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

Solid substrates employed in SSF processes are insoluble in water and act as a source of carbon, nitrogen, minerals as well as growth factors (Pandey, 1992). The bacterial and yeast cells grow by adhering to the surface of the solid substrate particles (Lonsane & Ramesh, 1990) while the filamentous fungi are able to penetrate deep into the solid substrate particles for nutrient uptake (Lonsane et al, 1985; Pandey & Radhakrishnan, 1993). Wheat bran is the most commonly used and widely accepted solid substrate for SSF eventhough other substrates such as rice bran, rice husk, saw dust, sugarcane pressmud, coir pith, mango peels, lignocellulosic wastes, cabbage and banana wastes, mustard oil cake, tapioca peels, maize bran, gram bran and many more naturally occurring substances are also being tried as substrates for SSF. (Aidoo et al, 1982; Pandey, 1992; Satyanarayana, 1994, Krishna & Chandrasekaran, 1995, 1996).
Since the yield of product from any fermentation process, submerged or solid state, is governed by the environmental variables, it becomes mandatory to optimize these parameters in order to obtain maximum product yield. In the present study, an effort was made to optimize the important physical, chemical and nutritional parameters that influence the production of glutaminase by marine *Vibrio costicola* under SSF using natural, synthetic and mixed substrates.

4.1. L-Glutaminase production under SSF using natural substrates

Most of the process parameters tested had significant influence on glutaminase production by *V. costicola*. Interestingly, the nature of the solid substrate was observed to influence the optimal requirements of several bioprocess conditions as indicated by the results obtained in the present investigation. The initial pH of the medium, incubation temperature and inoculum concentration required for maximal glutaminase production were similar irrespective of the nature of the substrates used. Since these environmental parameters are independent in exerting their influence on the growth and performance of bacteria (Chandrasekaran *et al.*, 1991), they did not vary in their optima for glutaminase yield despite the differences in the nature of the substrate used for SSF (Nagendra Prabhu & Chandrasekaran, 1996 a).
The critical importance of moisture content on microbial growth and product yield in SSF has been emphasized earlier (Ramesh & Lonsane, 1990). Microbiological activity on a substrate will progressively decrease at lower water contents finally ceasing at or near 12%. Moisture is reported to cause swelling of the substrates thereby facilitating better utilization of substrates by the microorganism (Kim et al, 1985). But high moisture content led to reduced product yield, during SSF, due to reduction in interparticle spaces, decreased substrate degradation and impaired oxygen transfer (Zadrazil & Brunert, 1981; Sandhya Xavier & Lonsane, 1984). Further the physical nature and water holding capacity are important criteria for a solid substrate for its use in SSF processes (Aidoo et al, 1982) and the absorbency of the substrates will determine the moisture at which free water becomes apparent (Smith & Aidoo, 1988). Normally, a moisture content of 30-80 % is required for a significant level of enzyme production with an optimum between 50-60 %, depending on the material (Oriol et al, 1988).

In the present study, it was observed that the level of initial moisture content in the different substrates viz. wheat bran, rice husk, copra cake powder, groundnut cake powder and saw dust significantly influenced the rate of glutaminase synthesis and overall enzyme yield. The optimum moisture content required for maximal glutaminase yield was 60 % for wheat bran and rice husk, 70 % for saw dust and copra cake powder, and 40 % for groundnut cake powder.
Similar observations were made by earlier investigators for different substrates. While an initial moisture content of 65% was optimal for α-amylase production by *Bacillus licheniformis* M 27 with wheat bran (Ramesh & Lonsane, 1990), a moisture content of 50% was needed by *Aspergillus niger* for the production of citric acid using coffee husk and sugar cane press-mud (Shankaranand & Lonsane, 1993, 1994). The variation in the levels of influence of the moisture content on glutaminase yield, observed with respect to different substrates used in the present study, could be probably due to the differences in the physico-chemical nature and water holding capacity of the substrates, which normally vary from one type of substrate to another.

Initial pH of the medium is another important parameter which affects the growth and product formation by microorganisms under both SmF and SSF. Although pH is one of the critical factors, the monitoring and control of pH during fermentation is not usually attempted in SSF. Good buffering capacity of some of the substrates used in SSF help in eliminating the need for pH control during fermentation (Lonsane *et al.*, 1985). This advantage is therefore exploited in the initial adjustment of the pH of the solids using the moistening media of the desired pH. However, local changes in pH of the agglomerates, produced if the organism develops in the form of a film on the solids, cannot be checked and result in low productivity.
in unagitated fermenter (Knapp & Howell, 1980). The results of the present study suggest that the organism prefers an optimum pH of 7.0 for maximal enzyme yield, while the organism had a growth pH optima of 6.0 (Renu, 1991). However, significant levels of enzyme could also be recovered at pH 6.0 and 8.0.

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

In the present study, the substrate particle size required for maximal glutaminase production varied according to the type of the substrate. Wheat bran and rice husk of 0.6 - 1.0 mm particle size supported maximum enzyme yield after 24 h compared to all other substrates, where a larger particle size of 1.0 - 1.4 mm favoured maximal enzyme yield after 36 h. The variation, among the particle size of substrates, with respect to support for maximal enzyme production may be attributed to their difference in water holding capacity and surface area for colonization by bacteria.
Temperature is directly related to the metabolic activities of the microorganism and it affects proper growth and product formation by the organism (Lonsane et al., 1985). Every organism has its optimum temperature at which it grows best resulting in higher yield of the desired product and hence temperature should be maintained at the optimum of the microorganism that is used for SSF. It was found that V. costicola used in the present study required 35°C for maximal glutaminase production, which is also its optimal growth temperature (Renu, 1991). It is inferred that the bacteria requires its optimal growth temperature for maximal level of enzyme production.

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

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

The variation in optimum incubation time required for maximal enzyme yield with the substrates could be attributed to the differences in their physical nature and biochemical status which consequently influence the aeration rate and nutrient availability for the bacteria during SSF. Preferential utilization of native carbon and nitrogen sources of copra cake powder and groundnut cake powder in place of glutamine may be the cause of delayed and reduced glutaminase yield compared to wheat bran and rice husk which have relatively lesser protein and carbohydrate contents. (Nagendra Prabhu & Chandrasekaran, 1996 a). Extended incubation time required for maximal enzyme production with saw dust may be accounted to its comparatively nutritionally inert and complex nature. The reduction
in glutaminase yield from all the substrates after 48 h is probably due to enzyme denaturation by the protease secreted by the bacteria (Nagendra Prabhu & Chandrasekaran, 1996 a). Similar results were obtained during α-amylase production by B. megaterium 16 M under SSF (Ramesh & Lonsane, 1987).

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

The addition of nitrogen sources, including organic, inorganic and amino acids, also did not have any major impact on glutaminase yield. This could also be due to the reasons mentioned above. In the case of organic nitrogen sources such as peptone, yeast extract and amino acids, there could have been a competitive preference of these sources over L-glutamine and hence a consequent reduction in glutaminase yield. On the other hand, addition of
mineral salts, enhanced glutaminase yield. Use of ammonium sulphate, sucrose, ammonium nitrate, potassium ferrocyanide and EDTA as nutritional supplements were reported to enhance the production of citric acid by SSF using different substrates (Shankaranand & Lonsane 1993, 1994).

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

A comparison of all the five organic solid substrates with respect to glutaminase production during SSF under optimal conditions testifies the advantages of wheat bran and rice husk over the other three substrates. Wheat bran is the most commonly used organic solid substrate for SSF processes aimed at producing microbial exoenzymes, organic acids, antibiotics, food flavours etc (Lonsane, 1994). Rice husk, saw dust and a large variety of materials have been used for various SSF experiments (Bharat Bhushan et al, 1994). Whereas copra
cake powder was used for lipase production and ground nut cake powder has not yet been tried as substrate for SSF (Pandey, 1992).

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

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

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

The results of the scanning electron microscope studies presented in Plates II and III adds evidence to the fact that marine bacteria adsorb on to solid particles and colonize rapidly. It was
observed that *V. costicola* grew by adsorbing on to the polystyrene beads and utilizing the nutrients supplied in the moistening medium.

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

The growth and enzyme production by the bacterium was maximum when both mineral salts glutamine medium and aged sea water containing 1 % (w/w) L-glutamine were used, prior to optimization of SSF conditions. The aged sea water glutamine medium was selected for further studies not only due to the ease of preparation and the nonrequirement of expensive chemicals, but also it serves the requirements of a marine bacterium with respect to several trace elements and mineral salts, which are naturally present in it.
The substrate media ratio, which determines the moisture content of substrates, played a critical role in the SSF process, as observed with wheat bran and other natural substrates tried in the present study. Since the water absorbency of polystyrene was low (2 gm/100 cm³; Brydson, 1982), increase in the proportion of media in the ratios above 1 1 (w/v; approximately 50 - 60 % moisture content) resulted in the existence of free water and a consequent reduction in the product yield (Nagendra Prabhu & Chandrasekaran, 1996 b). Zhu et al, (1994), in a similar study, used a moisture content of 60 % for the SSF production of nuclease P 1 by *Penicillium citrinum* using polyurethane foam as inert support.

The initial pH, incubation temperature and substrate (L-glutamine) concentration required by the bacteria with polystyrene system were very similar to that obtained in the present study with nutritionally rich substrates. As discussed earlier, these environmental parameters are independent in exerting their influence on the growth and performance of bacteria (Chandrasekaran et al, 1991) and were not influenced by the nature of the substrates used for SSF (Nagendra Prabhu & Chandrasekaran, 1996 a). In fact, the physico-chemical nature of polystyrene has not affected the physiology of the bacteria, but served as a substrate for surface colonization.
The effect of inoculum concentration on L-glutaminase production under SSF on polystyrene system was also very similar to that obtained with the natural substrates, recording a maximum glutaminase yield with an inoculum concentration of 0.750 mg dry wt/10 gds. The optimum incubation time was 24 h after standardization. The reason for the decrease in enzyme yield after 48 h might be attributed to the inactivation of glutaminase by the protease secreted by the bacteria (Nagendra Prabhu & Chandrasekaran, 1996 a,b). A similar observation was made during SSF on polyurethane foam for the production of nuclease P 1 by *P. citrinum*, where the nuclease yield was reduced after 3 days of fermentation due to enzyme inactivation by protease (Zhu *et al*, 1994). Ramesh and Lonsane (1987) also observed reduction in α-amylase production by *B. megaterium* under SSF on wheat bran after 52 h of incubation and postulated that this may be due to poisoning, denaturation and/or decomposition of the enzyme as a result of interaction with other components in the medium.

Incorporation of additional carbon sources enhanced the enzyme yield. Among the carbon sources tested, maltose incorporated at 1 % (w/w) level in the sea water glutamine medium could promote maximal enzyme yield compared to others. The increase in enzyme yield could be attributed to the rapid growth accomplished by the easy availability of additional carbon sources along with glutamine (Nagendra Prabhu & Chandrasekaran, 1995). On the other hand, it was observed that nitrogen sources, including amino acids, had a negative
impact on glutaminase yield. This could be due to the preferential utilization of these nutrients in place of glutamine (Nagendra Prabhu & Chandrasekaran, 1996 b).

All the mineral salts tested in the present investigation enhanced glutaminase yield significantly when compared to the control. \( \text{KH}_2\text{PO}_4 \) effected the maximum enzyme yield when compared to the control. \( \text{NaH}_2\text{PO}_4 \) followed by \( \text{MgSO}_4 \) could also enhance enzyme yield significantly. Results emphasize the critical role of phosphates and magnesium in the enhanced secretion of glutaminase by this bacteria. Zhu et al (1994) have achieved increased product yield of nuclease P 1 with polyurethane foam impregnated with a media that simulated the chemical composition of wheat bran. Whereas in the present study, maximal yield of the target product was obtained with the use of aged sea water media containing L-glutamine, maltose and \( \text{KH}_2\text{PO}_4 \), indicating enormous scope for economic production of this enzyme.

Media optimization studies in SSF could be accurately conducted only by using substrates which are nutritionally inert than with conventional substrates which contain native carbon and nitrogen sources besides other growth factors. It is difficult to optimize the exact nutritional requirements of the organism in an SSF system involving nutrient-rich substrates, because one cannot be sure about the increase/decrease in microbial growth and product formation resulted due to the added nutrient. The fermentation media must not
only meet the nutritional requirements of the microorganisms but also should be economical for an industrial process since factors such as its cost, efficiency of utilization and its effect on downstream processing are all important factors in the design of a fermentation medium (Stanbury, 1987). In this context the nutritionally inert polystyrene beads, as solid supports, has facilitated the design of an economic medium for enhanced product yield with minimal presence of undesired proteins.

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

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

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

In this context, use of synthetic, nutritionally inert supports such as polystyrene eliminates all these problems associated with product recovery, since the water absorbency of the polystyrene beads is very low (2 gm/cm³; Brydson, 1982), and consequently very little solvent will remain absorbed to the solids. This facilitates 92 - 96 % recovery of the leachate compared to 82 - 85 % recovery

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

The leachate from the polystyrene system was not only less viscous (mean viscosity 0.966 Ns/m²) but also showed high specific activity for glutaminase. Further, the leachate was free from undesired proteins unlike that from the wheat bran or other nutritionally rich substrates which were highly viscous and contained amylase and cellulase, besides glutaminase (Nagendra Prabhu & Chandrasekaran, 1995, 1996 b). These features are highly beneficial and desirable in the enzyme industry, as they eliminate the problems caused by the viscous nature of the leachate from natural substrates (Ramesh & Lonsane, 1989).
4.4. Biomass estimation in SSF

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

Though a number of methods have been developed, such as measurement of cell constituents like chitin (Aidoo et al., 1981), ergosterol (Seitz et al., 1979), nucleic acids (Bajracharya & Mudgett, 1980; Koliander et al., 1984), protein by Kjödhal method; biological activity such as ATP (West et al., 1986), enzyme activity (Barak & Chet, 1986), respiration rate (Sakurai et al., 1985), immunological activity (Frankland & Bailey, 1981), and nutrient consumption (Matcham et al., 1984), their wider applications are limited due to a number of factors such as chemical composition of the substrate, degree of interference from the substrate and the desired degree of sensitivity. Most of the techniques are standardized with respect to one particular
substrate and hence subject to variation with other solid substrates. In most cases, the nature of the substrate dictates the choice of the method and other practical considerations like rapidity of the assay and availability of the instruments (Lekha & Lonsane, 1994). According to Mitchell (1992), these indirect methods developed for biomass estimation are of questionable reliability.

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

Results of the present study clearly indicates the feasibility of polystyrene system with respect to the estimation of bacterial biomass by measuring the dry weight and/or cell protein. The polystyrene beads are nutritionally inert and does not contain any nutrients of its own and provides only surface for attachment of the bacteria during fermentation. Further all the nutritional components added in the medium are soluble and can be washed out. With the feasibility of direct biomass estimation, in this inert support system of SSF, more direct and close monitoring and control of the physiological development of the bacteria such as the growth and product formation becomes possible. The polystyrene system could be used to analyze the relationship between biomass and related
parameters and derive appropriate models to understand the similar situations of SSF on wheat bran or other substrates such as that observed with polyurethane foam (Zhu et al, 1994). Results of the present study clearly and strongly advocates the use of nutritionally inert solid supports for SSF studies by virtue of their suitability for easy separation of biomass from the fermentation medium and its direct estimation.

4.5. L-Glutaminase production under SSF using mixed substrates

Use of mixed substrates, derived from different nutritionally rich natural sources, as solid substrates for fermentation production of enzymes have yielded varying results as documented under review of literature (section 1.2.2.4). To quote a few while enhanced yield of glucoamylase by A. niger was obtained using wheat bran and corn flour at a ratio of 9 1 (Pandey & Radhakrishnan, 1993), protease production by Rhizopus oligosporus decreased when wheat bran was mixed at various combinations with wheat flour, soy flour, soy oil, rice bran etc. (Ikasari & Mitchell, 1994). In contrast to these studies, when a mixed substrate system was prepared using nutritionally inert and rich substrates in the present study, varying degrees of glutaminase yield was recorded. Mixtures containing high proportions of polystyrene and low amounts of wheat bran/rice husk supported increase in glutaminase yield compared to mixtures having more amount of wheat bran/rice husk than polystyrene.
Unlike the earlier reports, mentioned above, the inert support was observed to favour yield of glutaminase, only at similar levels, under mixed substrate systems having low proportions of wheat bran/rice husk compared to the yield obtained when used individually. It was also observed that both wheat bran and rice husk could record significant levels of enzyme yield when used individually compared to that obtained in the mixed condition. In fact, as such, the results of the present study advocates use of inert supports alone than as mixed substrates in combination with natural substrates, when viscosity and enzyme profile of the leachate is considered important. Nevertheless, since the enzyme yield is at comparable levels with the individual substrates, the mixed substrates, which attribute nutritionally rich solid substrate media, may be preferred for glutaminase production. Further, the mixed substrate studies using other natural substrates in combination with inert supports could result in the development of a more efficient SSF system which combines the beneficial aspects of both the conventional as well as SSF systems using inert supports.

4.6. Impact of mutation on L-glutaminase production

Mutation of *Torulopsis famata*, with NTG treatment resulted in a 3 fold increase in glutaminase yield (Kakinuma *et al*, 1987). Spontaneous mutation of Streptomycin sensitive strains of *E. coli* led to a 60 % decrease in L-glutaminase and L-asparaginase activities
Whereas in the present investigation, NTG treatment and UV irradiation of *V. costicola* yielded only 48% and 24% increase over the control respectively. The results indicate scope for further improvements in L-glutaminase yield through genetic manipulation. Since no reports are available on similar studies with any marine microorganism, no detailed discussion or comparison could be made.

4.7. Purification and characterization of L-glutaminase

An overall yield of 42% was obtained after 100 fold purification of glutaminase-asparaginase from *Achromobacteraceae* (Roberts *et al.*, 1972), while a 6000 fold purified glutaminase with 40% yield was obtained from *E. coli* (Prusiner *et al.*, 1976). Glutaminase from *Pseudomonas sp.* was purified with a yield of 40-50% (Roberts, 1976), whereas the enzyme from *P. acidovorans* was purified 2205 fold with 19% yield. 4% and 0.04% yield of 1620 and 190 fold purified glutaminase I and II from marine *Micrococcus luteus* was reported (Moriguchi *et al.*, 1994). Both intra and extra cellular glutaminase from *A. oryzae* with 6.2% and 3.2% recovery and 1100 and 730 fold purity was also reported (Yano *et al.*, 1988). In the present study, enzyme with high specific activity (597 U/mg protein) could be recovered with 43.68 fold purification. The final yield after gel-filtration chromatography was 30.62%. Electrophoretic studies revealed a single band upon polyacrylamide gel electrophoresis for
both SDS-PAGE and native PAGE. The subunit molecular weight was in
the range of 36,000 to 40,000 daltons. The native protein had an
approximate size of 140,000 to 150,000 daltons.

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

The studies on the temperature tolerance of the purified
glutaminase show that the enzyme has an optimum temperature of 40°C
for its activity and retained 85 % activity at 45°C. The enzyme was
most stable at 40°C and retained 95 % of the activity at 45°C. The
presence of substrate (L-glutamine) at 0.01 M level and NaCl at 10 %
level enhanced the thermostability of the enzyme up to 50°C (95 - 98 % activity) and enabled the enzyme to retain about 80 % activity at 60°C. Whereas the absence of L-glutamine or NaCl, led to a reduction in stability at 60°C.

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

The purified enzyme demonstrated typical Michaelis-Menten kinetics at low substrate concentration. Maximal activity was obtained with 0.04 M L-glutamine and further increase in substrate concentration did not have any change in the enzyme activity. Glutaminase from P. acidovorans recorded a relatively low Km value of 2.2 x 10^{-5} M (Davidson et al, 1977). The intra and extracellular glutaminase from A. oryzae also had low Km values of 9.1 x 10^{-5} M and 9.6 x 10^{-5} M respectively (Yano et al, 1988). Whereas the glutaminase
I and II from marine Micrococcus had a relatively high Km value of 4.4 and 6.5 mM respectively compared to others mentioned above (Moriguchi et al, 1994). In the present study also the enzyme recorded a Km value of $7.4 \times 10^{-2}$ M which is slightly higher than that reported for marine Micrococcus luteus.

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

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

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

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

A major outcome of the present investigation is the development of a new and ideal bioprocess for the production of L-glutaminase, using the polystyrene beads. The new system has facilitated the design of a simple aged sea water medium supplemented with L-glutamine, maltose and \( \text{KH}_2\text{PO}_4 \) for enhanced product yield with
minimal presence of undesired proteins. Leachate with low viscosity and high specific activity for the target product could be recovered from this system. 92 - 96 % of the leachate could be recovered from the inert support SSF system which is impossible with conventional SSF systems using natural substrates. A new protocol was also developed for the rapid and direct estimation of biomass during SSF using polystyrene. This may have tremendous application in the scaling-up studies and development of an ideal SSF system using both nutritionally rich and inert substrates.

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

The enzyme produced by the organism also has several beneficial properties needed for an industrial enzyme. It shows activity and stability over a wide range of pH and temperature and is not inactivated in presence of high NaCl concentrations. The substrate specificity towards L-glutamine is also very high which means that it could be used in low amounts to achieve the desired effect.
In order to sustain interest in SSF processes and to utilize its manifold advantages over the conventional SmF, the inherent problems of SSF should be overcome. Our results clearly suggest that SSF using nutritionally inert solid support materials could lead to further improvement of this already advantageous process. However, scale-up studies are needed for the development of an economic bioprocess for commercial exploitation. Further, this study conclusively proves that marine microorganisms could be exploited through solid state fermentation, for obtaining useful products such as industrial enzymes, organic acids, antibiotics and other secondary metabolites as experienced with their terrestrial counterparts. Economic exploitation of these vast untapped resources, employing solid state fermentation will yield many products beneficial for mankind and warrants serious and immediate attention.