACMR 267	11.72	23.49	46.99	46.99	46.99	41.12
Vibrio cholerae ACMR 347	17.62	17.62	23.49	41.12	41.12	35.24

Table 22: Effect of additional carbon sources on glutaminase production by *Pseudomonas* sp. and *Vibrio* sp. in SSF

(Enzyme recovery expressed as u/g)

Organisms	Glutamine	Additional Carbon Sources					
		Glucose	Maltose	Starch	Lactose	Na_2CO_3	Trisodium citrate
Pseudomonas fluorescens ACMR 43	41.12	29.37	46.99	41.12	29.37	35.24	35.24
Pseudomonas fluorescens ACMR 171	46.99	58.74	46.99	46.99	41.12	29.37	41.12
Vibrio costicola ACMR 267	41.12	29.37	64.67	29.37	29.37	35.24	41.12
Vibrio cholerae ACMR 347	35.24	29.37	46.99	29.37	29.37	23.49	35.24

Table 23: Effect of additional nitrogen sources on glutaminase production by *Pseudomonas* sp. and *Vibrio* sp. in SSF

(Enzyme recovery expressed as u/g)

Organisms	Glutamine	Additional Nitrogen Sources						
		Beef extract	Yeast extract	Peptone	Glutamic acid	Lysine	KNO_3	$NaNO_3$
Pseudomonas fluorescens ACMR 43	41.12	29.37	11.72	29.37	35.24	46.99	29.37	35.24
Pseudomonas fluorescens ACMR 171	46.99	23.49	17.62	41.12	35.24	46.99	35.24	41.12
Vibrio costicola ACMR 267	41.12	23.49	23.49	17.62	41.12	41.12	29.37	35.24
Vibrio cholerae ACMR 347	35.24	23.49	17.62	23.49	35.24	35.24	23.49	29.37

Table 24: Comparative analysis of glutaminase production in SmF and SSF with reference to different environmental variables

(a) pH

pH	*P. fluorescens* ACMR 43		*P. fluorescens* ACMR 171		*V. costicola* ACMR 267		*V. cholerae* ACMR 347	
	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g
4	0.704	29.37	1.408	35.24	0.938	17.625	1.644	29.37
5	2.348	46.95	1.878	52.87	1.408	41.12	2.818	35.23
6	2.584	52.87	2.114	46.95	2.114	52.87	3.268	46.99
7	2.114	35.24	1.878	46.95	1.644	46.99	3.00	41.12
8	1.878	23.45	1.760	41.12	1.174	41.12	2.348	23.49
9	0.938	17.62	0.704	23.45	0.704	23.49	1.878	11.72
10	0.469	ND	ND	5.85	ND	5.85	0.938	ND
11	ND	--	--	--	--	--	--	--

ND : Not Detectable

(b) Temperature

Temperature (°C)	P.fluorescens ACMR 43		P.fluorescens ACMR 171		V.costicola ACMR 267		V.cholerae ACMR 347	
	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g
15	0.939	11.72	0.587	17.62	1.174	23.45	1.292	17.62
25	2.348	29.37	1.057	35.24	2.114	29.37	3.054	35.24
30	2.48	33.93	1.527	37.11	2.20	35.24	3.104	35.24
35	2.584	41.12	2.114	46.99	2.348	46.95	3.268	41.12
45	1.644	23.49	1.409	29.37	0.936	23.45	1.409	29.37
55	0.58	5.85	0.822	5.85	0.469	5.85	0.587	5.85

(c) Substrate concentration

Substrate concentration (%)	P.fluorescens ACMR 43		P.fluorescens ACMR 171		V.costicola ACMR 267		V.cholerae ACMR 347	
	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g
0.25	1.408	17.62	0.938	23.49	0.822	17.624	1.409	23.49
0.5	2.818	29.37	1.644	58.74	1.174	23.493	2.584	29.37
1.0	2.348	41.12	2.348	46.99	2.114	46.991	3.168	41.12
2.0	1.057	35.24	1.409	35.24	1.292	41.12	1.644	29.37
3.0	0.587	29.37	1.409	23.49	0.938	23.40	1.527	17.62

(d) NaCl concentration

NaCl concentration (%)	P.fluorescens ACMR 43		P.fluorescens ACMR 171		V.costicola ACMR 267		V.cholerae ACMR 347	
	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g
0	1.644	29.37	0.938	29.37	0.234	5.87	3.538	58.7
1.0	2.114	41.12	1.878	46.99	1.527	23.49	2.79	41.12
3.0	2.584	35.24	3.459	52.86	3.054	46.99	1.409	29.37
5.0	1.408	23.49	2.348	35.24	1.409	35.24	1.292	23.49
7.0	0.469	11.72	0.882	23.49	0.882	29.37	0.822	5.87
10.0	0.234	--	0.234	--	0.469	--	0.117	--

(e) Additional carbon sources

Carbon sources	P.fluorescens ACMR 43		P.fluorescens ACMR 171		V.costicola ACMR 267		V.cholerae ACMR 347	
	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g
Glutamine	2.584	41.12	2.114	46.99	2.114	41.12	3.09	35.24
Glucose	3.054	29.37	3.524	58.74	3.648	29.37	3.838	29.37
Maltose	2.584	46.99	2.584	46.99	1.878	64.67	1.408	46.99
Starch	2.348	41.12	2.114	46.99	1.878	29.37	1.878	29.37
Lactose	1.878	29.37	1.644	41.12	2.114	29.37	1.878	29.37
Na_2CO_3	2.114	35.24	1.878	29.37	1.878	35.24	1.878	23.49
Trisodium citrate	2.668	35.24	2.114	41.12	2.348	41.12	3.114	35.24

(f) Additional nitrogen sources

Nitrogen sources	P.fluorescens ACMR 43		P.fluorescens ACMR 171		V.costicola ACMR 267		V.cholerae ACMR 347	
	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g
Glutamine	2.584	41.12	2.114	46.99	2.114	41.12	3.09	35.24
Beef extract	2.818	29.37	2.348	23.49	1.878	23.49	1.408	23.49
Yeast extract	2.348	11.72	2.114	17.62	2.114	23.49	0.704	17.62
Peptone	1.408	29.37	0.938	41.12	3.054	17.62	1.408	23.49
Glutamic acid	1.644	35.24	1.644	35.24	2.818	41.12	3.054	35.24
Lysine	2.584	46.99	2.114	46.99	3.288	41.12	3.524	35.24
KNO_3	2.114	29.37	1.644	35.24	1.878	29.37	1.644	23.49
$NaNO_3$	2.114	35.24	1.878	41.12	2.114	35.24	1.878	29.37

(g) Inoculum concentration

Inoculum concentration (%)	*P. fluorescens* ACMR 43		*P. fluorescens* ACMR 171		*V. costicola* ACMR 267		*V. cholerae* ACMR 347	
	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g
1.0	2.584	41.12	1.878	46.99	2.348	41.12	0.921	35.24
3.0	2.348	41.12	2.348	52.86	2.584	52.86	1.26	52.86
5.0	2.348	35.24	2.114	41.12	2.114	41.12	1.124	46.99
7.0	2.114	29.37	2.114	41.12	2.114	35.24	1.30	29.37

(h) Incubation time

Incubation time (hours)	P.fluorescens ACMR 43		P.fluorescens ACMR 171		V.costicola ACMR 267		V.cholerae ACMR 347	
	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g	SmF u/ml	SSF u/g
6	0.704	29.37	1.292	17.62	0.587	23.49	0.587	17.32
12	1.408	35.24	1.878	29.37	1.408	35.24	2.348	29.37
18	1.878	41.12	2.348	52.86	2.114	35.24	3.46	35.24
24	2.348	52.86	2.114	46.99	1.878	32.86	3.20	46.99
30	2.114	46.99	2.114	46.99	1.644	41.12	2.81	46.99
36	1.878	46.99	1.878	41.12	1.644	41.12	2.50	41.12
48	1.587	41.12	1.878	41.12	1.408	35.24	2.00	41.12

4. DISCUSSION

Glutaminase producing bacteria were present in higher levels both in the water and sediments of marine as well as estuarine environments. These results very clearly indicate that these environments are potential sources of glutaminase producing bacteria when compared to terrestrial environments. Of course no such comparative studies have been either carried out in the past or been attempted in the present study. Marine environments in general are unique by virtue of their salinity, wide range of mineral content and well knitted ecosystem when compared to terrestrial environments which is constantly disturbed by human activities. The marine bacteria have not been experimentally tried for their potential in many of the human endeavours in which they would have a major role to play, for example in the field of health and medicine and in industry. Hence an attempt has made in the present study to screen the glutaminase producers from the marine environments, unlike the other investigations carried out in the past by others. Since no similar reports on quantitative distribution of glutaminase producing bacteria in marine environments are available in the literature, no comparison could be made possible.

The qualitative distribution of glutaminase producing bacteria was rather limited to few genera that included species of Pseudomonas, Aeromonas, Vibrio, Alcaligenes, Acinetobacter, Bacillus and Planococci. Interestingly no member of Enterobacteriaceae was isolated from both water and sediment samples. While Pseudomonas was the dominant flora in marine samples, Acinetobacter and Bacillus were dominant in estuarine environments. These results although could not be directly compared with any other similar results on glutaminase producing bacteria, the dominance of these species is comparable to their predominance in the heterotrophic flora reported for marine and estuarine environments of Cochin (Chandrasekaran, 1985; Brightsingh, 1986).

It may be noted that earlier reports on the isolation of glutaminase producing bacteria were mainly restricted to environments and the isolates mainly belonged to the species of E.coli, Proteus morganii, Xanthomonas juglandis, Erwinia carotovora, Serratia marcescens, Pseudomonas fluorescens, P.aeruginosa, Aeromonas hydrophila, (Imada et al., 1973), Acinetobacter glutaminasificans (Roberts et al., 1972), Clostridium welchii (Hughes & Williamson, 1952) Bacillus licheniformis (Cook et al., 1981).

Potential strains for glutaminase production were selected for further studies mainly based on their ability to produce enhanced levels of glutaminase in mineral salts medium supplemented with 1% glutamine as the sole carbon source. Unlike the earlier investivations which employed nutrient medium for the selection and testing of glutaminase producers. The objectives of using the mineral media added with glutamine for selection was mainly to identify the organism that can produce glutaminase in larger quantity, probably as an induced enzyme which could be also secreted into the medium. The earlier investigations have attempted growth of the cell using a nutrient medium, isolated the enzyme after breaking the cell and tested enzyme activity using the cells, as such (Ramadan et al., 1964a; Hartman, 1968; Prusiner et al., 1976). Hence a different approach was desired to look for new avenues in the source of enzyme.

The attempt was so fruitful in that several high enzyme yielding strains could be recovered. However, only the top four ranking strains which belonged to species of Pseudomonas fluorescens (ACMR 171 and ACMR 43), Vibrio costicola (ACMR 267) and Vibrio cholerae (ACMR 347) were used for further investigations.

Among these, species of P.fluorescens isolated from soil had already been reported as glutaminase producers (Imada et al., 1973; Yokotsuka et al., 1987). However, no reports are available on Vibrio sp. as potential producers of glutaminase in the literature. V.cholerae which is generally known as a cholerae causing pathogen, was recovered in this study as a high glutaminase producing bacteria. No pathogenicity tests have been conducted to assertain this as a pathogen. However, there are ample indirect evidences to assume that this is not a pathogen since V.cholerae particularly of environmental origin do not produce detectable enterotoxin and appear to be non-pathogenic (Spira et al., 1979; Spira & Daniel, 1980).

Optimal levels of the different environmental variables namely temperature, pH, NaCl concentration, substrate concentration, additional carbon and nitrogen sources, inoculum concentration and period of incubation were determined for attaining maximal enzyme production by selected strains. These tests were carried out using same isolation medium under submerged fermentation conditions (SmF). The results highlighted in the previous chapter throws more light on the nature of organisms studied in terms of their responses to changes in the environmental variables besides

indicating their optimum requirements for maximal enzyme production. The environmental pH of marine water generally varies from 7.00-8.43; temperature, from 25-38.5°C; and salinity from 0.39×10^{-3} to 28.75×10^{-3} (Bright Singh, 1986).

The optimal pH required for the production of maximal glutaminase production was reported as pH 7-7.6 for Acinetobacter sp., Pseudomonas sp., E.coli, Clostridium welchii, Aspergillus oryzae (Katsumata et al., 1972; Roberts et al., 1972; Prusiner et al., 1976; Yano et al., 1988) while Bacillus licheniformis produced glutaminase maximally at two pH ie., pH 7 and 9 (Cook et al., 1981). In the present study all the species could produce maximal levels of glutaminase at pH 6 although they could produce significant levels at pH ranging from pH 5-8. Despite their isolation and cultivation at pH 7, all the strains preferred pH 6 for their maximal enzyme production. Nevertheless they recorded significant levels of enzyme production at pH 7 also.

The optimal temperature for maximal glutaminase production was reported as 25-30°C for Pseudomonas (Katsumata et al., 1972; Soda et al., 1972; Prusiner et al., 1976). whereas Acinetobacter produced glutaminase maximally at 25°C (Roberts et al., 1972). In the present study all the four

strains produced maximal levels of enzyme at 35°C, inspite of their initial isolation and cultivation at 30°C. However, significant levels of enzyme were recorded at temperatures varying from 25-45°C.

Although many of the earlier investigators have used general nutrients like glucose, yeast extract, peptone, meat extract for glutaminase production (Kozlov et al., 1972; Novak & Philips, 1974; Prusiner et al., 1976), basal synthetic medium containing glutamic acid (Ramadan et al., 1964a; Roberts et al., 1972; Soda et al., 1972; Roberts, 1976) or glutamine (Katsumata et al., 1972; Cook et al., 1981) were also reported. L-glutamic acid was used at 1 to 4% concentration and L-glutamine at 20 mM to 0.5% levels for maximal enzyme production by Acinetobacter and Pseudomonas. However, in the present investigation, except P.fluorescens ACMR 43 which preferred 0.5% glutamine for their maximal enzyme production, all the others required 1% substrate concentration and could also produce significant levels of glutaminase at substrate concentrations ranging from 0.5-3%.

Earlier studies on glutaminase production did not indent for the effect of NaCl concentration on enzyme production owing to the isolation of the strains from soil.

However, Roberts, (1976) used 6 mg of NaCl/litre of growth medium along with the substrate for the enzyme production by Pseudomonas. Whereas in the present investigation, the effect of NaCl concentration on the growth and glutaminase production by bacteria assumes paramount importance owing to their isolation from marine environments where normally salinity levels undergo frequent changes. It is not a surprise that 3% NaCl was required by the bacteria for their maximal enzyme production, except V.cholerae which did not require NaCl for maximal enzyme production, as they are originally isolated from saline environments. Growth pattern of V.cholerae in the presence of different concentrations of NaCl is more often used to assign their taxonomic position (Bergey's Manual of Systematic Bacteriology, 1984). However, the V.cholerae of the present study were able to produce significant levels of enzyme production at 1 to 5% NaCl concentrations probably owing to their native habitat being estuarine sediments.

Glucose was the only carbon source other than L-glutamic acid and L-glutamine (Prusiner et al., 1976) used for the production of glutaminase by bacteria, especially by E.coli. Effect of glucose in the growth medium for the production of glutaminase was observed to vary from bacteria

to bacteria. Thus it was observed that presence of low concentrations of glucose was effective in initiating a rapid growth of bacteria before they depended on amino acids as a source of energy (Wade et al., 1971) while inhibiting the glutaminase production totally in Pseudomonas (Roberts, 1976) and partially in Bacillus licheniformis (Cook et al., 1981). Whereas, in the present study, glucose enhanced enzyme yield by all strains when used as an additional carbon source along with glutamine. All other carbon sources tested did not yield any valuable information towards the improvement of enzyme yield since they did not influence the enzyme production either positively or negatively.

Beef extract, yeast extract, peptone, meat extract, caesin hydrolyzate and glutamic acid were widely employed as media constituents along with other components for glutaminase production by Clostridium welchii, Pseudomonas aeruginosa (Hughes & Williamson, 1952; Kozlov et al., 1972; Soda et al., 1972). But their effect on enzyme production was not monitored and analyzed. However, inclusion of yeast extract and tryptone to the growth medium with L-glutamic acid was reported to minimize glutaminase production by Acinetobacter glutaminasificans

(Roberts et al., 1972). Whereas in the present study, while beef extract enhanced maximal enzyme production in both P.fluorescens of marine origin, it repressed both the Vibrio sp. Lysine promoted higher enzyme yield in all the strains when compared to other substrates. Peptone and glutamic acid while inducing enzyme production in V.costicola ACMR 267, repressed all other strains. Yeast extract, along with KNO_3 and $NaNO_3$, did not influence enzyme production to an appreciable level. Hence it is very difficult to draw a generalised conclusion on the possible role of these substrates on glutaminase production by bacteria. Moreover, the molecular mechanism of the utilisation of these substrates as nitrogen sources and their phenomenal role in enzyme induction and repression warrants further studies for appropriate inferences.

According to Wade et al., (1971), inspite of the induction of initial growth of many species of bacteria by glucose at 0.1% concentration, in general at higher concentrations carbohydrates displayed repressing effects on enzyme production. Thus in Pseudomonas presence of glucose at 0.1-0.5% level in the medium along with glutamic acid was proved to be inhibitory (Roberts, 1976). Whereas in the present investigation, glucose at concentrations varying from

0.5-1% enhanced maximal enzyme production by all the four strains. However, as Wade et al., (1971) stated, increased levels of glucose concentration above 1% resulted in a decline in enzyme production by all the strains which might be probably due to the well known 'Glucose effect' phenomenon.

In the present investigation all the strains exhibited significant levels of enzyme production at 1 to 7% inoculum concentration. However, except for Pseudomonas fluorescens ACMR 43 which required 1% inoculum concentration for their maximal level of enzyme production, for all other strains, 3% inoculum concentration was necessary. Kozlov et al., (1972) reported that Clostridium welchii could produce maximal glutaminase only at 10% inoculum level. In the present study, results indicated very clearly that low levels of inocula are more than enough to produce higher yields of enzyme by all the bacteria.

Maximal glutaminase production was observed in the late exponential phase of growth of Pseudomonas and Bacillus licheniformis (Roberts, 1976; Cook et al., 1981) and in the early stationary phase of E.coli (Hartman, 1968; Prusiner et al., 1976). P.aeruginosa produced maximal levels of

glutaminase after 18 hours of incubation (Soda et al., 1972), while Acinetobacter glutaminasificans required only 6 hours of incubation for the same (Roberts et al., 1972). However, in the present study all the strains produced maximal level of enzyme only during their stationary phase of growth after completing their exponential phase by 12 hours of growth itself. Nevertheless all the strains registered significant levels of enzyme production during the exponential phase of growth also.

Glutaminases have been reported to be produced as both extracellular and intracellular fractions. Arima et al., (1972) observed extracellular secretion of L-glutaminase by Pseudomonas dacunhae, P.ovalis, P.aureofaciens, P.chlororaphis, P.schuylkilliensis. In contrast with Pseudomonas sp. glutaminase activities were hardly found in the culture filtrates of yeast and fungi. Imada et al., (1973) reported that P.aureofaciens, P.schuylkilliensis, Alcaligenes facealis possessed little L-glutaminase activity in their culture filtrate after 40 hours of incubation and certain fungal species also recorded extracellular glutaminase. Whereas, Yano et al., (1988) observed that Aspergillus oryzae could produce both intra and extracellular glutaminases.

There is a belief that L-asparagine and L-glutamine are deamidated only intracellularly, although the reason for such specific localization inside the cell has been left unexplained. So apart from the above cited reports, no detailed accounts are available on the occurrence of extracellular glutaminase among bacterial genera. In this context the present investigation throws more evidence for the extracellular glutaminase for the production in higher titres than intracellular fraction during growth in all 3 types of media tested, by P.fluorescens, V.cholerae and V.costicola.

A comparative analysis of glutaminase production suggest that extracellular fractions are produced 2.6-6.8 times higher than that of intracellular fraction. Mineral media supported the production of both intra and extracellular glutaminase while nutrient broth supported only growth. However, addition of glutamine to nutrient broth did effect marginal induction of both extra and intracellular glutaminase. Extracellular glutaminase production in minimal media by marine bacteria thus deserve due attention by industry.

Glutaminase from Acinetobacter sp., Pseudomonas sp., Clostridium welchii and E.coli was purified earlier by $(NH_4)_2SO_4$ fractionation, chromatography upon DEAE cellulose, sephadex, and electrophoresis (Ramadan et al., 1964a; Kozlov et al., 1972; Roberts, 1976; Prusiner et al., 1976). Present investigation also employed $(NH_4)_2SO_4$ fractionation, dialysis and chromatography upon seralite (anion and cation) for the purification of glutaminase.

Homogeneous preparations of glutaminase from Pseudomonas differed in specific activity from 36 IU/mg of protein - 160 IU/mg of protein (Ramadan et al., 1964a; Katsumata et al., 1972; Roberts, 1976). Purified glutaminase with a specific activity of 3.8 u/mg of protein (Meister, 1956) and 1,520 μ moles per min per mg of protein (Hartman, 1968) was obtained from E.coli. Similarly purified glutaminase from Acinetobacter glutaminasificans was shown to possess a specific activity of 160 IU/mg of protein (Roberts et al., 1972). However, in the present study, specific activities of glutaminase obtained after purification ranged from 60-90 IU/mg of protein for all the strains.

Over all yield of purified glutaminase from various bacteria were reported to be 40% for E.coli and Clostridium welchii (Hughes & Williamson, 1952; Hartman,

1968; Kozlov et al., 1972; Prusiner et al., 1976), 40-60% for Acinetobacter (Roberts et al., 1972) and 40-50% for Pseudomonas (Roberts, 1976). In the present study purified glutaminase were obtained with an overall yield of 35-45%.

Glutaminase from E.coli were purified 6000 fold (Prusiner et al., 1976) whereas isozymes from P.aeruginosa were purified 200 and 170 fold. Whereas in the present investigation only upto 40-60 fold purified glutaminase preparation could be obtained after purification from all the four strains. The purified preparation of glutaminase possessed comparatively a very low level of asparaginase activity eliminating the doubt that the enzyme could be of a glutaminase-asparaginase nature. However, homogenity of the prepared enzyme is yet to be confirmed by electrophoresis.

Glutaminase isolated from various organisms were reported to prefer different ranges of pH for their optimal activity. Enzyme from Pseudomonas were active over a pH range of 5-9 with an optimum at pH 7 (Ramadan et al., 1964a; Roberts, 1976). While isozymes from P.aeruginosa were active over a pH range of 7.5-9 (Soda et al., 1972) and glutaminase from P.fluorescens exhibited an optimum pH range of 7.5-9.5 (Yokotsuka et al., 1987). Acinetobacter glutaminase were

active over a pH of 6-9 (Roberts et al., 1972) whereas, while E.coli glutaminase A was active below 5, glutaminase B was active at pH 7.1-9. However, both were maximally stable at pH 7 (Hartman, 1968; Prusiner et al., 1976). Clostridium welchii glutaminase was most active at pH 5-5.2 and at 4.5 (Hughes & Williamson, 1952; Kozlov et al., 1972), whereas Cryptococcus albidus glutaminase exhibited activity over a wide pH range of 5.5-8.5 (Yokotsuka et al., 1987). Both extra and intracellular glutaminase of Aspergillus oryzae were most active and stable at pH 9 (Yano et al., 1988) while enzyme from A.sojae were maximally active at 7.5-8.5 (Yokotsuka et al., 1987).

Glutaminase of all the strains tested could demonstrate stability and appreciable activity over a wide range of pH (pH 4-9) besides recording maximal activity and maximal stability at the same pH. Relatively they were more stable and active around pH 6 except P.fluorescens ACMR 171 which was maximaly active and stable at pH 8. Glutaminase activity in the clayey sediments in marine environments of Porto Novo was reported to show two pH optima of 5.6 and 8.4 (Dharmaraj et al., 1977). The pH optima of glutaminase from organisms isolated from water and sediment samples of marine and estuarine environments of Cochin share closer similarity

with the earlier reports. A possible reason might be due to the similarity in their origin, ie., marine environment.

The optimal temperature for maximal activity of glutaminase varied among the microorganisms widely. Thus glutaminase of Acinetobacter (Roberts et al., 1972) and Pseudomonas (Ramadan et al., 1964b; Roberts, 1976) were maximally active at 37°C while isozymes of P.aeruginosa were active at 30°C (Soda et al., 1972). Whereas enzyme from Clostridium welchii was maximaly active at 40°C (Hughes & Williamson, 1952) and both the intra and extracellular glutaminases from Aspergillus oryzae were shown to prefer 45°C for their maximal activity (Yano et al., 1988). Enzyme from Pseudomonas was reported to be unstable beyond 37°C (Ramadan et al., 1964b), whereas glutaminase from Clostridium welchii got inactivated beyond 60°C (Hughes & Williamson, 1952; Kozlov et al., 1972) and those from Aspergillus oryzae lost their activity at 55°C (Yano et al., 1988). From the present study, it is inferred that glutaminase of all the strains were active and stable at temperatures varying from 30-60°C with their maximal activity and stability at 40°C. A further increase in temperature to 70°C resulted in a decrease in the activity and stability of enzymes of all the strains.

All the four strains uniformly reacted sharply to an increase in substrate concentration from 0.01 M to 0.04 M by rapid increase in activity. However P.fluorescens ACMR 43 and V.costicola ACMR 267 preferred 0.06 M substrate concentration while P.fluorescens ACMR 171 and V.cholerae ACMR 347 required 0.04 and 0.08 M substrate concentration for their maximal enzyme yield. According to Prusiner et al., (1976) glutaminase of E.coli exhibited an intermediary plateu region between 8 and 13 mM glutamine concentration.

Km of glutaminases from Pseudomonas was influenced by the presence of phosphate where in its presence km was $7x10^{-}3$ M and in its absence it was $8x10^{-3}$ M (Ramadan et al., 1964b). Km values for glutaminase from Acinetobacter glutaminasificans was $5.8\pm1.5x10^{-6}$ M (Roberts et al., 1972) and Clostridium welchii was 10^{-3}M (Kozlov et al., 1972). Isozymes from Pseudomonas aeruginosa possessed km of $1.1x10^{-4}$ M and $1.8x10^{-4}$ M (Soda et al., 1972) whereas enzymes from Pseudomonas 7A exhibited a Km of $4.6x10^{-4}$ M (Roberts, 1976) while intra and extracellular glutaminases of Aspergillus oryzae exhibited a Km of $9.1x10^{-}5$ M and $9.6x10^{-5}$ M respectively (Yano et al., 1988).

Glutaminase purified in the present study showed a Km of $1.0x10^{-4}$ M for P.fluorescens ACMR 171; $4.6x10^{-5}$ M for P.fluorescens ACMR 43; $9.54x10^{-5}$ M for V.costicola ACMR 267; $1.5x10^{-5}$ M for V.cholerae ACMR 347. Results observed in the present study are very similar to those recorded for the glutaminases of A.oryzae, Pseudomonas 7A and P.aeruginosa. This indicates that glutaminase as an enzyme has more or less similar characteristics irrespective of their source, especially with reference of Km.

Glutaminase of various organisms have reported to be impaired by the presence of NaCl to a great extent. Glutaminase from E.coli, P.fluorescens, Cryptococcus albidus, A.sojae recorded only 65, 75, 65 and 6% respectively of their original activity in the presence of 18% NaCl (Yokotsuka et al., 1987). Cryptococcus albidus, Candida utilis, Torulopsis candida possessed only 68, 61 and 86% of their optimal activity in the presence of 17.5% of salt (Kakinuma et al., 1987). While the activity of both extra and intracellular glutaminase from Asperagillus oryzae were reduced to half in the presence of 5% NaCl (Yano et al., 1988). Glutaminase from clayey sediments of marine environment was observed to be unaffected by 10% NaCl concentration (Dharmaraj et al., 1977).

In the present investigation, NaCl concentration of 0-5% did not influence the level of activity of enzyme of all the strains tested. Further, interestingly, even when the concentration of NaCl was increased upto 25%, the level of enzyme activity was not drastically affected. Thus V.cholerae glutaminase retained 33% of their optimal activity at 25% NaCl concentration while glutaminase of others could retain 49.96-58.33% of their activity. This observation testifies the fact that organisms isolated from marine environment are of halophilic in nature. Although V.cholerae recorded maximal enzyme production in the absence of NaCl the results obtained for other NaCl concentrations leads one to believe that V.cholerae glutaminase have high levels of NaCl tolerance.

A 15-20 min. of incubation was reported to yield maximal activity for the glutaminase of Pseudomonas (Soda et al., 1972; Roberts, 1976) and for Acinetobacter (Roberts et al., 1972). While 60 min. was required for glutaminase of Aspergillus oryzae (Yano et al., 1988). In the present study also, the time required for the maximal activity of glutaminase was observed to be 10-15 min. and further incubation failed to enhance enzyme activity.

Glutaminase A and B from E.coli exhibited a high degree of substrate specificity, hydrolyzing only the deamidation of L-glutamine (Prusiner et al., 1976). Glutaminase from Pseudomonas catalysed the hydrolysis of L-glutamine and D and L-asparagine (Ramadan et al., 1964b) while enzymes from Pseudomonas and Acinetobacter hydrolyzed both D and L isomers of glutamine and asparagine. An isomolar mixture of L-glutamine and L-asparagine was hydrolyzed at a rate less than that of L-glutamine alone which consequently leads one to conclude that both the substrates compete for the same activity site (Ramadan et al., 1964b; Roberts et al., 1972; Roberts, 1976). Both intra and extracellular glutaminase from Aspergillus oryzae catalysed the hydrolysis of only L-glutamine and L-glutamyl derivatives (Yano et al., 1988).

In the present investigation, glutaminases of both Pseudomonas and Vibrio were observed to prefer glutamine while hydrolysing L-asparagine at an insignificant level. It was evident that presence of asparagine (0.04 M) along with glutamine (0.04 M) resulted in a minimal enzyme activity by glutaminase of all strains except ACMR 171, when compared to that of L-glutamine alone. Further this observation adds evidence for the earlier held assumption that there exists a

competition for same active site by both glutamine and asparagine. However, interestingly P.fluorescens ACMR 171, recorded enhanced activity in the presence of asparagine along with L-glutamine. Perhaps only after a detailed study, any reason could be assigned for this variation in that strain.

Heavy metals were reported to affect, qualitatively, the glutaminase activity. E.coli glutaminase were inhibited totally by Hg (100% at 0.1 mM) and partially by Ag (35%, at 0.1 mM), Pb (57% at 1 mM) and Cu (19% at 1 mM) while unaffected by Mg, Mn, Zn, Cd, Co, Fe and Ca at 1 mM level (Hartman, 1968). Whereas glutaminase from P.aeruginosa was partially inhibited by Hg (Soda et al., 1972). Hg (10^{-3} M) and Fe (10^{-3} M) were observed to arrest complete activity of glutaminase of Pseudomonas while divalent cations such as Ca, Mg, Co, Mn, Zn, Cd and Cr were observed to enhance the level of glutaminase activity (Ramadan et al., 1964b). It was also reported that 50% of the optimal activity of glutaminase from Aspergillus oryzae was lost when incubated with Hg, Cr, Fe and 30% loss incurred with Pb (Yano et al., 1988). Whereas in the present investigation all of the heavy metals tested inhibited glutaminase activity significantly.

A variety of substances are known to modify the activity of glutaminase. Glutaminase activity of Pseudomonas was stimulated by certain divalent anions such as phosphate (0.4 M) and borate (0.2 M), they were inhibited by phthalein dyes such as phenolphthalein (10^{-4} M) and bromocresol purple (10^{-4} M) showed strong inhibition (Ramadan et al., 1964b). Whereas E.coli glutaminase was not inhibited by EDTA and phthalein dyes (Hartman, 1968). Glutaminase from Acinetobacter glutaminasificans was not affected by L-glutamate (30 mM) L-aspartate (30 mM) alpha ketoglutarate (5 mM) and EDTA (0.1 mM) while bromocresol green inhibited the activity at 1 mM level (Roberts et al., 1972). Similarly glutaminase from Pseudomonas was also not affected by L-glutamate, L-aspartate (30 mM each) and EDTA (0.1 M) (Roberts, 1976). Phosphate was reported to have no influence on the activity of glutaminase of Pseudomonas aeruginosa (Soda et al., 1972).

In the present study also, glutamate, aspartate (30 mM) and EDTA (0.1 mM) did not influence the activity of glutaminase while phosphate (0.4 M), and tris ion (0.2 M) enhanced the activity of glutaminase. The compounds which imparted the inhibition of glutaminase include alpha keto-glutarate (2 mM), phenolphthalein (1 mM) and bromocresol purple (1 mM) and borate (0.2 M) however on a lesser scale.

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

Solid substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitrogen, minerals and as well as growth factors. The bacterial and yeast culture grow by adhering to the surface of solid substrate particles (Lonsane & Ramesh, 1990) while filamentous fungi are able to penetrate deep into the solid

substrate particles (Lonsane et al., 1985) for nutrient uptake. A commonly used solid substrate was wheat bran which contained a total nitrogen, 2.33%, starch, 14.1%, sugar, 7.6% and cellulose, 35.2% (Ramesh, 1989). Solid state fermentation technique was employed for the production of amylases by Bacillus licheniformis (Ramesh, 1989).

Except for the reports on the production of extra and intracellular glutaminase by Aspergillus oxyzae from SSF (Yano et al., 1988; Tomita et al., 1988). No detailed investigations have been conducted on glutaminase production by SSF techniques. Further there is an absolute lack of knowledge on the possibility of employing solid state fermentation techniques for the large scale production of glutaminase by bacteria. Hence an attempt was made to produce glutaminase through SSF. The results strongly indicate that SSF method would be advantageous for glutaminase production.

Kumar and Lonsane, (1987) standardised extraction parameters to obtain maximal enzyme recovery of gibberellic acid from solid state fermentation. They have stressed the need for the development of efficient extraction techniques for the recovery of products from bacterial wheat bran of

SSF for effective commercial exploitation. Kumar and Lonsane, (1987) extracted gibberellic acid from SSF with aqueous solution of ethanol as solvent in 1:3 ratio at 25-40°C with a contact time of 15-60 min. Ramesh, (1989) extracted amylase from B.licheniformis with phosphate buffer of pH 7.2 in a 1:5 volume ratio of bran to buffer at 4-6°C with the contact time of 60 min. Since no reports are available on this aspects of glutaminase, an attempt was made to standardise the extraction for maximal recovery of glutaminase.

Maximal glutaminase recovery was obtained from all strains at drying temperatures of the bacterial wheat bran ranging from 30-40°C and phosphate buffer (pH 6 or 8, varied for the strains) as extraction media, in 1:5 ratio of bran to buffer. Glutaminase was recovered in maximal levels when the buffer with the optimum pH was allowed to be in contact with the bacterial wheat bran for 60-90 min. at 35°C. These results are at comparable levels with the standardised extraction parameters reported for amylases from Bacillus licheniformis through SSF (Ramesh, 1989) and also the procedures employed for the extraction of gibberellic acid (Kumar & Lonsane, 1987).

As the yield of product from any fermentation process, irrespective of the process, submerged or solid state, is governed by the environmental variables it becomes mandatory to optimize these variables in order to obtain maximal yields of enzyme. In the present study an effort was made to optimize moisture content, particle size, pH temperature, substrate concentration, NaCl concentration, carbon sources, nitrogen sources, inoculum concentration and incubation period of SSF. Glutaminase has been earlier reported to be produced by Aspergillus oryzae employing SSF on wheat bran (Yano et al., 1988). They were grown at a pH of 7.2 and 28°C for 72 hours and intracellular and extracellular glutaminase produced were later extracted, purified and characterized. Both the fractions showed weak activities and poor stabilities at pH 5 and exhibited only less than 15% of the total activity at a NaCl concentration of 16%.

The critical importance of moisture content of the medium and its control during fermentation are extensively documented for solid state fermentation processes involving fungal cultures (Lonsane et al., 1985). Moisture content of 40-70% is required for a significant level of enzyme production by all the strains with an optimum between

50-60%. Present results indicated a relationship between moisture content and enzyme production for all the strains upto their optimum moisture level. The optimum water requirement for solid state fermentation production of alpha amylase was reported as 65% for Bacillus licheniformis (Ramesh & Lonsane, 1990). The present results are similar to their observation emphasizing the critical role of moisture content.

Wheat bran of particle size between 0.2-0.8 cm was used for the production of amylase by B.licheniformis (Ramesh & Lonsane, 1989). They observed high yields of amylase using wheat bran of these size. Whereas in the present investigation maximal enzyme production was obtained from P.fluorescens (ACMR 171 and ACMR 43) by using a wheat bran of particle size less than 1.20 mm size while Vibrio sp. (ACMR 267 and ACMR 347) preferred a particle size between 1.41-2.06 mm for the same.

Major advantage of solid state fermentation over submerged fermentation is increased product yield (Lonsane & Karanth, 1990). Results obtained in this study adds evidence to this statement, since the enzyme production was many fold higher in SSF than in submerged fermentation.

P.fluorescens (ACMR 171) exhibited a slight shift in their optimum pH of 5 in SSF to pH 6 in SmF. This might be due to the difference in the complexity of environmental variables that vary between SSF and SmF, such as moisture content and particle size which could have influenced the pH of the medium. But incubation temperature did not influence the level of enzyme production in both the fermentations as one may expect to be the same since temperature is not normally influenced by other variables. The variation in the requirement for optimal substrate concentration shown by both the P.fluorescens strains where ACMR 43 requiring only a less concentration of glutamine in SSF and ACMR 171 requiring a higher level in SmF, leads one to assume that wheat bran components had played a significant role in altering the requirement for substrate for the production of exoenzymes. However, this warrants experimental confirmation. Another probable causative factor could be attributed to unoptimized water content in SmF.

Likewise P.fluorescens ACMR 43 requires 3% NaCl concentration in SmF and 1% concentration in SSF. Since the strain was isolated from marine water where it was exposed to 3% NaCl concentration, it might have preferred 3% level of NaCl for maximal enzyme production. Whereas in SSF since

the organisms exist in some kind of immobilised state absorbed to particles, NaCl would not have exerted significant influence on the bacteria unlike in submerged condition where the bacteria is under constant contact with changing concentration of NaCl in their microenvironment. However, this needs further studies for confirmation.

Glucose enhanced the level of enzyme production by all the strains in SmF while reducing the enzyme production in SSF except in P.fluorescens ACMR 171. Since the wheat bran contains 7.6% sugar, addition of glucose could have accrued a higher level of final glucose concentration made available to the organism. This may be a possible factor that could have reduced enzyme levels in SSF compared to SmF.

In general, the reults obtained from both SmF and SSF studies with reference to optimization of environmental variables that normally influence the metabolic state of bacteria strongly indicated that these bacteria are stable in their optimal requirement for glutaminase production irrespective of the medium, they are provided with, for enzyme production.

5. SUMMARY

5.1 Glutaminase producing bacteria were quantitatively and qualitatively enumerated on mineral salts medium added with glutamine and the potential strains were studied in detail for maximal enzyme production. Glutaminase producing bacteria were present in higher levels varying from 10^5-10^7/ml or g in the water and sediment samples of both marine and estuarine environments of Cochin.

5.2 L-glutaminase producing bacteria isolated during the study included mainly the species of Pseudomonas Aeromonas, Vibrio, Alcaligenes, Acinetobacter, Bacillus and Planococci.

5.3 Among the 500 isolates obtained, four strains identified as Pseudomonas fluorescens ACMR 171, Pseudomonas fluorescens ACMR 43, Vibrio costicola ACMR 267 and Vibrio cholerae ACMR 347 were selected for further studies based on their efficiency to produce maximal levels of glutaminase in minimal medium using glutamine as carbon source.

5.4 All the four strains were initially characterized for the optimal requirements of pH, temperature and NaCl

concentration, substrate concentration, additional carbon and nitrogen sources, inoculum concentration and incubation time for maximal growth and enzyme production.

5.5 They could grow and produce maximal glutaminase at a wide range of parameters ranging from pH 5 to 8, temperatures 25-45°C, NaCl concentrations 0-5%, substrate concentrations 0.5-3%, within 18-24 hours of incubation. However, the maximal enzyme production was recorded at pH 6 and at a temperature of 35°C. A 3% NaCl concentration in the medium led to the maximal production of glutaminase by all strains except V.cholerae ACMR 347 which required no NaCl for the same. Similarly all the strains produced maximal level of enzyme at 1% substrate concentration except P.fluorescens ACMR 43 which preferred 0.5% for their maximal enzyme production. Both Vibrio sp. and P.fluorescens ACMR 171, recorded maximal enzyme production at 3% inoculum concentration, while P.fluorescens ACMR 43 required only 1% inoculum concentration for the same.

5.6 Glucose enhanced significant level of enzyme production in all strains followed by trisodium citrate which favoured glutaminase production in all except P.fluorescens ACMR 171 which responded to maltose. 0.5%

glucose concentration favoured maximal enzyme production in all strains except V.costicola ACMR 267 which preferred 1% glucose concentration for the same.

5.7 Beef extract, followed by lysine, peptone and glutamic acid were observed to possess a boosting effect on the level of enzyme production by the strains while KNO_3 and $NaNO_3$ exhibited a repressive effect.

5.8 Growth curves of the strains indicated that they spend about 3-5 hours initially in the lag phase and about 7 hours in the logarithmic phase which extends over to 10-12 hours of growth. Generation time for all the strains except V.cholerae ACMR 347 were found to be longer in mineral media (72 min. for P.fluorescens ACMR 171, 60 min. for P.fluorescens ACMR 43, 84 min. for V.costicola ACMR 267) than in nutrient broth (54 min. for P.fluorescens ACMR 171, 48 min. for P.fluorescens ACMR 43, 60 min. for V.costicola ACMR 267) whereas V.cholerae ACMR 347 possess a longer generation time in nutrient broth (42 min.) than in mineral media (38 min.).

5.9 Extracellular glutaminase fraction from all the strains were in higher titres (2.6-6.8 times) than

intracellular enzymes during growth in mineral media, nutrient broth and nutrient broth added with glutamine. Further about 2.5 fold production of glutaminase could be achieved in mineral media added with glutamine than in nutrient broth with glutamine. Nutrient broth did not favour intracellular synthesis of glutaminase by all the strains.

5.10 Glutaminase isolated from Pseudomonas sp. and Vibrio sp. were purified and recovered after $(NH_4)_2SO_4$ fractionation followed by dialysis. Maximal yield of purified glutaminase was recorded for P.fluorescens ACMR 43 (45.84%) followed by P.fluorescens ACMR 171 (42.31%) V.costicola ACMR 267 (39.28%) and V.cholerae ACMR 347 (35.79%). However, maximal folds of recovery of glutaminase was from V.costicola ACMR 347 (56.09) followed by V.cholerae ACMR 267 (47.84) P.fluorescens ACMR 171 (47.5) and P.fluorescens ACMR 43 (40.22). The maximal specific activities were obtained for P.fluorescens ACMR 43 (88.49 u/mg of protein) followed by P.fluorescens ACMR 171 (80.75 u/mg) V.cholerae ACMR 347 (71.77 u/mg of protein) and V.costicola ACMR 267 (61.7 u/mg of protein).

5.11 The purified glutaminase from all the strains recorded activities and stabilities over wide ranges of

pH (pH 4-9) and temperature (30-60°C). However, the optimal activity and stability of all the strains were recorded at pH 6 except P.fluorescens ACMR 171 which recorded pH 8 for maximal activity and stability. At 40°C, all strains exhibited maximal activity and stability.

5.12 Optimal substrate concentration for maximal activity of glutaminase of each strain varied from 0.04-0.08 M and 0.04 M substrate concentration could promote significant levels of glutaminase activity in all the strains.

5.13 Glutaminases purified from the strains possessed a km of 1.0×10^{-4} M for P.fluorescens ACMR 171, 4.6×10^{-5} M for P.fluorescens ACMR 43, 9.54×10^{-5} M for V.costicola ACMR 267 and 1.5×10^{-5} M for V.cholerae ACMR 347.

5.14 Glutaminase was not influenced drastically by high concentrations of NaCl (upto 25% NaCl) except for the enzyme from V.cholerae ACMR 347 whose activity was decreased rapidly to 33.28% of optimal activity at 25% NaCl concentration, enzymes of all other strains could retain 49.96-58.33% of their optimal activity at 25% NaCl concentration.

5.15 Maximum rate of hydrolysis of glutamine by glutaminase could be effected within 10-15 min.

5.16 Heavy metals viz., Hg, Fe, Ca, Mn, Pb, Co, Cu and Zn affected the enzyme activity adversely, especially Hg effected 100% inhibition of P.fluorescens ACMR 171 and V.costicola ACMR 267.

5.17 Glutamic acid, aspartic acid, and EDTA had not influenced the activity of glutaminase while phosphate ion and tris ion enhanced the activity of glutaminase. Those compounds which attributed inhibition of glutaminase included alpha ketoglutarate, phenolphthalein bromocresol purple and borate ion.

5.18 Glutamine was identified as the preferential substrate for glutaminase from all strains. Asparagine was deamidated by glutaminases of all tested strains except P.fluorescens ACMR 43 however, at a lesser magnitude and a combination of L-asparagine and L-glutamine resulted in a reduced enzyme activity.

5.19 In solid state fermentation all the strains could produce glutaminase in a wide range of operational parameters

varying from a moisture content of 40-70%, pH of 4-10, temperature of 15-45°C, substrate concentration of 0.25-3%, NaCl concentration of 0-5% within 18-24 hours of incubation. However, the optimum conditions for enzyme production were a moisture content of 50-60%; pH 6 for all strains except P.fluorescens which preferred pH 5; a temperature of 35°C; substrate concentration of 1% for all except P.fluorescens ACMR 171 which required only 0.5% and 3% NaCl concentration for P.fluorescens ACMR 171 and V.costicola ACMR 267, while P.fluorescens ACMR 43 required only 1% NaCl concentration and V.cholerae ACMR 347 exhibited maximal enzyme production by all strains.

5.20 Only maltose enhanced glutaminase production in SSF whereas all others tested did not promote enzyme production in all the strains except P.fluorescens ACMR 171 which showed enhancement of enzyme production in response to glucose.

5.21 Beef extract, yeast extract, peptone, KNO_3 and $NaNO_3$ led to minimal enzyme production by all the strains whereas lysine followed by glutamic acid marginally enhanced enzyme production.

5.22 Wheat bran of less than 1.20 mm size was preferred by both the strains of Pseudomonas (ACMR 171 and ACMR 43) and V.cholerae ACMR 347, whereas particles varying from 1.41-2.06 mm are required by V.costicola ACMR 267 for maximal enzyme recovery.

5.23 Maximal recovery of glutaminase from bacterial wheat bran of all strains obtained at the following conditions, a drying temperature of 40-50°C, phosphate buffer of optimum pH as the extraction media, a 1:5 ratio of bran to buffer, a contact time of 60-90 min. and at a contact temperature of 25-35°C.

5.24 A comparative analysis of data obtained for submerged fermentation and solid state fermentation production of glutaminase clearly indicated SSF technique to be the process that can yield many fold enzyme and holds promise for commercial application.

REFERENCES

1. Abdumalikov, A.kh. & Nikolaev, A.Ya. (1967) Biokhimiya 32: 859.

2. Abe, T., Takenaka, O. & Inada, Y. (1974) Biochimica et. Biophysica Acta. 358: 113.

3. Abell, C.S. & Uren, J.R. (1981) Antitumor Compounds of Natural Origin; Chemistry and Biochemistry (Aszalos, A., ed.), Chemical Rubber Company Press, Boca Raton, Florida, 123.

4. Abuchowski (1981) Cancer Treat.Rep. 65: 1077.

5. Adamson, R.H. & Fabro, S. (1968) Cancer Chemotherapy Rep. 52: 617.

6. Alazard, D. & Raimbault, M. (1981) Eur.J.Appl.Microbiol. Biotechnol. 12: 113.

7. Arima, K. (1964) Global Impacts of Applied Microbiology, (Starr, M.P. ed.), John Wiley & Sons, New York, 277.

8. Arima, K., Sakamoto, T., Araki, C. & Tamura, G. (1972) Agri.Biol.Chem. 36: 356.

9. Aunstrup, K. (1979) Appl.Environ.Microbiol. 45: 205.

10. Baskerville, A., Hambleton, P. & Benbough, J.E. (1980) Br.J.Exp.Pathol. 61: 132.

11. Bertino, J.R., O'Brien, P. & Mc cullough, J.L. (1971) Science 172: 161.

12. Blazek, R.,& Benbough, E.J. (1981) Biochem.Biophys.Acta. 677: 220.

13. Brightsingh, I.S. (1986) Ph.D.Thesis, Cochin University of Science and Technology, Cochin.

14. Broome, J.D. (1961) Nature, 191: 1114.

15. Broome, J.D. (1971) In Colloque Sur la L-asparaginase. Colloque du CNRS No.197, Paris, Edition du Centre National de la Recherche Scientific.

16. Buchanan, R.E. & Gibbons, N.C. (1974) Bergey's Manual of Systematic Bacteriology, 8th edn., Williams & Wilkins Co., Baltimore.

17. Burchenal, J.H. & Kranofsky, D.A. (1970) Cancer, 25: 241.

18. Capizzi, R.L., Bertino, J.R. & Handschumacher, R.E. (1970) Ann.Rev.Medicine, 21: 433.

19. Catane, R., Von Hoff, D.D. & Glaubiger, D.L. (1979) Cancer Treat.Rep. 63: 1033.

20. Cedar, H., & Schwartz, J.H. (1967) J.Biol.Chem. 242: 3753.

21. Chahal, D.S. (1983) Appl.Environ.Microbiol. 45: 205.

22. Chandrasekaran, M. (1985) Ph.D.Thesis, Cochin University of Science and Technology.

23. Chang, T.M.S. (1971) Nature, 229: 117.

24. Cook, W.R., Hoffman, J.H. & Bernlohr, R.W. (1981) J. Bacteriol. 148: 365.

25. Cooney, D.A. & Handschumacher, R.E. (1970) Ann.Rev. Pharmacol. 10: 421.

26. Cooney, D.A. & Rosenbluth, R.J. (1975) Adv.Pharmacol. Chemother. 12: 185.

27. Datar, R. (1986) Process Biochem. 21: 19.

28. Dharmaraj, K., Selvakumar, N., Chandramohan, D. & Natarajan, R. (1977) Indian J.Mar.Sci. 6: 168.

29. Dranoff, G., Gertrude, B.E., Friedman, H.S. & Bigner, D.D. (1985a) Cancer Res. 45: 4077.

30. Dranoff, G., Gertrude, B.E., Friedman, H.S. & Bigner, D.D. (1985b), Cancer Res. 45: 4082.

31. Eagle, H. (1955) Science, 122: 501.

32. Eagle, H., Oyama, V.I., Levy, M., Horton, C.I. & Felishman, R. (1956) J.Biol.Chem. 218: 607.

33. Ehrenfeld, E., Marble, S.J. & Meister, A. (1963) J.Biol. Chem. 238: 3711.

34. EL-Asmar, F.A. &Greenberg, D.M. (1966) Cancer Res. 26: 116.

35. Flickinger, M.C. (1985) In Comprehensive Biotechnology, (Young, M.M. ed.), Vol.3, Pergamon Press, New York.

36. Forage, A.J. &Righelato, R.C. (1974) Microbial Biomass, Economic Microbiology (Rose, A.H. ed.), Vol.4, Academic Press, London, 289.

37. Fukushima, Y. & Motai, H. (1990) J.Fermentn. Bioengg. 69: 189.

38. Furuya, T., Ishige, M., Uchida, K. &Yoshino, H. (1983) J.Agric.Chem.Soc. Japn. 57: 1.

39. Furuya, T., Noguchi, K., Miyaushi, K. &Uchida, K. (1985) Nippon Nogeikagaku Kaishi 59: 605.

40. Ghildyal, N.P., Lonsane, B.K., Sreekantaiah, K.R. & Murthy, V.S. (1985) J.Fd.Sci.Technol. 22: 171.

41. Godfrey, R.J. (1983) Industrial Enzymology, The Applications of Enzymes in Industry, The Nature Press, New York.

42. Greenberg, D.M., Bluementhal, G.H. &Ramadan, M.A. (1964) Cancer Res. 24: 957.

43. Greenstein, J.P. & Winitz, M. (1961) In Chemistry of the Aminoacids, Vol.2, John Wiley & Sons Inc., New York, London, 1929.

44. Hahn, H.B. (1986) Enzyme Microb.Technol. 8: 322.

45. Hakimi, J. &Bosman, H.B. (1979), Res.Commun.Chem.Pathol. Pharmacol. 26: 581.

46. Hambleton, P., Benbough, J.E. &Baskerville, A. (1980) Br.J.Exp.Pathol. 61: 200.

47. Hammer, R.A. & Hartman, S.C. (1968) J.Biol.Chem. 243: 864.

48. Hartman, S.C. (1968) J.Biol.Chem. 243: 853.

49. Hartman, S.C. (1971) The Enzymes, Vol.4, 3rd edn., (Boyer, P.D. ed.), 79.

50. Hersh, E.M. (1971) Science, 172: 736.

51. Hesseltine, C.W. (1977) Process Biochem. 12: 24.

52. Holcenberg, J.S., Roberts, J. & Dolowy, W.C. (1971) Nature 232: 23.

53. Holcenberg, J.S., Teller, D.C., Roberts, J. & Dolowy, W.C. (1972) J.Biol.Chem. 247: 7750.

54. Holcenberg, J.S., Roberts, J. & Dolowy, W.C. (1973) In the Enzymes of Glutamine Metabolism (Prusiner and Stadtman ed.), Academic Press, New York, 277.

55. Holcenberg, J.S., Schmer, G. & Teller, D.C. (1975) J. Biol.Chem. 250: 4165.

56. Holcenberg, J.S. & Ericsson, L. (1976) Fed.Proc.Fed.Amer. Soc.Exp.Biol. 35: 282.

57. Holcenberg, J.S., Teller, D.C. & Roberts, J. (1976) J. Biol.Chem. 251: 5375.

58. Holcenberg, J.S., Ericsson, L. & Roberts, J. (1978) Biochem. 17: 411.

59. Holcenberg, J.S., Camitta, B.M. & Borella, L.D. (1979a) Cancer.Treat.Rep. 63: 1025.

60. Holcenberg, J.S., Borella, L.D. & Camitta, B.M. (1979b) Cancer Res. 39: 3145.

61. Hughes, D.E. (1949) Biochem.J. 45: 325.

62. Hughes, D.E. (1950) Biochem.J. 46: 231.

63. Hughes, D.E. & Williamson, D.H. (1952) Biochem.J. 51: 45.

64. Imada, A., Igarasi, S., Nakahama, K. & Isono, M. (1973) J.Gen.Microbiol. 76: 85.

65. Iwasa, T., Fujii, S. & Yokotsuka, T. (1972) Proc.Ann. Meet.Agric.Chem.Soc. Jap. 102.

66. Iwasa, T., Fujii, S. & Yokotsuka, T. (1987) J.Jap.Soc. Savee.Res.Inst. 13: 120.

67. Jacobs, S.P., Wodinsky, I., Kensler, C.J. & Venditti, J. (1969) J.Proc.Amer.Assoc. Cancer Res. 10: 43.

68. Kabanova, E.A., Lebedeva, Z.I. & Berezov, T.T. (1985) Vopr.Med.Khim. 31: 31.

69. Kabanova, E.A., Lebedeva, Z.I. & Berezov, T.T. (1986) Biokhimiya 51: 432.

70. Kakinuma, T., Fujii, S., Iwasa, T. & Yokotsuka, T. (1987) J.Jap.Soysauce Res.Inst. 13: 116.

71. Katsumata, H., Katsumata, R., Abe, T., Takenaka, O. & Inada, Y. (1972) Biochimica et. Biophysica Acta. 289: 405.

72. Kien, L.C., Anderson, A.J. & Holcenberg, J.S. (1985) Cancer Res. 45: 4876.

73. Kidd, J.G. (1953) J.Exp.Med. 98: 565.

74. Kikuchi, M., Hayashida, H., Nakano, E. & Sakaguchi, K. (1971) Biochemistry 10: 1222.

75. Knox, D.E., Horowitz, G. & Friedell, H. (1969) Cancer Res. 29: 669.

76. Kovalenko, N.A., Kozlov, E.A.& Gerasimova, A.V. (1970) Biokhimiya 35: 670.

77. Kovalenko, N.A., Kozlov, E.A.& Mardashev, S.R. (1971) Biokhimiya 36: 1198.

78. Kozlov, E.A., Kovalenko, N.A.& Mardashev, S.R. (1972) Biokhimiya 37: 56.

79. Krieg, R.N.& Holt, G.J. (1984) Bergey's Manual of Systematic Bacteriology, Vol.1, Williams & Wilkins Co., Baltimore.

80. Kumar, P.K.R.& Lonsane, B.K. (1987) Process Biochemistry, 139.

81. Kuroshima, E., Oyama, Y., Matsuo, T.& Sugimori, T. (1969) J.Ferment.Technol. 47: 693.

82. Lebedeva, Z.I., Kabanova, E.A.& Berezov, T.T. (1986) Biokhimiya, 51: 293.

83. Levintow, L. (1954) J.Natl.Cancer Inst. 15: 347.

84. Lonsane, B.K., Ghildyal, N.P. & Murthy, V.S. (1982) In Technical Brochure Symposium on Fermented Foods, Food Contaminants, Biofertilizer and Bioenergy, Assocn.of Microbiologists of India, Mysore, 1.12.

85. Lonsane, B.K., Ghildyal, N.P., Budiatman, S.& Ramakrishna, S.V. (1985) Enzyme Microb.Technol. 7: 258.

86. Lonsane, B.K.& Karanth, N.G. (1990) National Symposium on Current Trends in Biotechnology, Proceedings, Nov., 28-30, 39.

87. Lonsane, B.K.& Ramesh, M.V. (1990) Adv.Appl.Microbiol. 35: 1.

88. Lowry, O.H., Rosebrough, N.J., Farr, A.L.& Randall, R.J. (1951) J.Biol.Chem. 193: 265.

89. Mardashev, S.R., Gerasinomova, A.V.& Kozlov, E.A. (1966), Microbiologiya, 35: 223.

90. Mardashev, S.R., Eremenko, V.V.& Nikolaev, A.Ya. (1970) Microbiology 39: 6.

91. Marsh, J.W., Davis, J. & Wriston, J.C. (1977) J.Biol.Chem. 252: 7678.

92. Mauer, A.M. & Simon, J. (1976) Cancer Treat.Rev. 3: 17.

93. Mc Ilwain, H. (1948) J.Gen.Microbiol. 2: 187.

94. Meister, A. (1955) In Methods of Enzymology (Mosbach, K. ed.), Vol.2, 380.

95. Meister, A., Levintow, L., Greenfield, R.E. & Abendschein, P.A. (1955) J.Biol.Chem. 215: 441.

96. Meister, A. (1956) Physiol.Rev. 36: 103;

97. Miller, H.K. & Balis, E.M. (1969) Biochem. Pharmacol. 18: 2225.

98. Mugnetsyan, E.V. & Stepanayan, K.P. (1987) Biol.Zh.Arm. 40: 325.

99. Munoz, R.G., Valencia, T.J.R., Sanches, S. & Farres, A. (1991) Biotech.Lett. 13: 277.

100. Nakadai, Y.& Nasuno, S. (1989) J.Ferment.Bioengg. 52: 564.

101. Nasuno, S.& Ohara, T. (1972) Seasoning Sci. 19: 41.

102. Newman, R.E.& Mc Coy, T.A. (1956) Science 124: 124.

103. Novak, E.K.& Philips, A.W. (1974) J.Bacteriol. 117: 593.

104. Nuzhina, A.M. (1970) Vop.Onkol. 16: 99.

105. O'Mahony, M.& Ishi, M. (1987) A Basic Taste (Kawamura and Kare, ed.), Marcel Dekker, New York.

*106. Ohshima, M. (1976) Ph.D. Thesis, Kyoto University, Japan.

107. Oldham, S. (1967) Mfg.Chem.Aerosol News, Aug. 47.

108. Owen, E.E.& Robinson, R.R. (1963) J.Clin.Invest. 42: 262.

* Original not refered.

109. Pekhov, A.A., Zanin, V.A., Margretova, N.V. & Berezov, T.T. (1985) Byull. EKSP. Biol. Med. 99: 557.

110. Pitts, R.F. (1971) N.Eng.J.Med. 284: 32.

111. Prusiner, S. & Stadtman, E.R. (1971) Biochem.Biophys.Res. Commun. 45: 1474.

112. Prusiner, S. (1973) In the Enzymes of Glutamine Metabolism (Prusiner and Stadtman, ed.) Academic Press, New York, 293.

113. Prusiner, S., Davis, J.N. & Stadtman, E.R. (1976) J.Biol. Chem. 251: 3447.

114. Puntus, T.P., Vinogradov, B.D., Ryekin, S.S. & Merkulov, M.F. (1979) Deposited Doc. 579.

115. Ramadan, M.E.A., El.Asmar, F.A. & Greenberg, D.M. (1964a) Archives of Biochemistry and Biophysics 108: 143.

116. Ramadan, M.E.A., El.Asmar, F.A. & Greenberg, D.M. (1964b) Archives of Biochemistry and Biophysics 108: 150.

117. Ramesh, M.V.& Lonsane, B.K. (1987a) Biotech.Letts. 9: 323.

118. Ramesh, M.V.& Lonsane, B.K. (1987b) Biotech.Letts. 9: 501.

119. Ramesh, M.V. (1989) Ph.D.Thesis, Mysore University.

120. Ramesh, M.V.& Lonsane, B.K. (1989) Biotech.Letts. 11: 49.

121. Ramesh, M.V.& Lonsane, B.K. (1990) Appl.Microbiol.Biotechnol. 3: 29611.

122. Riley, V. (1970) Pathologic.Biologic. 121: 757.

123. Roberts, E. (1960) In the Enzymes (Boyer, P.D., Lardy, H., Myrback, K. eds.), Vol.IV, Academic Press, New York, 285.

124. Roberts, E.& Simonsen, D.G. (1960) In Aminoacids, Proteins, Cancer Biochemistry (Edstall, J.T. ed.), Academic Press, New York, 121.

125. Roberts, J., Holcenberg, J.S.& Dolowy, W.C. (1970) Nature, 227: 1136.

126. Roberts, J., Holcenberg, J.S.& Dolowy, W.C. (1971) Life Sciences 10: 251.

127. Roberts, J., Holcenberg, J.S.& Dolowy, W.C. (1972) J. Biol.Chem. 247: 81.

128. Roberts, J. (1976) J.Biol.Chem. 251: 2119.

129. Roberts, J., Schmid, F.A., Old, Z.J.& Stockert, E. (1976) Cancer Biochem. Biophys. 1: 175.

130. Schmid, F.A.& Roberts, J. (1974) Cancer Chemother. Rep. 58: 829.

131. Schrek, R., Holcenberg, J.S., Roberts, J.& Dolowy, W.C. (1971) Nature, 232.

132. Shalhoub, R., Webber, W., Glabman, M., Canessa Fischer, M., Klein, J., de Haus, J.& Pitts, R.F. (1963) Am.J. Physiol. 204: 181.

133. Shikata, H., Yasui, T., Ishiyami, Y.& Omori, K. (1978) Nippon Shoyo Ken Kyusho Zasshi. 4: 48.

134. Shikata, H., Yasui, T., Ishiyami, Y.& Omori, K. (1979) Nippon Shoyo.Ken Kyusho Zasshi 5: 21.

135. Sizer, I.M. (1972) Applied Microbiology, 3: 1.

136. Smirnova, I.P., Lebedeva, Z.I.& Bexezov, T.T. (1977) Probl.Zlokach.Rosta. 80: 4.

137. Soda, K., Uchiyama, K.& Ogata, K. (1966a) Agri.Biol.Chem. 30: 541.

138. Soda, K., Uchiyama, K.& Ogata, K. (1966b) Agri.Biol.Chem. 30: 547.

139. Soda, K., Ohshima, M.& Yamamoto, T. (1972) Biochemical and Biophysical Res.Commun. 46: 48.

140. Spiers, A.S.D.& Wade, H.E. (1979) Cancer Treat.Rep. 63: 1019.

141. Spira, W.M., Daniel, R.R., Ahmed, G.S., Yusuf, A.H.A.& Sack, D.A. (1979) Proc. 14th Joint Conf.Cholerae, U.S.-Japan Coop.Med.Sci.Prog. 137.

142. Spira, W.M.& Daniel, R.R. (1980) Proc. 15th Joint Conf. Cholerae U.S.-Japan Coop Med.Sci.Prog. 440.

143. Squires, E.J., Hall, D.E.& Brosnan, J.T. (1970) Biochem. J. 160: 125.

144. Steinkraus, K.H. (1984) Acta.Biotechnol. 4: 19.

145. Sudha, V. (1981) Ph.D.Thesis, Annamalai University.

146. Tarnowski, G.S., Mountain, I.M.& Stock, C.C. (1969) Proc.Amer.Assoc.Cancer Res. 10: 92.

147. Tarnowski, G.S., Mountain, I.M.& Stock, C.C. (1970) Cancer Res. 30: 1118.

148. Teramoto, A., Ishige, M., Furuya, T., Uchida, K.& Yoshino, H. (1985) Nippon Nogei Kagaku Kaishi 59: 245.

149. Tomita, K., Yano, T., Kumagai, H. & Tochikura, T. (1988) J.Ferment.Technol. 66: 299.

150. Ushijima, S. & Nakadai, T. (1983) Proc.Soc.Ferment. Technol.Japan. 260.

151. Varner, J.E. (1960), In the Enzymes (Boyer, P.D., Lardy, L., Myrback, K. ed.), Vol.IV, Academic Press, New York, 234.

152. Wade, H.E. & Rutter, D.A. (1970) Science 6: 62.

153. Wade, H.E., Robinson, H.K. & Philips, B.W. (1971) J.Gen. Microbiol. 69: 299.

154. Wagner, O., Irion, E., Arens, A. & Baver, K. (1969) Biochemical & Biophysical Res.Commun. 37: 383.

155. Warell, R.P.Jr., Chov, T.C. & Gordon, C. (1980) Cancer Res. 40: 4546.

156. Wlodawer, A., Hodgson, O.K. & Bensch, K. (1975) J.Mol. Biol. 99: 295.

157. Wlodawer, A., Roberts, J.& Holcenberg, J.S. (1977) J. Mol.Biol. 112: 515.

158. Wriston, J.C. (1971) In the Enzymes (P.D.Boyer, ed.), Vol.IV, Academic Press, New York, 101.

159. Wriston, J.C.& Yellin, T.O. (1973) Adv.Enzymol. 39: 185.

160. Yamamoto, K. (1974) Cited in Yokotsuka (1988b), Asian Food Conference, Food Science & Technology in Industrial Development, Bangkok, October 24-26, 8.

161. Yamamoto, S.& Hirooka, H. (1974a), J.Ferment.Technol. 52: 564.

162. Yamamoto, S.& Hirooka, H. (1974b), J.Ferment.Technol. 52: 570.

163. Yano, T., Ho, M., Tomita, K., Kumagai, H.& Tochikara, T. (1988) J.Ferment.Technol. 66: 137.

164. Yokotsuka, T., Iwasa, T., Fujii, S.& Kakinuma, T. (1972) Proc.Ann.Meet.Agric.Chem.Soc.Japan, April, 333.

165. Yokotsuka, T., Hashimoto, H., Fujii, S.& Iwasa [redacted]1974) (Unpublished).

166. Yokotsuka, T. (1986) Adv. In Food Res. Vol.130 (Cheichester, C.O. ed.), Academic Press, New York, 195.

167. Yokotsuka, T. (1987) Pro Pak Asia 87 Conference, Singapore, April 8-10.

168. Yokotsuka, T., Iwasa, T., Fujii, S.& Kakinuma, T. (1987) J. Jap.Soy sauce Res.Inst. 13: 18.

169. Yokotsuka, T. (1988a) International Symposium on Application of Biotechnology for Small Industries Development in Developing Countries, Bangkok, Thailand, September 21-24.

170. Yokotsuka, T. (1988b) Asian Food Conference, Food Science and Technology in Industrial Development, Bangkok, Thailand, October 24-26.