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.