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.