6. SUMMARY AND CONCLUSIONS

*Pseudomonas* sp. BTMS-51 isolated from the marine sediments of Cochin coast and available in the culture collection of the Department of Biotechnology, Cochin University of Science and Technology was used in the present study. The bacterial cells were immobilized by gel entrapment in Ca-alginate and by physical adsorption on three nutritionally inert supports - polystyrene, polyurethane foam and nylon web. The conditions, which favour maximum enzyme production by immobilized cells, were optimised under batch mode, and the efficiency for enzyme production was determined under the optimised conditions. Repeated batch operations were performed to evaluate the potential for reuse of the immobilized cells. The operational conditions which supported maximal enzyme yield, productivity and substrate conversion were standardised for continuous operation in plug flow reactors and operational stability was determined by extended operation for a minimum of 72h.

The optimal conditions for production of stable Ca-alginate immobilized beads were standardised initially. 3% (w/v) sodium alginate concentration, a 1% (w/v) concentration of 18h old cells, 0.15M CaCl₂, and 2h curing time were ideal for production of stable beads with maximal enzyme yield. The ideal medium for bead activation was 1% glutamine in aged seawater, and conditions optimal for bead activation included pH 5, 28 ± 2°C, and 12h incubation. Enzyme production by the Ca-alginate immobilized beads was most effective in seawater glutamine medium containing 2% (w/v) glutamine and 1% (w/v) glucose in aged seawater. A medium pH of 6, incubation temperature of 35°C, and retention time of 12h supported the maximum enzyme yield. Incorporation of nitrogen sources other than glutamine in the medium led to a decrease in the enzyme production. Seawater with the minimal added nutrients could serve as an efficient enzyme production medium by the Ca-alginate entrapped cells.

Consistent enzyme yield (21-33U/ml) was obtained over the 20 cycles of repeated batch operation and there was no decay in enzyme production indicating the suitability of entrapped cells for repeated use.

Upon continuous operation in a packed bed reactor or circulating bed reactor, increase in dilution rates generally resulted in a reduction in the yield and percent
substrate conversion but at the same time, an increased volumetric productivity. Increase in substrate concentration could bring about an increase in yield and productivity and a reduced rate of substrate conversion. The operation conditions for maximal product yield were 2% (w/v) substrate concentration and a dilution rate of 0.64-h⁻¹ for the PBR, while it was 2% (w/v) substrate concentration and a dilution of 0.24-h⁻¹ for the CBR. The conditions that supported maximal productivity were a dilution rate of 1.27-h⁻¹ and 0.56-h⁻¹ respectively for PBR and CBR, at a substrate concentration of 2% (w/v) for both. Maximal rate of substrate conversion was at a substrate concentration of 0.5% (w/v), and at dilution rates 0.64-h⁻¹ and 0.24-h⁻¹, respectively for PBR and CBR. Both the reactors showed an extended operational stability with out decay in productivity.

Physical adsorption of the cells on inert supports was achieved through growth of the cells in an immobilization medium containing the support particles. The medium that supported maximal adsorption of cells was Zobell's broth irrespective of the type of support. Incubation time required for maximal cell adsorption was identical (36h) for the three supports. Initially, mineral salts glutamine medium (MSG) and seawater glutamine medium (SWG) was tested as the basal enzyme production medium and the ideal retention time for maximal enzyme production was determined. SWG was the better medium in terms of enzyme yield and 12h was the ideal retention time.

The medium composition and conditions for maximal enzyme production by cells immobilized on the three supports were optimised using a response surface Box-Behnken experimental design. The interactions between the various operational parameters were determined from computer generated three dimensional response graphs.

When polystyrene (PS) beads were used as the support system for immobilization, temperature had a statistically significant effect on enzyme yield. pH of the medium and operation temperature had effect independent of other parameters and the optimal range was found to be pH 5.5-6.5 and 30-32.5 °C for maximal enzyme yield. Interactions between other parameters produced differences in enzyme yield by the immobilized cells. The optimal levels of the operational parameters were determined, while fixing the operation temperature at 35 °C, using a software package and was pH 6.33, 1.5 % (w/v) glutamine n, 1.5% (w/v) glucose and 0.11%
(w/v) yeast extract. The observed enzyme yield 15.51 (U/ml) was 37.21% lower than the predicted yield (24.7U/ml) as validated by batch operation in shake flasks.

Temperature and glutamine concentration had significant effects on enzyme production by cells immobilized on polyurethane foam (PUF). The dependence of enzyme production by cells on temperature was independent of the interactions and the optimal operating range was 30-32.5 °C. The effect of glutamine concentration on enzyme production was different upon interaction with the different parameters. The optimal pH range was 5.5-6.5. Interactions between other parameters also affected the enzyme production by PUF immobilized cells. The predicted optimal conditions for maximal enzyme production were 1.5% (w/v) glutamine, 0.5% (w/v), and a pH of 6.01 when the operating temperature was fixed at 35 °C. Maximal enzyme production conditions required that yeast extract be absent from the medium. The production conditions were validated by batch studies and the observed enzyme yield was 46.27% lower than that predicted.

Temperature, both alone and upon interaction with yeast extract concentration, had significant effects on enzyme production when nylon web (NW) was the adsorbent, for cell immobilization. pH dependence for enzyme production was unaffected by interactions with other parameters, and similar to polystyrene or PUF systems, the ideal range was 5.5-6.5. The optimal range of operation temperature was 30-35 °C. Enzyme production by cells immobilized on nylon web was influenced by interactions between other parameters. The predicted levels of operational parameters were pH 6.06, 1.5% (w/v) glutamine, 1.42% (w/v) glucose and 0.02% (w/v) yeast extract, for maximum enzyme yield, when the operation temperature was fixed at 35 °C. The observed enzyme yield of 22.92 (U/ml) was 16.35% lower than the predicted value (27.4U/ml).

Reusability of the cells immobilized by adsorption on the three supports was evaluated by repeated batch operation for 20 cycles in each case. All the three systems supported consistent enzyme yield over the several cycles of operation and no decay in productivity or cell loading on the supports was observed upon prolonged operation. The mean enzyme yield obtained were 13.88, 18.79 and 17.99 U/ml respectively for the PS, PUF and NW systems.

Suitability of the cells immobilized on beds of PS, PUF and NW for continuous production of the enzyme was evaluated in a packed bed reactor. Bacteria
were immobilized on the bed surface by circulation of an actively growing culture aerated in an external loop, through the reactor column for 36h and enzyme production was achieved by circulation of production medium through the bed. The conditions that favoured maximum yield, productivity and rate of substrate conversion were determined subsequently.

Irrespective of the nature of support matrix, lower yield and percent substrate conversion rate were observed at higher dilution rates. Higher dilutions in general, resulted in increased volumetric productivities, which was also dependent on substrate concentration and availability. Concentration of substrate positively affected the yield and productivity, and showed a negative influence on the rate of substrate conversion.

Irrespective of the nature of support matrix, the conditions required for maximal enzyme yield under continuous culture were the lowest dilution rate and highest substrate concentration. Whereas, the conditions for maximal productivity were the highest substrate concentration and highest dilution rate for PUF and NW systems. For the PS system, the productivity decreased marginally at the highest dilution rate tried. Highest percentage of substrate conversion was obtained at the lowest substrate concentration and dilution rates in all the three systems.

Continuous operation of the reactors with any of the three bed materials was stable for an extended operation period of 72h and did not show any signs of decay in terms of enzyme yield or cell loading, indicating the potential for continuous production of glutaminase by cells immobilized on these support matrices.

Conclusions

Based on the data obtained in the present study, it is concluded that marine bacteria are ideal candidates for immobilization using either Ca-alginate entrapment or physical adsorption on to synthetic inert supports and the process of immobilization does not negatively influence them. Thus, Ca-alginate entrapment of the bacteria was found to be well suited for reuse of the biomass and extended operational stability during continuous operation. Adherence of the bacterium to inert supports was observed to be strong and it imparted minimal stress on the
immobilized bacterium and allowed detachment and relocation on the supports which enabled the formation of a dynamic equilibrium maintaining a stable cell loading. This is particularly desirable in the industry for extended operational stability and maintenance of consistently higher outputs.

Response surface analyses of the enzyme production data obtained with various combinations of process variables, throws more light on the nature of interactions of various operational parameters among themselves, and on extracellular enzyme production by immobilized marine bacteria. Further, it also enabled the investigation process to evolve a statistically sound optimisation, which consequently facilitated a near accurate recognition of optimal conditions for maximal enzyme production by immobilized marine bacteria.

Information generated on the continuous production of glutaminase by bacteria adsorbed on inert supports, in a packed bed reactor, indicate scope for further scale up and application in industrial production. Moreover, the data obtained on parameter interactions suggest a scope for further development of an ideal model for industrial production of extracellular enzyme employing marine bacteria.

It is proposed that marine *Pseudomonas* sp. BTMS-51 is ideal for industrial production of extracellular L-glutaminase and immobilization on to synthetic inert support such as polyurethane foam could be an efficient technique, employing packed bed reactor for continuous production of the enzyme.

May be the present study is the first of its kind in making several pioneering attempts with respect to marine bacteria in employing different immobilization techniques, synthetic carriers, reactor systems, use of sea water as fermentation medium, and response surface methodology for optimisation of operational parameters for production of extracellular enzymes.