CHAPTER 3

IV. IMMOBILIZATION FOR IMPROVED PROTEASE PRODUCTION

4.1. INTRODUCTION

Biotechnology is undergoing impressive advances in the synthesis of biocompatible surfaces for the immobilization of a range of biomolecules, with important applications in biosensing and medicine (Sadjadi et al., 2009). There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play a crucial role in cellular metabolic processes but also paid considerable attention in the industrial applications. The extracellular proteases are of commercial value and find multiple applications in various industrial sectors. The demand for industrial enzymes, particularly of microbial origin, is increasing due to their applications in a large number of fields such as the food, dairy, pharmaceutical, laundry detergent, textile and cosmetic industries (Kembhavi et al., 1993). These enzymes contribute to the development of high added value applications of product using enzyme-aided digestion (Kumar and Takagi, 1999). Although there are many microbial sources available for proteases production, only a few are recognized as commercial producers. Microorganism serves as an important source of protease mainly due to their
shorter generation time, the ease of bulk production and the ease of genetic and environmental manipulation (Patel et al., 2005). Pezynska-Czoch and Mordarshi (1988) have reported that *Streptomyces* species are the most industrially important actinomycetes, due to their capacity to produce numerous secondary metabolites and particularly antibiotics. The ability of these bacteria to produce large amounts of enzymes, as proteases with varied substrate specificities offers another potentially interesting use.

Majority of the industrial sectors are continuously trying to identify the protease enzymes that have the potential industrial applications, either to use them directly or to create modified enzymes that have enhanced catalytic activity and well adapted for large scale industrial processes (Glaser, 2000). However these new enzymes would have to offer a competitive advantage over existing products. One example of a potential candidate for a detergent protease was found to be *Teredinobacter turnirae*, a shipworm bacterium showing a symbiotic relationship with a marine shipworm, *Psiloteredo healdi* (Greene et al., 1989). It has been reported that *T. turnirae* produces proteolytic enzyme of which more than 80% are extracellulars (Greene, 1994). Moreover, the increasing demand for protease as a detergent supplement justifies a study aiming at a search for proteolytic enzymes from various microbial sources. For industrial applications, the immobilization of protease on a solid support can offer several advantages, including repeated usage of enzyme, ease of
product separation, improvement of enzyme stability and continuous operation in packed-bed reactors. The activities of the immobilized proteases so far reported are rather low when compared to those reported for other enzymes (Kise and Hayakawa, 1991; Gauthier et al., 1991; Hyndman et al., 1992).

Cell immobilization technology is often studied for its potential to improve fermentation processes and bioremediation (Beshay et al., 2002; Abd-EL-Haleem et al., 2003). The use of immobilized cells in the production of metabolites by culture of microorganisms are one of the most interesting techniques proposed during decades for improvement of fermentation process (Longo et al., 1992). It offers various advantages, such as increase of productivity due to the high cell concentration within the reactor and prevention of washout in continuous operation among others. Immobilization of whole cells for the production of extracellular enzymes offer many advantages such as the ability to separate cell mass from the bulk liquid for possible reuse, facilitating continuous operation over a prolonged period and enhanced reactor productivity (Zhang et al., 1989). However, proper selection of immobilization techniques and supporting material is needed to minimize the disadvantages of immobilization. The immobilization also presents some disadvantages such as disruption on immobilization by cell growth, gas evolution and disruption due to shear stresses. The most remarkable disadvantages are the diffusion limitation, which often occur when
immobilized cells are applied (Webb, 1986). Thus, it is important to know how the immobilizing materials affect the intra-gel transport of large molecules, such as proteases. The immobilization techniques can be divided into four groups based on the physical mechanism causing immobilization: physical entrapment within a porous matrix, attachment or adsorption to a pre-formed carrier, self aggregation by flocculation (natural) or cross linking agents (artificially induced) and cell contained behind barrier. Among the numerous techniques and support material that have been proposed, entrapment in polysaccharide gel (such as alginate or K-carrageenan) is commonly used for immobilization (Bucke, 1986).

Immobilization of microbial cells using sodium alginate is very simple and cheap. Sodium alginate is readily available and it is a non-toxic biological material. Therefore, it is suitable matrix for immobilization of bio-molecules and microorganisms (Mattiasson et al., 1993). Sadjadi et al. (2009) reported that the beads of calcium alginate are prepared under mild conditions and have been extensively used for microencapsulation and entrapment of bacterial cells for the protease production. The advantages of immobilized enzymes over their counterparts in solution include enhanced temperature and stability of the biocatalyst and ease of separation from the reaction medium and enabling multiple reuses. In the last decade, nanosized materials have been widely used as support for immobilization. Among the nanomaterials,
gold and titanium oxide are very popular when used in conjunction with biological materials including proteins, peptides, enzymes, antibodies and nucleic acids, because of their unique characteristic properties. A number of templates which have been used for enzyme immobilizations are silica nanotubes, phospholipid bilayers, self-assembled monolayers, Langmuir-Blodgett films, polymer matrices, galleries of a-zirconium phosphate, mesoporous silicates such as MCM-41, silica nanoparticles, and thermally evaporated lipid films, each with its characteristic advantages and disadvantages (Sadjadi et al., 2009).

Enzymes being thermostable have presented the problem of their preservation; therefore it is necessary to increase the life span of biologically important enzymes. The enzymes after immobilizations undergo certain changes in the spectrum of their activity. Fortunately many enzymes remain significantly active after immobilization. Ziegler (1992) and Renner (1998) found that immobilized DNAsel can be used to remove contaminating genomic DNA from RNA samples isolated from single cells for differential display analysis. Rai and Taneja (1998) reported that immobilized d-hydentoinase exhibit 80% enzyme production and 80% protein content. Furthermore, the immobilization of the preparation does not change its optimum pH, temperature or affinity but increases its self life, thermostability and stability in various organic solvents. Saleemuddin (1999) advocated that bioaffinity
based immobilizations are usually reversible fascinating the reuse of support matrix, orient the enzymes favorably and offer enzyme immobilization directly from partially or even cell lysates.

The productions of some enzymes, mainly carbohydrate-hydrolysing and proteolytic enzymes have been studied using immobilized growing fungal cells (Federici, 1993). Several researchers have studied on the possibility of producing phosphatases with immobilized bacterial and yeast cells, especially with polyacrylamide gel-immobilized *Citrobacter* sp. (Butler *et al*., 1991) and *Saccharomyces cerevisiae*, immobilized in polyaldehyde-hardened gelatin (Parascondola *et al*., 1993). The filamentous fungus *Humicola lutea* can be successfully used as a highly active acid phosphatase producer (Aleksieva and Micheva-Viteva, 2000; Micheva-Viteva *et al*., 2000) when cultivated in orthophosphate-free medium containing casein as nitrogen and phosphate source. The ability of microbial acid phosphatases to provide mild hydrolysis of casein can be of great significance for food technology and nutrition (Sviridenko *et al*., 1987). Grigorov *et al*. (1983) have reported that the mutant fungus *Humicola lutea* (strain 72) as a high producers of acid proteinases. Michailova *et al*. (1984) previously reported that fine structural changes of submerged mycelia during maximal enzyme production in batch experiments. Semicontinuous production of acid proteinase by immobilized *Humicola lutea* mycelia was reported using polyacrylamide

Enzymes are well-known green catalysts that possess a high degree of specificity. The specificity involves discrimination between substrates (substrate specificity), similar parts of molecules (regiospecificity) and optical isomers (stereospecificity). The mildness and specificity of enzymes endow them with a high efficiency for applications in fine-chemical synthesis of pharmaceutical industry, food processing, biosensor fabrication, bioremediation, and protein digestion in proteomic analysis. However, the applications of enzymes are limited by their instability and nonreusability. Enzyme immobilization is an effective way to overcome these limitations to some extent. First, the multiple-point attachment to the support can restricts the undesirable conformational change of enzyme proteins in unfriendly environments. Second, insoluble supports can be recycled much more easily than soluble enzymes. The result of immobilization is strongly depending upon the properties of supports, which are usually referred to as material types, compositions and structures etc. So far, different nanostructure materials have been used as supports, such as mesoporous silica, nanotubes, nanoparticles, and nanofibers. They stand out of other supports because of their extremely high surface area-to-volume ratios, which provides large
specific surface of areas for highly efficient immobilization as well as to stabilize the enzymes. The enzyme-immobilized nanofibrous membranes have functions of biocatalysis and separation simultaneously which is generally accepted as the fundamental requirement for enzymatic membrane-bioreactor (Zhen et al., 2009). Michailova el al. (1984) reported that the structural changes of submerged mycelia were observed during maximal enzyme production in batch experiments.

Though, few reports evidenced the noteworthy protease production from the fish gut bacteria, report on immobilization induced protease production by fish gut isolate still stands as a lacunae. In this context, the present chapter was studied to test the efficiency of immobilized bacterial cells on protease production by using various matrices such as agar-agar, polyacrylamide gel, carrageenan, sodium alginate and gelatin.
4.2. MATERIALS METHODS

4.2.1. Preparation of inoculum

The protease producing bacterial strains (*B. flexus* and *Pseudomonas* sp.) were subcultured and the pure culture was maintained on nutrient agar slant at 4°C for further study. Five ml of distilled water was added to a 24 h old slant of protease producing bacterial strains. The cells were scrapped from the slant and suspended into sterile distilled water and the resulted cell suspension at 10% level was transferred aseptically into 250mL Erlenmeyer flasks containing 45ml of sterile enrichment medium, beef extract (0.3 %), peptone (0.5 %), NaCl (0.5 %) and glucose (0.5 %) at pH 7 for 24 h and then 10 % of enriched culture was inoculated in 250 mL flask containing 45 mL Basal medium containing (g/l) - (NH₄)₂SO₄ - 2g ; K₂HPO₄ - 1g ; KH₂PO₄ - 1g ; MgSO₄.7H₂O - 0.4g ; MnSO₄.H₂O - 0.01g ; FeSO₄.7H₂O - 0.01g ; Yeast extract - 1g ; Peptone - 10g at pH 7. The culture was then incubated for 2 days by reciprocal shaking (120 rpm) at 35°C. The cells were then harvested by centrifugation at 10000 rpm for 15 min and the supernatant was used for further protease assay. The cell suspension was used as inoculum for immobilization as well as for free-cell fermentations (control).
4.2.2. Estimation of protease production by immobilized bacterial cells

The assay process consists of following ingredients such as 1.25 ml Tris buffer (pH 7.2), 0.5 ml of 1% aqueous casein solution and 0.25 ml culture supernatant. Appropriate control was also maintained. The mixture was incubated for 30 min at 30°C. Then 3 ml of 5% TCA was added to this mixture and placed at 4°C for 10 min to form precipitate. Then it was centrifuged at 5000 rpm for 15 min. From this, 0.5 ml of supernatant was taken, to this 2.5 ml of 0.5M sodium carbonate was added, mixed well and incubated for 20 min. Then it was added with 0.5 ml of folin phenol reagent and the absorbance was read at 660 nm using UV-Vis Spectrophotometer (TECOMP 8500). The amount of protease produced was estimated and expressed in microgram of tyrosine released under standard assay conditions.

4.2.3. Immobilization of whole cell in sodium alginate

To study the effect of whole cell immobilization on protease production by the candidate species, it was immobilized with sodium alginate (Sigma, Mumbai, India) solution. For this, different concentrations of sodium alginate (1.5%, 2.0%, 2.5%, 3.0% and 3.5%) were used. The alginate entrapment of cells was performed according to the method described by Johnson and Flink (1986). Alginate was dissolved in boiling water and autoclaved at 121°C for 15 min. Cells were harvested during the mid-logarithmic growth phase by
centrifugation (5000g, 10 min), resuspended in 2 ml of saline and added to 100 ml of sterilized alginate solution. This alginate / cell mixture (with stirring) was extruded drop by drop into a cold, sterile 0.2 mM CaCl₂ solution through a sterile pipette. Gel beads of approximately 2 mm diameter were obtained. The beads were hardened by resuspending into a fresh CaCl₂ solution for 24 h at 4°C with gentle agitation. Finally these beads were washed with distilled water to remove excess calcium ions and nonentrapped cells. Then approximately 100 beads were transferred to 100 ml production medium for the protease production. The protease production was carried out by above mentioned assay procedure.

4.2.4. Immobilization of whole cell in k-carrageenan

To study the effect of whole cell immobilization of tested candidate bacteria using K- carrageenan (Sigma) for the protease production, different concentrations of K-carrageenan such as 1%, 1.5%, 2.0%, 2.5%, 3.0% and 3.5% were weighed out and added to 18 ml of 0.9% sodium chloride. They were dissolved by gentle heating and sterilized in autoclave. The cell suspension was added to the molten K-carrageenan solution maintained at 40°C, mixed well, and poured into sterile flat bottom 4-inch diameter petriplates. After solidification, the K-carrageenan blocks were cut into equal size cubes (4 mm³) and added to sterile 2% potassium chloride solution and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4
times with sterile distilled water. Then, the protease production was carried out by above said method.

4.2.5. Immobilization of whole cell in agar-agar

The various concentrations of agar-agar (Hi-media, India) such as 4%, 5%, 6%, 7%, 8% and 9% were weighed and dissolved in 18 ml of 0.9% sodium chloride solution to get final concentration of 2%. The cell suspension was added to the molten agar-agar maintained at 40°C. Then it was shaken well for few seconds (without foam formation) and poured into sterilized petriplates and allowed to solidify. The solidified agar block was cut into equal size cubes (4 mm$^3$), added to sterile 0.1 M phosphate buffer (pH 7.0), and kept in the refrigerator (1 h) for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water for 3 to 4 times. The protease production was carried out by above mentioned assay procedure.

4.2.6. Immobilization of whole cell in polyacrylamide

A cell suspension (2 ml) was prepared by adding 10 ml of chilled sterile distilled water. To another 10 ml of 0.2 M sterile phosphate buffer (pH 7.0), the following chemicals were added: various concentrations of acrylamide and bisacrylamide such as 6%, 8%, 10%, 12%, 14% and 16%, 10 mg ammonium persulphate, and 1 ml TEMED (NNN1N1 Tetra Methyl
Ethylene Diamine). The of cell suspension (2ml) and the above mentioned phosphate buffer mixture was mixed well and poured into sterile flat bottom 10 cm-diameter petriplates. After polymerization (solidification), the acrylamide gel was cut into equal size cubes (4 mm³), transferred to 0.2 M phosphate buffer (pH 7.0) and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in phosphate buffer (pH 7.0) at 4°C until use. The protease production was carried out by above stated assay procedure.

4.2.7. Immobilization of whole cell in gelatin

2ml of cell suspension was added to 15 ml of different concentrations of such as 5%, 6%, 7%, 8%, 9% and 10% sterile gelatin (Hi-media), maintained at 45°C, and poured into a sterile petridish. The gel was over layered with 10 ml of 5% glutaraldehyde for hardening at 30°C. The resulting block was cut into small-size cubes (4 mm³) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde. The cubes were washed 3 to 4 times with sterile distilled water and stored in Tris buffer (pH 7.2) at 4°C until use. The protease production was carried out by above mentioned assay procedure.
4.2.8. Production of protease by repeated batch process with immobilized cells

The once used immobilized beads/blocks (cells equivalent to 0.03 g DCW) were transferred into 50 ml of production medium in 250-ml Erlenmeyer flasks. The composition of production medium was (g/L): glucose, 5; peptone, 7.5, and salt solution, 5% (MgSO₄·7H₂O, 5 g/L; KH₂PO₄, 5 g/L; and FeSO₄·7H₂O, 0.1 g/L) with a pH of 9.0. The flasks were incubated at 37°C for 48 h. Samples were withdrawn at regular intervals of 24 h and assayed for protease production under sterile conditions by following the above described procedure. Production of protease by repeated batch process one of the advantages of using immobilized biocatalysts is that they can be used repeatedly and continuously. Therefore, the reusability of bacterial strains with immobilized matrix was examined. After attaining the maximum production of protease (24 h), the spent medium was replaced with fresh production medium (50 ml) and the process was repeated for several batches until the beads/blocks started disintegrating.
4.3. RESULTS

4.3.1. Production of protease by cells immobilized in Agar-agar

The results indicated that protease production started from 24 h and it reached maximal level at 48 h. It was observed that the production with the immobilized cells in agar agar was less than that of immobilized cells with other matrix like sodium alginate, gelatin and K-carrageenan. The maximum protease production was attained at 48 h of incubation after which there was no considerable change. The highest enzyme production was recorded in *B. flexus* (327.26 ± 1.52 U/ml) and *Pseudomonas* sp. (272.75 ± 1.22 U/ml). The optimized concentration of agar agar for the protease production was 7% for both the bacterial strains (Fig 11a and b).

Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates as a function of incubation time and concentration of agar – agar was highly significant in *Pseudomonas* sp. (P<0.001) and *B. flexus* (P<0.001).

4.3.2. Production of protease by cells immobilized in sodium alginate

Figures 12 a and b show the amount of protease yield by microbial cell mass entrapped in sodium alginate matrix. The protease production was found to be increased gradually upto 48 h of incubation after which there was no appreciable change. The enzyme production was studied from 24 h to 72 h.
with immobilized cells and reached maximum level in *Pseudomonas* sp
(550.13 ± 1.63 U/ml) and *B. flexus* (563.99 ± 1.63 U/ml) at 48 h. On further incubation, enzyme production gradually decreased. The optimized concentration for the protease production was 2.5% for both tested bacterial species.

Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates as a function of incubation time and concentration of sodium alginate was highly significant in *Pseudomonas* sp. (P<0.001) and *B. flexus* (P<0.001).

### 4.3.3. Production of protease by cells immobilized in K- carrageenan

Figures 13 a and b show the amount of enzyme production by the bacterial cell mass entrapped in K-carrageenan gel matrix. The maximum protease production was attained at 48 h of incubation afterwards the enzyme yield decreased gradually. The highest enzyme production of 341.28 ± 2.04 U/ml was recorded in *B. flexus* and 312.44 ± 1.63 U/ml in *Pseudomonas* sp. This production was less when compared to that of the values registered in sodium alginate matrix and at the same time it was found to be high when compared to the production registered in free cells. The optimized concentration for the protease production was 2.5% for both the tested bacterial strains.
Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates due to incubation time was highly significant in *Pseudomonas* sp (P<0.001) and *B. flexus* (P<0.001).

4.3.4. Production of protease by cells immobilized in polyacrylamide

A gradual increase in protease production was noticed due to increase in fermentation time. The maximum protease production was obtained in 48 h of incubation after which the enzyme production decreased marginally. The enzyme production was studied from 24 to 72 h with immobilized cells and the highest enzyme production of 300.68 ± 1.63 U/ml was recorded in *B. flexus* and 348.14 ± 2.45 U/ml in *Pseudomonas* sp. It was found to be a lower enzyme production when compared with the production registered in alginate matrix. The optimized concentration for the protease production was 12% for both the tested bacterial species (Fig 14 a and b).

Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates as a function of fermentation time was highly significant in *Pseudomonas* sp. (P<0.001) and *B. flexus* (P<0.001).
4.3.5. Production of protease by cells immobilized in gelatin beads

A remarkable protease production was observed in cells immobilized in gelatin beads. Here the maximum level of 496.22 ± 1.63 U/ml by *Pseudomonas* sp. and 555.59 ± 1.63 U/ml by *B. flexus* were registered during 48 h of incubation. The protease production obtained with this carrier was low when compared with the production recorded in sodium alginate matrix. But the protease production was high when compared with the protease production by free cells and the immobilized cells of carrageenan, agar-agar and polyacrylamide. The optimized concentration of gelatin for the protease production was 7% for both tested bacterial species (Fig 15 a and b).

Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates due to incubation time was highly significant in *Pseudomonas* sp. (P < 0.001) and also in *B. flexus* (P < 0.001).

4.3.6. Repeated batch fermentation with free and immobilized cells

The semi-continuous fermentation was terminated to investigate the stability of the biocatalysts and their ability to produce protease under repeated batch cultivation conditions. The results revealed that the amount of enzyme production with immobilized cells gradually decreased from the end of first batch. Thus the repeated batch fermentation performed well with all beads carried out in two batches.
4.3.6.a. Repeated batch fermentation of protease by cells immobilized in agar-agar

Results on the protease production in second batch fermentation by the test bacterial strains immobilized in agar – agar are shown in Figures 16 a and b. In this experiment the significant protease production was obtained at 48 h of incubation and the further incubation found to decrease the protease production. The highest enzyme production recorded in *B. flexus* and *Pseudomonas* sp. were; 212.46 ± 2.04 U/ml and 187.29 ± 1.22 U/ml respectively. It was observed that the production with the cells immobilized in agar agar was less than that of other matrix like sodium alginate, gelatin and K-carrageenan matrix. The optimum concentration of agar agar for the protease production was 7% for both bacterial strains.

Statistical analysis indicated that, the variation in protease production due to incubation time by the tested bacterial isolates was highly significant for both *Pseudomonas* sp. (P<0.001) and *B. flexus* (P<0.001).

4.3.6.b. Repeated batch production of protease by cells immobilized in sodium alginate

The results indicated that the protease production gradually increased from 24 h to 48 h of incubation and afterwards the yield was found to be decreased gradually. The protease production was at maximal level in
Pseudomonas sp. (252.65 ± 1.63 U/ml) and B. flexus (366.99 ± 1.66 U/ml) during 48 h during the second batch (Fig 17 a and b).

Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates due to fermentation duration was highly significant in Pseudomonas sp (P<0.001) and B. flexus (P<0.001).

4.3.6.c. Repeated batch production of protease by cells immobilized in K-carrageenan

The protease production by the immobilized cells in K-carrageenan inferred that the 48 h of incubation was suitable and further increase in incubation subsequently decreased the enzyme production. The highest enzyme production was recorded in B. flexus (251.76 ± 1.22 U/ml) and Pseudomonas sp. (206 ± 2.04 U/ml). Among the tested carrier, the K-carrageenan showed low yield when compared with sodium alginate matrix. The optimized concentration of K-carrageenan for the protease production was 2.5% for both the bacterial strains (Figures 18 a and b).

Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates due to incubation time was highly significant for both Pseudomonas sp. (P < 0.001) and B. flexus (P < 0.001).
4.3.6.d. Repeated batch production of protease by cells immobilized in polyacrylamide

In repeated batch the enzyme production was studied from 24 h to 72 h with immobilized cells. The maximum protease production was obtained in 48 h of incubation and then the yield decreased with further incubation. The highest enzyme yield recorded by *B. flexus* was 240.63 ± 2.04 U/ml and in *Pseudomonas* sp. was 226.98 ± 2.45 U/ml. It was found to be a lower enzyme production compared to sodium alginate matrix. The optimized concentration of polyacrylamide for the protease production was 12% for the both the bacterial species.

Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates due to the fermentation duration was highly significant in *Pseudomonas* sp. (P < 0.001) and also in *B. flexus* (P < 0.001).

4.3.6.e. Repeated batch protease production by cells immobilized in gelatin beads

Results on protease production in repeated fermentation by the test bacterial species immobilized in gelatin beads revealed that it was maximum (279.89 ± 1.63 U/ml) in *Pseudomonas* sp and *B. flexus* (342.57 ± 1.22 U/ml) during 48 h of incubation. The protease production obtained with this carrier was very low when compared to the protease yield with sodium alginate
matrix. But the protease production was high when compared to the protease production by the immobilized cells of carrageenan, agar-agar and polyacrylamide (Figures 20 a and b).

Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates due to fermentation duration was highly significant in *Pseudomonas* sp. (P < 0.001) and *B. flexus* (P < 0.001).
4.4. DISCUSSION

Proteases represent one among the three largest groups of industrial enzymes and have extensive applications in food and pharmaceutical industries. Alkaline proteases are important in detergent and leather industries (Rao et al., 1998). Microbial proteases are preferred in view of the rapid growth of microbes, limited space required for their cultivation and their ready accessibility to genetic manipulation. Increased thermostability is an important factor for the suitability of an enzyme in industrial applications. The strategies used for improving the thermostability of proteins and chemical modification or cross linking of proteins (Braxton and Wells, 1992). In all these strategies, however, the recovery of yield and reusability of free enzymes as industrial catalysts are quite limited. Therefore, an increased attention has been paid to enzyme immobilization which offers advantages over free enzymes in choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme removal from the reaction mixture and adaptability to various engineering designs (Zoborsky, 1973). The fungus Conidiobolus macrorosporus (NCIM 1298) produces high yields (30 U/ml) of alkaline protease in short fermentation cycles (48 h) comparable to the bacterial enzyme. The present findings were also in consonance with the above statement and the maximum protease production was found at 48 h of incubation for both the tested bacterial strains.
Cell immobilization is one of the common techniques for increasing the overall cell concentration and productivity. The separation of products from immobilized cells is easier when compared with suspended cell systems. Immobilization of cells may allow continuous operation of cultivation processes at high dilution rates. Immobilization is a strategy for protecting cells from shear forces. Many different techniques for immobilizing cells have been proposed (Beshay et al., 2003). Earlier researcher reported that the whole cell immobilization on Bacillus mycoides was considerably move stable than the free enzyme and could be stored for extended periods in both wet and dry forms before use (Abdel Noby et al., 1998). Various reports confirm that the thermal stability of immobilized protease depend on the application of immobilization methods (Kise and Hayakawa, 1991; Hyndman et al., 1992).

The experiment on the effect of immobilization using agar-agar showed 4 to 5 folds enhanced production over the free cells. The optimized concentration for this matrix was 7% and the protease productions registered were 272.75 ± 1.22 U/ml and 327.26 ± 1.52 U/ml after 48 h respectively by Pseudomonas sp. and B. flexus. This report is in accordance with the earlier report of Adinarayana et al. (2005). They reported that the high yield of alkaline protease with immobilized cells of Bacillus subtilis using agar-agar matrixes. Also this study was supported by earlier study of Anna et al. (2003), they reported that the use of agar agar entrapped cells of Bacillus circulans
ATCC 21783 for cyclodextrin glucanotransferase production in a fluidized bed reactor led to maximum enzyme production (180 U/ml) after 48 h of incubation.

Polyacrylamide was successfully used for immobilization of many enzyme systems (Kim et al., 1994). In this study, the maximum protease production was observed at 48 h by Pseudomonas sp. (348.14 ± 2.45 U/ml) and B. flexus (300.68 ± 1.63 U/ml) and the optimized suitable concentration for this matrix was 12%; whereas, further increase in polyacrylamide concentration subsequently decreased the enzyme production. This result is consistence with the positive influence of these matrixes on fermentative production of protease by Bacillus subtilis reported by Adinarayana et al. (2005). Abdel naby et al. (1998) also reported that the production of alkaline protease from Bacillus mycoides was maximum at 5% acrylamide. The decrease in yield with increase in cross link might have been due to the decrease in the porosities of gel matrix which caused diffusion limitation of the substrates. The immobilized enzymes retained 15.78% of the specific protease activity.

A few reports on immobilization of Streptomyces fradiae and Pencillium chrysogenum cells for the production of tyrosin and penicillin respectively were available in the literature about the use of K-carageenan as
an entrapment matrix (Deo et al., 1998). The present study on the effect
K-carrageenan on protease production revealed that 2.5% was the suitable
concentrations for the protease productions and the production registered
were; 312.44 ± 63 U/ml and 339.15 ± 1.93 U/ml respectively for B. flexus and
Pseudomonas sp. This same phenomenon was reported earlier by Adinarayana
et al. (2005) and they inferred that the production of alkaline protease was
more in Bacillus subtilis by using K. carrageenan as a matrix. Ramakrishna
and Prakasham (1999) also reported that K. carrageenan immobilized
Brevibacterium flavum attained high stability against several denaturing
chemicals.

The natural polymer such as agar, agarose, pectin and gelatin were also
employed for cell immobilization of protease production (Ramakrishna and
Prakasham, 1999). In the present study, the protease production was studied
by using gelatine as a carrier material and the protease production with this
carrier was also high compared with other matrixes such as agar-agar,
K-carrageenan and polyacrylamide. The suitable optimized concentration for
the protease production was 5% for both the tested bacterial species.
Concurrent to this study Ramakrishna and Prakasam (1999) reported that
gelatin as a carrier material for the production of B-galactosidase and
penicillin acylase by the immobilized E.coli cells.
Production of protease by immobilized cells in sodium alginate showed that the maximum amount of protease production was registered at 48 h of incubation and values recorded were; 550.13 ± 1.63 U/ml for *Pseudomonas* sp. and for *B. flexus* was 563.99 ± 1.63 U/ml. On further incubation, enzyme production gradually decreased. Similarly Samia *et al.* (2008) also reported that the high yield of alkaline protease production by *Bacillus licheniformis* cells immobilized with sodium alginate matrix and the optimized concentration of this matrix was 2%. Ramakrishna *et al.* (1992) reported that immobilization of *Bacillus cereus* in calcium alginate by using paused – bed reactors continuously synthesized thermostable amylase at a higher level. The decreased protease production at higher concentration may be due to decrease in gel porosity at higher gel concentration and limited diffusion (Ahmed *et al.*, 2008).

In repeated batch fermentation, the strength of the biocatalysts and their ability to produce protease under consecutive batch fermentation was studied. The results showed that the amount of protease production with immobilized cells gradually reduced during the end of first batch. However the repeated batch fermentation performed well with all beads done in two batches. Romo and Perezmartinez. (1997) have reported that the viability of microbial cells over a period of 18 months under entrapped conditions and it was considered as one of the potential applications. The overall results inferred
that the protease production with cells immobilized in alginate matrix was found to maximum and was closely followed by gelatin beads when compared with the other tested beads such as K-carrageenan, agar agar and polyacrylamide. However, all the beads were found to yield better protease yield than the free cells of *B. flexus* and *Pseudomonas* sp. (control).