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CELL IMMOBILIZATION OF *Bacillus flexus* FOR THE PRODUCTION OF MODERATELY EXTRACELLULAR PROTEASE

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Abstract

Immobilization methods provide a special microenvironment in which cells always have different behaviors compared with free cells. Protease production by *Bacillus flexus* with free and immobilized cells was examined in this study. Entrapment method of immobilization was used with K-carrageenan, gelatin, polyacrylamide, sodium alginate and agar matrices for protease production free cells. Sodium alginate immobilized cells showed remarkable operational stability and maximum levels of protease production. Repeated batch fermentation showed specific volumetric productivity of 563.99 ± 1.63 U/ml, 555.59 ± 1.63 U/ml, 341.28 ± 2.04 U/ml, 300.68 ± 1.63 U/ml, 327.26 ± 1.52 U/ml with sodium alginate, gelatin, K-carrageenan, polyacrylamide and agar-agar respectively. Apart from sodium alginate, Gelatin and K-Carrageenan immobilized bacterial cells agar agar showed the maximum average cell leakage. Thus, the alginate-immobilized cells of bacterial cells proved to be an effective biocatalyst for long-term usage and maximum enzyme production.

Key words: Sodium Alginate, Haloalkaliphilic Protease, K-Carrageenan, Polyacrylamide and Gelatin.

1. Introduction

Proteolytic enzymes catalyze the cleavage of peptide bonds in proteins (Godfrey, 1996). They conduct highly selective and specific modification of proteins i.e. zymogenic form of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, processing and transport of proteins across the membrane. These proteolytic enzymes catalyze various steps in tumor invasion or in infection cycle of a number of pathogenic microorganisms. Their involvement in the life cycle of disease causing organisms has led to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS (Rao *et al*., 1998). This makes proteases valuable target for new pharmaceuticals. Production of proteases was present in several microorganisms such as protozoa, bacteria, yeast and fungi. The inability of the plant and animal proteases to meet the current world demands (due to extensive use in food, pharmaceutical and detergent industry) has led to an increased interest in microbial proteases (Beg *et al*., 2012). Among the microbes, the bacterial strains of *Bacillus* are producers of the most important alkaline protease because of their ability to produce huge amount of protease having significant proteolytic activity and stability at high pH and temperature (Sen and Sathyanarayana, 1993).

Immobilization of bacterial cells using various matrices technology is often studied for its potential to develop fermentation processes and

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bioremediation (Beshay *et al*., 2002; Abd-El-Haleem *et al*., 2003). Whole cell immobilization by entrapment is a widely used and simple technique. They stated viability of microbial cells over a period of 18 months under entrapped conditions using various types of matrices and it was considered as one of the potential applications (Romo and Perezmartinez, 1997). The uses 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. The whole cells immobilization for the yield of extracellular proteolytic enzymes offer many advantages such as the facility to separate cell mass from the bulk liquid for probable reuse, facilitating continuous operation over a prolonged period and enhanced reactor productivity. With this background, the present study has a goal to immobilize the *Bacillus flexus* cells by entrapment method using different matrices and to compare the protease production with free cells.

2. Materials and Methods

Preparation of inoculum

The production of moderately halophilic protease producing bacterial isolate from estuarine fish gut was used for the preparation of inoculums. The bacterial cell was crumbed from the slant with sterile distilled water and the resulted cell suspension at 10 % level was transferred aseptically into 250 ml Erlenmeyer flasks containing 45 ml of sterile inoculum composed in the medium with beef extract - 0.3 %; peptone - 0.5 %; NaCl - 0.5 % and glucose - 0.5 % at pH 7 for 24 hrs. Then, 10 % of enriched culture was inoculated in 250 ml flask containing 45 ml basal medium containing (g/l) - $(NH_4)_2SO_4$ – 2 g; K_2HPO_4 – 1 g; KH_2PO_4 – 1 g; $MgSO_4.7H_2O$ – 0.4 g; $MnSO_4.H_2O$ – 0.01 g; $FeSO_4.7H_2O$ – 0.01 g; yeast extract – 1 g and peptone – 10 g at pH 7. The culture was then incubated for 2 days by shaking at 32 ^oC. Then, the bacterial cell was collected by centrifugation at 10,000 rpm for 15 min and the supernatant was used for further experimental analysis. This cell suspension was used as inoculum for immobilization as well as for free-cell fermentations.

Strain identification

Genomic DNA extraction, Cloning and sequencing of 16S rRNA gene

The isolated bacterial strain was grown in 2 ml Zobell Marine Broth (Hi-Media) overnight at 27 ^oC. The culture was spun at 7000 rpm for 3 min. The pellet was resuspended in 400 µl of sucrose TE. Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1 hr at 37 °C. To this tube, 100 µl of 0.5 M EDTA (pH 8.0), 60 µl of 10 % SDS and 3 µl of proteinase K from 20 mg/ml were added and incubated at 55 ^0C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile distilled water. The amplified product (1,500 bp) was purified using GFX ™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's instruction. The 16S rRNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone^{TM} PCR Product Cloning Kit #K1214, MBI Fermentas). Full length sequencing of the rRNA gene (about 1500 bp) for the isolated bacteria was carried out in Macrogen (Seoul, Korea). The full length sequences obtained were matched with previously published sequences available in NCBI using BLAST (Altschul *et al*., 1997).

Protease assay

The assay system 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. Approximate controls were also made. The mixture was incubated for 30 min at 30 ^0C. Then 3 ml of 5 % TCA was added to this mixture and placed at 4 ^0C 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 0.5 M 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.

Whole bacterial cell immobilization by entrapment in sodium alginate

The immobilization of bacterial cells with alginate matrix was performed according to the method (Johnsen and Flink, 1986). The sodium alginate solutions (3 %) were prepared by dissolving sodium alginate in 100 ml boiling water which was autoclaved at 121 ºC for 15 minutes. Both alginate slurry and cell suspension (equivalent to 0.03 g dry cell weight) were mixed and stirred for 10 minutes to get a uniform mixture. Then, the slurry were taken into a sterile syringe and added drop wise into 0.2 M $CaCl_2$ solution from 5 cm height and kept for curing at 4 ºC for 1 hr. The alginate entrapped beads were washed with sterile distilled water for 3 to 4 times. Then, the prepared beads were preserved in 0.9 % sodium chloride solution in the refrigerator for further use. All operations were carried out aseptically under laminar flow unit to avoid contamination.

Effect of whole cells immobilization on protease production by the candidate species

The effect of alginate matrix on immobilized bacterial cells for protease production by the candidate species was studied (Sigma, Mumbai, India). For this, various concentrations of sodium alginate (1.5, 2.0, 2.5, 3.0 and 3.5 %) were prepared for the production of moderately halophilic protease. The preparation of alginate entrapment of cells was performed according to the method (Johnsen and Flink, 1986). Cells were collected during the mid-logarithmic growth phase by centrifugation (5000 g, 10 min), resuspended in 2 ml of saline and suspended to 100 ml of sterilized alginate solution. This alginate with bacterial cell mixture was added drop by drop into a cold sterile 0.2 M $CaCl_2$ solution through a sterile 1 ml pipette. A gel bead of approximately 2 mm diameter was obtained. The sodium alginate gel bead was hardened by resuspention into a fresh $CaCl_2$ solution for 24 hrs at 4 ^{0}C with mild stirring. At last these beads were cleaned with distilled water to eliminate surplus calcium ions and unentrapped cells. Finally 100 beads were transmitted to 50 ml production medium and repeated batch cultivations were completed. This process was carried out by decanting the spent medium and replacing it by fresh medium after washing the alginate beads with sterile saline.

Immobilization of whole cells in k- carrageenan

The effect of k-carrageenan matrix on immobilized bacterial cells for protease production by the candidate species was studied. The different concentrations of k-carrageenan were prepared and suspended to 18 ml of 0.9 % sodium chloride. Then, the k- carrageenan was dissolved by mild heating and sterilized by autoclave. The bacterial inoculum (2 ml equivalent to 0.03 g DCW) was added to the molten k- carrageenan solution maintained at 40 °C. Then, the mixture was uniformly 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 mm^3) and suspended into sterile 2 % potassium chloride solution and kept in the refrigerator for 1 hr for curing. Finally, cubes were washed 3 to 4 times with sterile distilled water.

Immobilization of whole cells in agar-agar

Different concentrations of agar agar was added in 18 ml of 0.9 % sodium chloride solution to get final concentration of 2 % and sterilized by autoclaving. The bacterial inoculums (2 ml equivalent to 0.03 g DCW) were added into the molten agar-agar incubated at 40 °C. Then, the mixture was stirred well for few seconds (without forming foam) and added into sterilized petriplates and allowed to solidify. The solidified agar block was cut into equal size pieces (4 mm^3), added to sterile 0.1 M phosphate buffer (pH 7.0) and kept in the refrigerator (1 hr) for curing. After curing, phosphate buffer was decanted and the pieces were washed with sterile distilled water for 3 to 4 times.

Immobilization of whole cells in polyacrylamide

The effect of polyacrylamde matrix (Sigma, Mumbai, India) on immobilized bacterial cells for protease production by the candidate species was studied. The bacterial inoculum was prepared by adding 0.03 g cells to 10 ml chilled sterile distilled water. To another 10 ml of 0.2 M sterile phosphate buffer (pH 7.0), the following chemicals were added: 2.85g acrylamide, 0.15g bisacrylamide, 10 mg ammonium persulphate, and 1 ml TEMED (NNN1N1 tetra methyl ethylene diamine). The bacterial cell inoculum and the above phosphate buffer mixture was mixed well and added into sterile flat bottom 10 cm-diameter Petriplates. A

After solidification, the acrylamide gel was cut into equal size (4 mm^3) and it immediately transferred to 0.2 M phosphate buffer (pH 7.0) and kept in the refrigerator for 1 hr for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile distilled water at 4 °C until use.

Immobilization of whole cells in gelatin

To study the effect of gelatin matrix on immobilized bacterial cells for protease production by the candidate species was studied. Five milliliters (0.06 % DCW) of bacterial inoculum was poured into 15 ml of 20 % sterile gelatin, maintained at 45 °C and added into a sterile petridish. The gel mixture was over layered with 10 ml of 5 % glutaraldehyde for hardening at 30 °C. The resulting block was cut into small-size cubes (4 mm^3) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde. Glutaraldehyde was used as a cross linking agent.

Production of protease by batch process with immobilized cells

The immobilized beads or blocks (cells equivalent to 0.03 g DCW) were transmitted 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 % ($MgSO_4.7H_2O$ - 5 g/l; KH_2PO_4 - 5 g/l and $FeSO_4.7H_2O$ - 0.1 g/l) with a pH of 9.0. The flasks were maintained at 37 °C for 48 hours. Samples were withdrawn at regular intervals of 6 hrs and assayed for protease production. Production of protease by repeated batch process is one of the advantages of using immobilized biocatalysts and can be used repeatedly and continuously. Therefore, the reusability of bacterial strains immobilized in matrix was examined. After attaining the maximum production of alkaline protease (24 hrs), 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. The enzyme titers and cell leakage of each cycle was studied.

3. Results

Identification of protease positive colony

With the morphological and biochemical tests followed by 16S rRNA analysis, the strain was identified as *Bacillus flexus* that has been found to produce moderately halophilic protease after 24 hrs incubation (Fig - 1). Extracellular moderately halophilic protease production by *Bacillus flexus* cells entrapped in different matrices started from 24 to 72 hrs of incubation under shaking conditions and it reached maximum level after 48 hrs of incubation. On further incubation, enzyme production was found to be gradually decreased. However, enzyme production by free cells of *Bacillus flexus* was found to be maximized only after 24 hrs of incubation.

Effect of whole cell immobilization on various matrices

Agar-agar

The results showed that the protease production started from 24 hrs and it reached maximum at 48 hrs. The production of moderately halophilic protease with the immobilized cells in agar-agar was less than that of immobilized cells with other matrix like sodium alginate, gelatin and k-carrageenan matrix. The highest protease production was obtained at 48 hrs of incubation after which there was no considerable increase. The maximum protease production recorded was

327.26 ± 1.52 U/ml in *Bacillus flexus* and the optimized concentrations for the protease production was 7 % (Fig. 2).

Sodium alginate

The production of protease from bacterial cell entrapped in sodium alginate matrix was increased gradually up to 48 hrs of incubation after which there was reduction. The protease production reached maximum level (563.99 ± 1.63 U/ml) at 48 hrs in 2.5 % sodium alginate immobilized cells. On further incubation, protease production decreased (Fig - 3).

K- carrageenan

The production of protease from bacterial cell entrapped in k- carrageenan gel matrix showed that the maximum protease production attained after 48 hrs of incubation beyond which there was no positive change. The maximum protease production registered in *Bacillus flexus* was 341.28 ± 2.04 U/ml and comparatively lesser than that of sodium alginate matrix and also it was found to be high amount of enzyme production compared to free cells. The optimized concentration for the protease production was 2.5 % (Fig - 4).

Polyacrylamide

The production of protease from bacterial cell entrapped in polyacrylamide gel matrix gradually increased in protease production after from 24 hrs. The maximum protease production was obtained in 48 hrs of incubation after which there was no sizeable change and the highest enzyme production recorded in *Bacillus flexus* recorded was 348.14 ± 2.45 U/ml. The optimized concentration for the protease production was 12 % (Fig - 5).

Gelatin beads

The production of protease from bacterial cell entrapped in gelatin beads A obvious protease yield was started at 24 hours and reached maximum level (555.59 ± 1.63 U/ml) in *Bacillus flexus* at 48 hours of incubations (Fig - 6). The production of protease was recorded with this carrier was very low compared with the protease production from the immobilized cells of the sodium alginate matrix (Fig - 3).

Comparison of protease production by immobilized cells in various matrixes by entrapment technique

Production of protease with the immobilized cells was found to be maximum at alginate matrix and it was followed by gelatin beads, whereas least amount of protease production was registered in agar - agar immobilized cells.

Repeated batch fermentation with free cells and immobilized cells

The semi-continuous fermentation was finished to study the permanence of the gel beads and their capability to yield protease under repeated batch cultivation. The results indicated that the production of protease with immobilized cells gradually decreased from the end of first batch. Thus the repeated batch fermentation performed well with all beads ran in two batches.

Agar-agar

The production of protease was progressively increased from 24 hrs and reached the optimum yield at 48 hrs of incubation after which there was no significant production in second batch. The production of protease with the immobilized cells in agar agar was less than that of immobilized cells with other matrix like sodium alginate, gelatin, polyacrylamide and K-carrageenan matrix. The highest enzyme production of *Bacillus flexus* was recorded as 212.46 ± 2.04 U/ml. The optimized concentration for the protease production was 7 % (Fig - 2).

Sodium alginate

The candidate bacterial cells entrapped in sodium alginate matrix indicated that the protease production was gradually increased from 24 hrs to 48 hrs of incubation after which there was no large yield (Fig - 3). The maximal level protease yield in *Bacillus flexus* was recorded (366.99 ± 1.66 U/ml) at 48 hrs in second batch.

k- carrageenan

The results revealed that the effect of bacterial cell mass entrapped in k - carrageenan gel matrix on protease production was recorded in 48 hours of incubation in second batch (Fig - 4). Afterwards there was no noteworthy development in protease yield at 72 hrs of incubation. The optimum enzyme production registered was 251.76 ± 1.22 U/ml. The optimized concentration for the protease production was 2.5 %.

Polyacrylamide

The results indicated that the bacterial cell mass entrapped in polyacrylamide gel matrix on protease yield for the period of second batch (Fig - 4). The protease production was started from 24 hrs and reached the maximum level at 48 hrs of incubation in the second batch. The maximum protease production recorded was 240.63 ± 2.04 U/ml. The optimized concentration for the protease production was 12 %.

Gelatin beads

There was protease production in successive period which indicated the maximum level was of 342.57 ± 1.22 U/ml in *Bacillus flexus* at 48 hrs of incubation. The protease production obtained with this carrier was very low when compared to the protease yield from the immobilized cells of the sodium alginate matrix. But, the protease production was very high when compared with the production of protease from free cells and the immobilized cells of carrageenan, agar - agar and polyacrylamide (Fig - 5).

4. Discussion

The immobilization method is one of the important techniques used for better productivity by protecting the cells from shear forces. In addition to this the product and cell separation is easy so that the cells can be repeatedly used for several times for batch fermentation. Among the immobilization of microbial cells, entrapments are the most suitable and general practice for enzyme production. Immobilization of bacterial cells using various matrices was known to be a simple technique and maintain the cells from adverse conditions i.e pH, temperature, etc., found in the surrounding media (Adinarayana *et al*., 2005; Sankaralingam *et al*., 2012).

The result of immobilization of candidate bacterial cell using agar-agar demonstrated that the beads gave 4 to 5 folds higher production of protease than the free cells. The optimized concentration for this matrix was 7%. They reported that the use of agar-agar entrapped cells of *Pseudomonas* sp ATCC 21783 for moderately halophilic protease production in semi-continues batch fermentation after 48 hours of incubation.

The viability of gel beads and enzyme production by *Halobacterium* sp. JS cells immobilized in polyacrylamide gel matrices was found to be greatly increased for 5 repeated cycles (15 days) (Kumar and Vats, 2010). In the present study, the optimum protease production was obtained at 48 hrs incubation in *Bacillus flexus* and the optimized suitable concentration for this matrix was 12 %. The report was consistent with the positive influence of these matrices on fermentative production of protease by *Pseudomonas* sp. The maximum production of protease by *Bacillus mycoidus* on polyacrylamide matrix at 2 % acrylamide concentration (Abdel-Naby *et al*., 1998). Low levels of alkaline protease production observed in cells with polyacrylamide carrier are due to the fact that polyacrylamide monomer was toxic to the cells (Adinarayana *et al*., 2005).

The *Brevibacterium flavum* cells immobilized with k-carrageenan attained high stability against several denaturing chemicals (Ramakrishna and Prakasham, 1999). In the present study, the effect of k- carrageenan on enzyme production revealed that 2.5 % was the suitable concentration for protease production and the production of protease was 339.15 ± 1.93 U/ml in *Bacillus flexus* (Deo *et al*., 1985). They have reported that the production of tyrosin and penicillin from *Streptomyces fradiae* and *Pencillium chrysogenum* respectively using k-carageenan as an entrapment matrix.

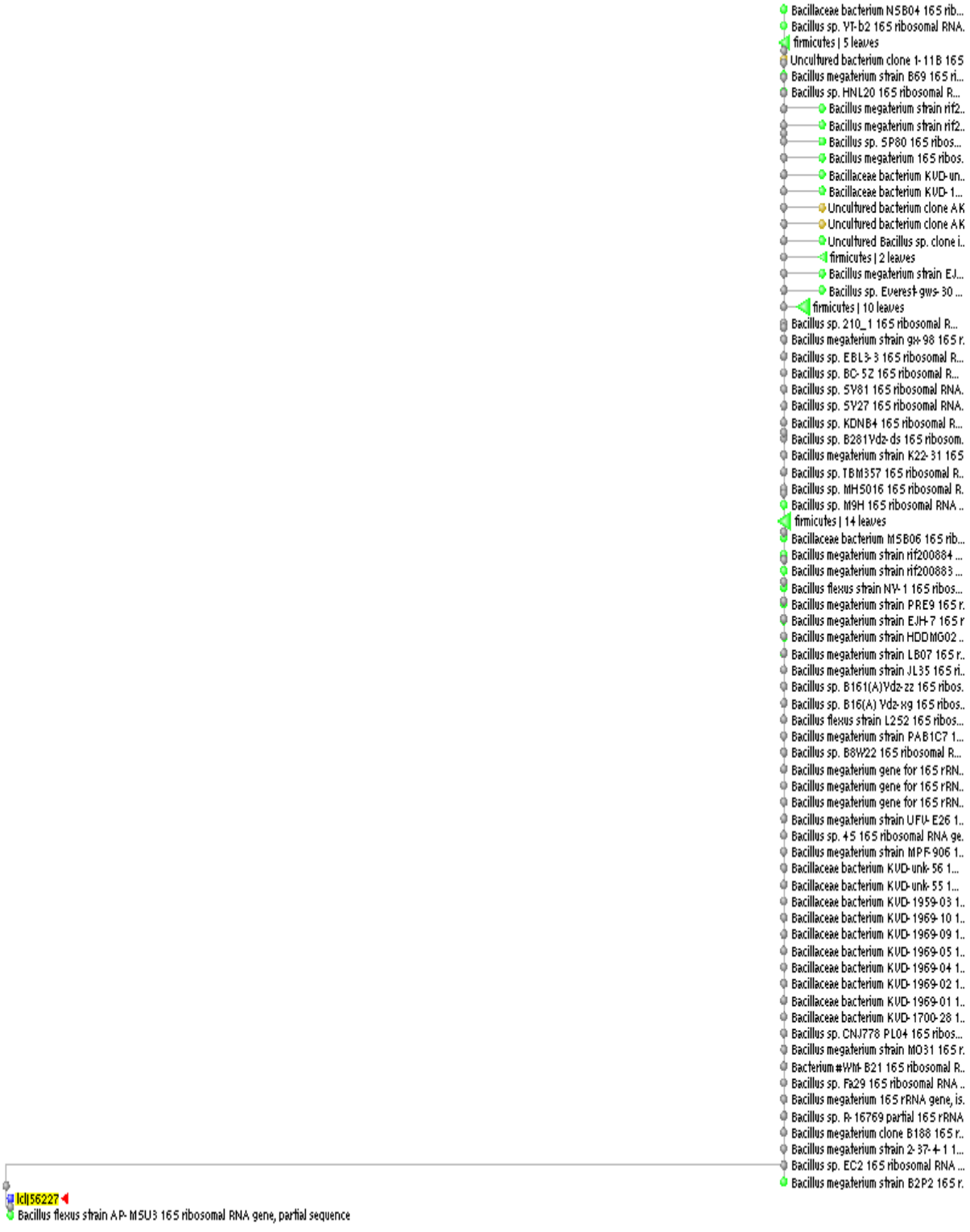

Figure - 1: Rectangular view of phylogenetic tree

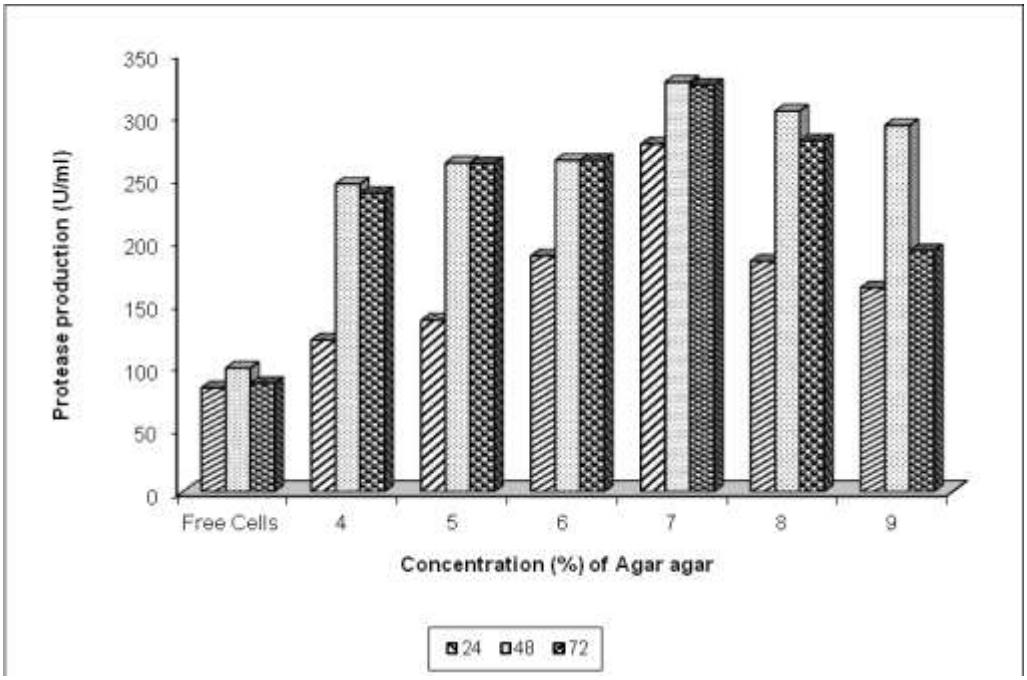

Figure – 2 (a): Immobilization of protease producing *Bacillus flexus* using agar-agar (Batch – I)

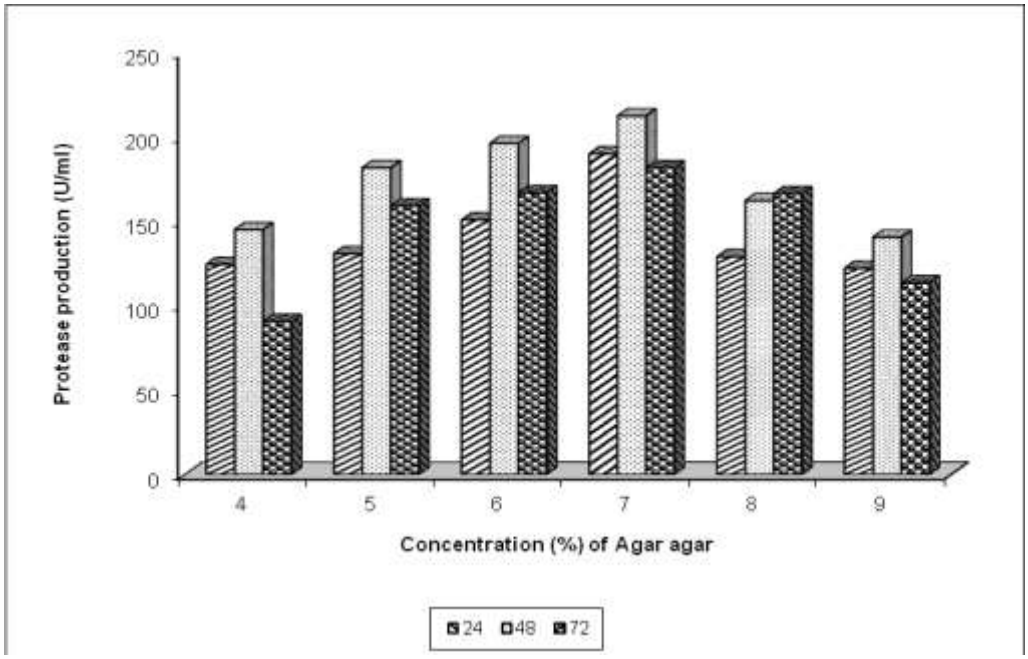

Figure - 2(b): Immobilization of protease producing *Bacillus flexus* using agar – agar (Batch – II)

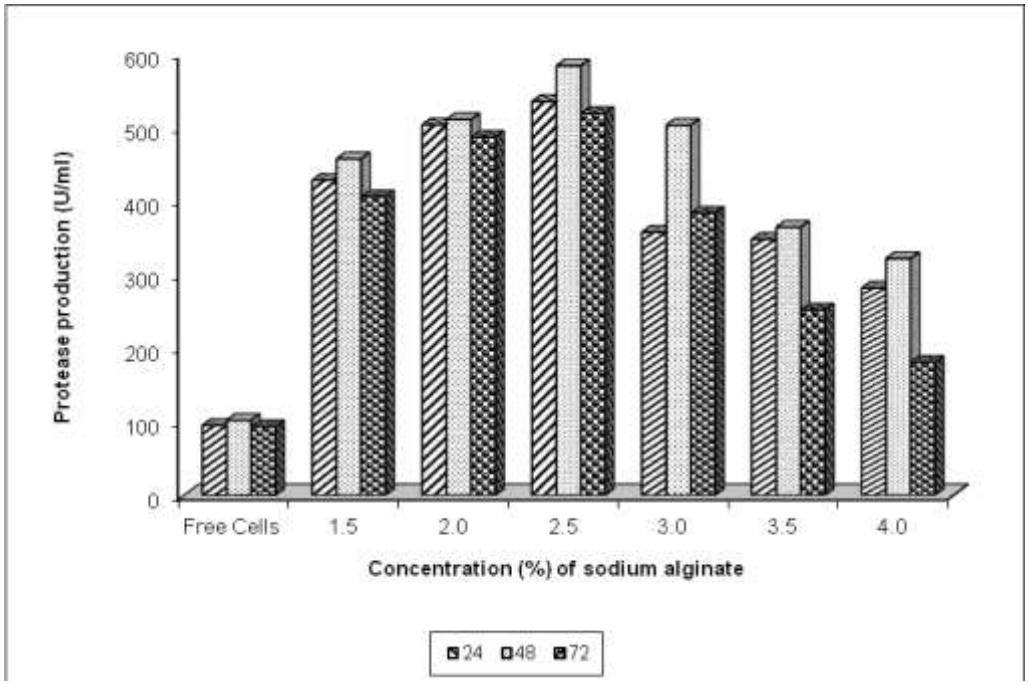

Figure - 3(a): Immobilization of protease producing *Bacillus flexus* using sodium alginate (Batch – I)

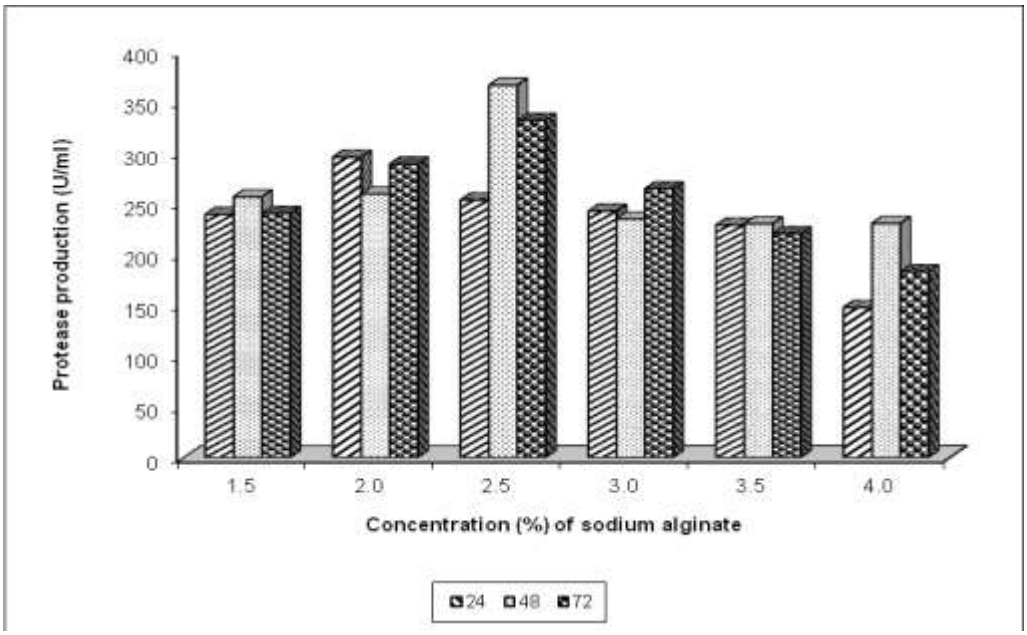

Figure - 3(b): Immobilization of protease producing *Bacillus flexus* using sodium alginate (Batch – II)

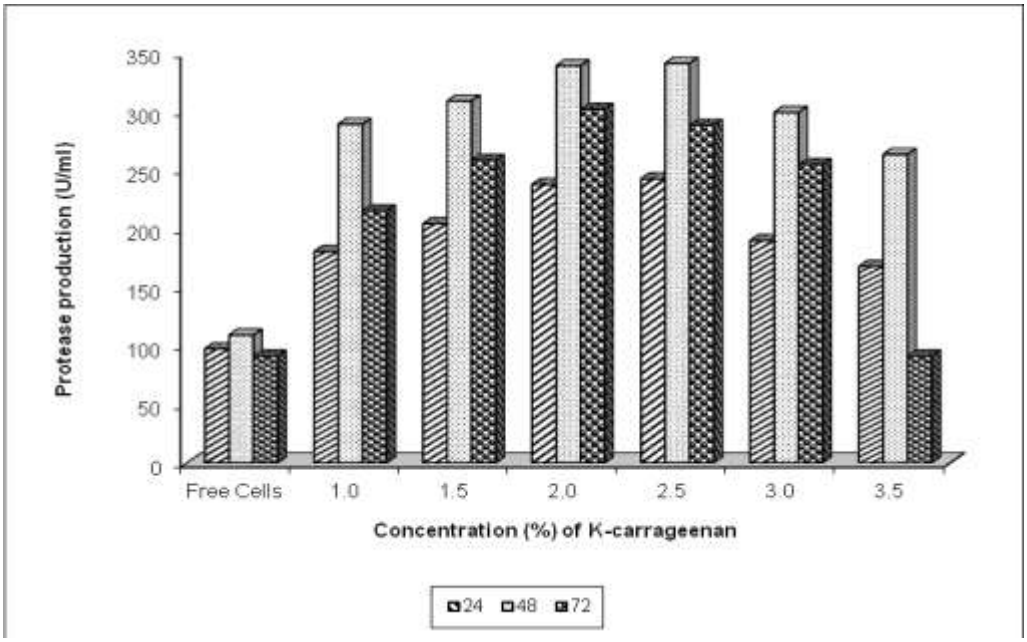

Figure – 4 (a): Immobilization of protease producing *Bacillus flexus* using K-carrageenan (Batch – I)

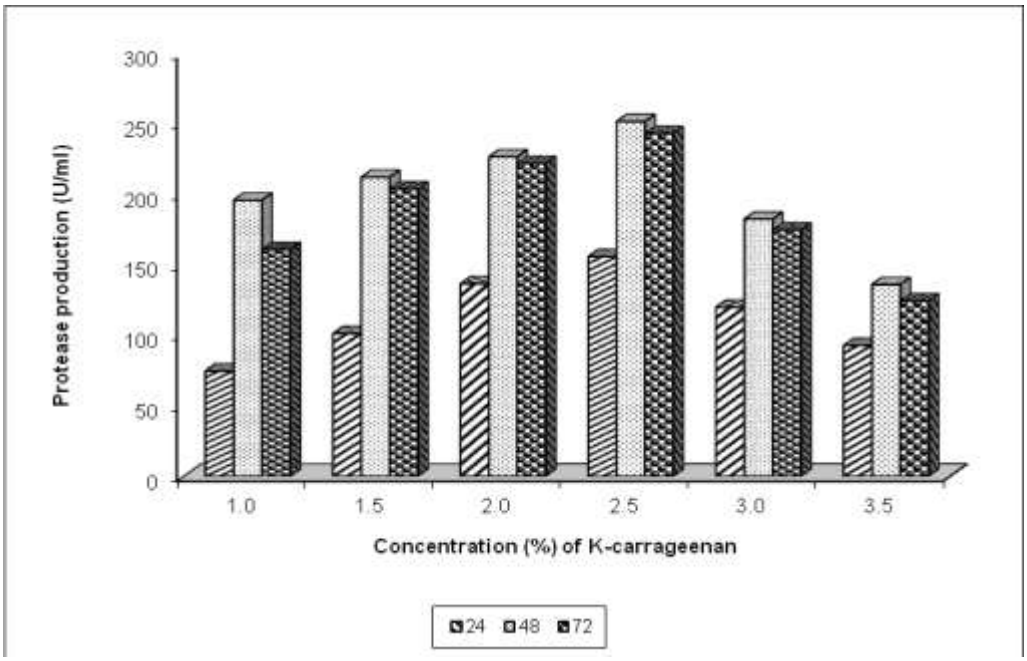

Figure - 4(b): Immobilization of protease producing *Bacillus flexus* using K-carrageenan (Batch – II)

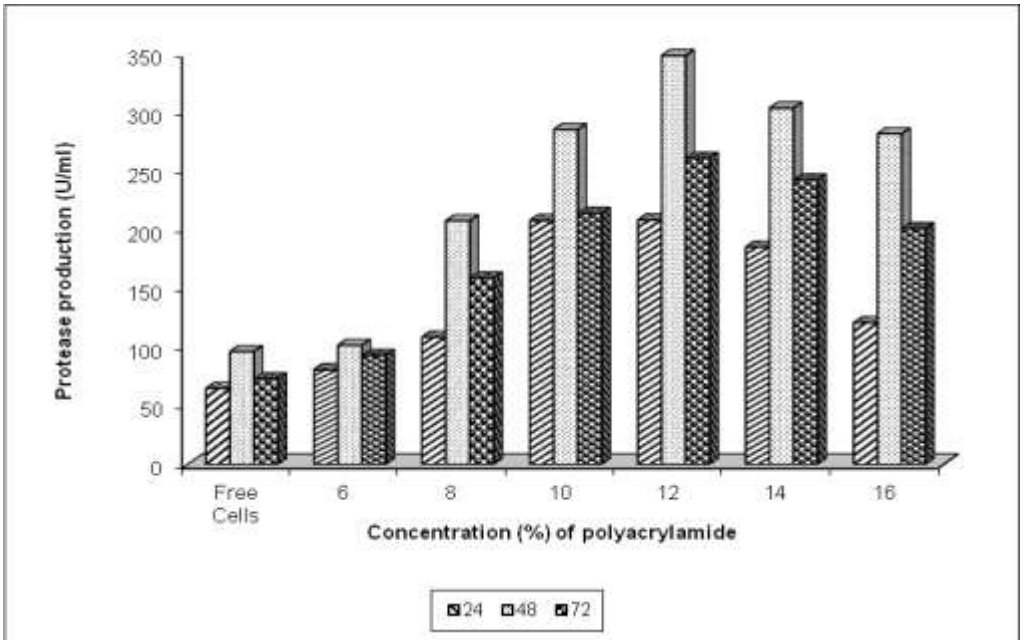

Figure - 5 (a): Immobilization of protease producing *Bacillus flexus* using polyacrylamide (Batch – I)

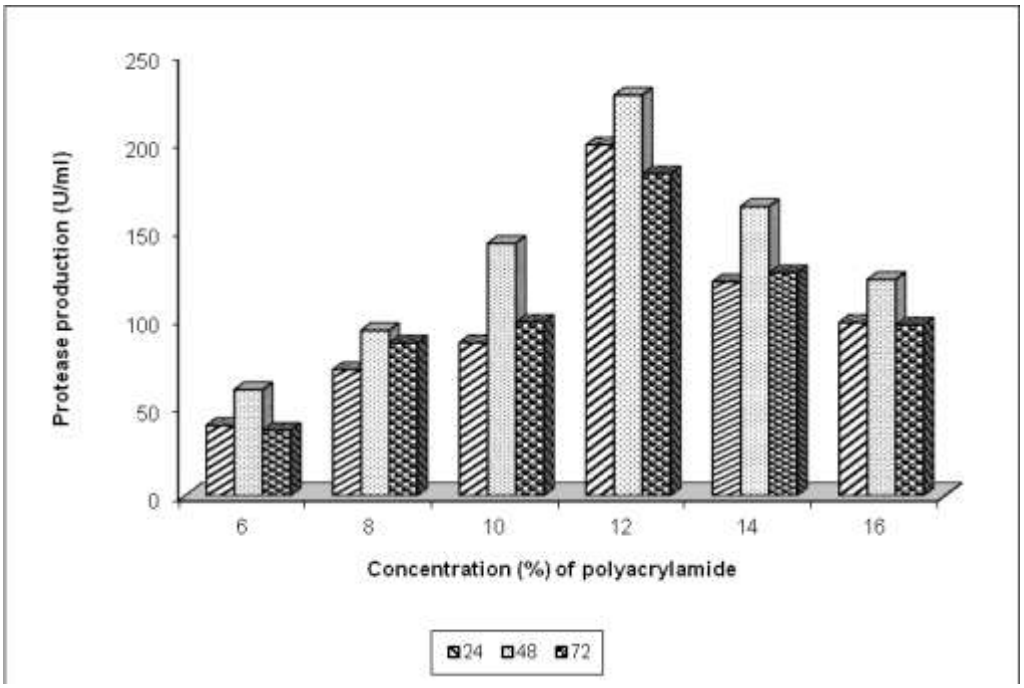

Figure – 5 (b): Immobilization of protease producing *Bacillus flexus* using polyacrylamide (Batch – II)

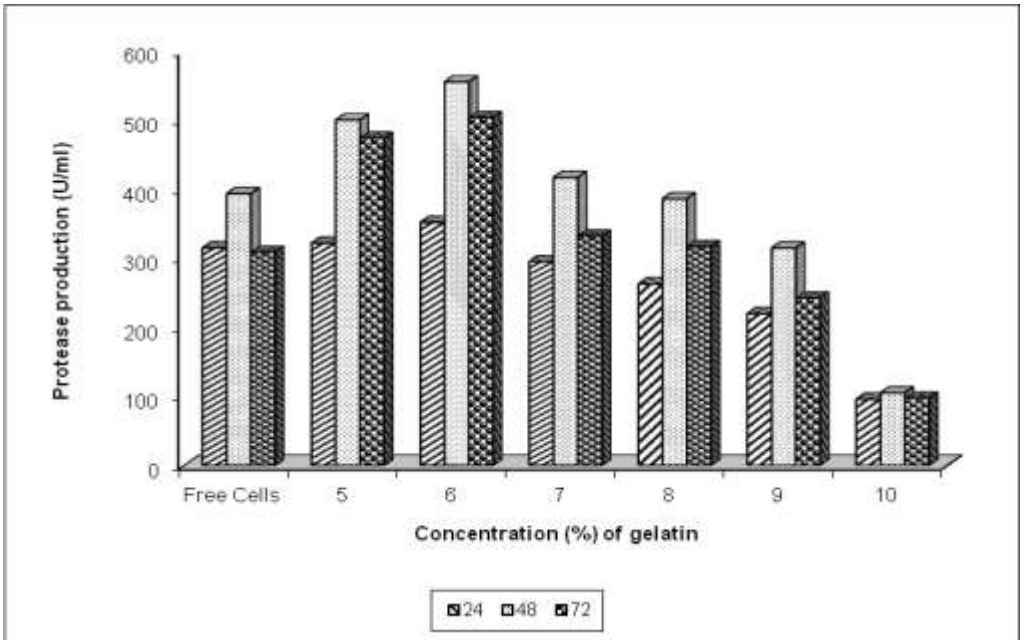

Figure – 6 (a): Immobilization of protease producing *Bacillus flexus* using gelatin (Batch – I)

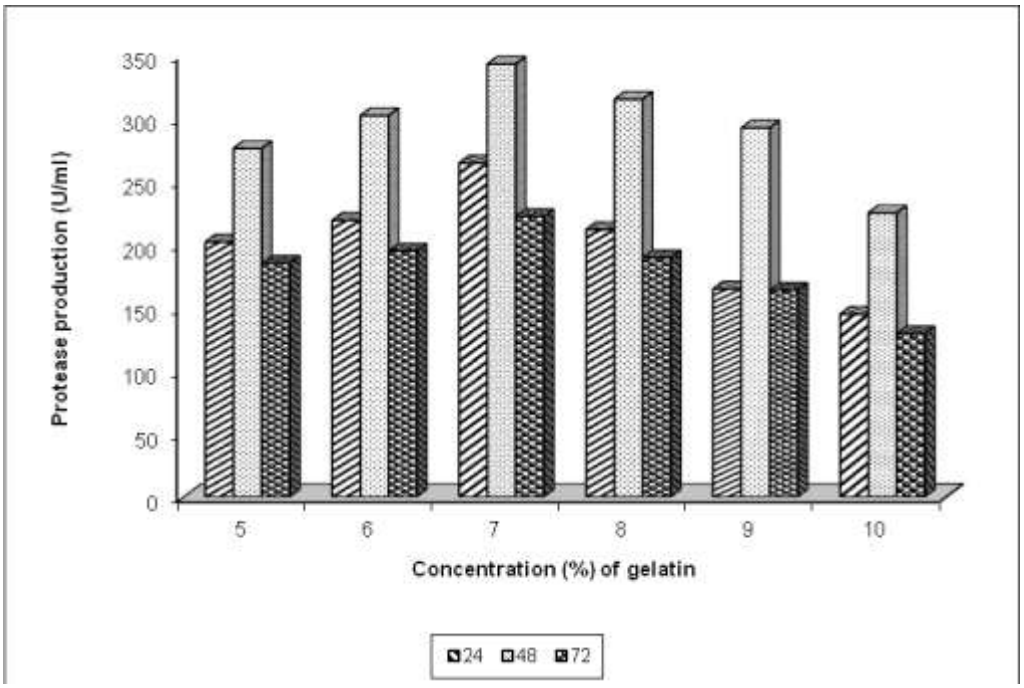

Figure – 6 (b): Immobilization of protease producing *Bacillus flexus* using gelatin (Batch – II)

The production of ß-galactosidase and penicillin acylase by *E. coli* by using gelatin as a carrier material (Ramakrishna and Prakasham, 1999). For this study, the production of protease was determined by using gelatin as a carrier material. The suitable optimizing concentration for the protease production was 5% by the candidate species. In our study while gelatin was used as the carrier, concurrent to this report, using gelatin as immobilization matrix for the maximum production of protease from *Bacillus subtilis* and for ß- amylase was already reported by Deo *et al*. (1985).

In this study, the enzyme production was studied from 24 to 72 hrs with cells immobilized with sodium alginate matrix. The maximum level in *Bacillus flexus* (550.13 ± 1.63 U/ml) reached at 48 hrs (Adinarayana *et al*., 2005). They have reported that the protease production with immobilized cells of *Bacillus subtilis* PE-11 by using alginate matrixes. The immobilization of *Pseudomonas* sp. produces maximum protease by sodium alginate after 48 hrs of incubation. The suitable optimal concentration for the protease productions was 2.5 %.

5. Conclusion

Immobilization techniques provide a special microenvironment in which cells always have different behaviors compared to free cells. Whole cell immobilization technique is generally used for higher productivity by protecting the cells from shear forces, in addition to this the product and cell separation is easy so that the cells can be reused several times. The highest enzyme production was recorded in cells immobilized with sodium alginate matrix and it was followed by gelatin, agar - agar, k-carrageenan and polyacrylamide. Repeated batch fermentation with free cells and immobilized cells revealed that there is a stability of the biocatalysts and their ability of protease production. The present study shows that the amount of protease yield with immobilized cells gradually decreased from the end of first batch due to cell leakage. Thus the repeated batch fermentation performed well with all beads ran in two batches. Protease production with the cells immobilized in sodium alginate matrix was recorded the highest and it was followed by other matrices.

6. References

1) Abdel - Naby M. A., A. M. S. Ismail., S. A. Ahamed and A. F. Fattah. 1998. Production and Immobilization of alkaline protease from *Bacillus mycoides*. *Bioresource Technology*, 64: 205 - 210.
2) Abd-El-Haleem D., U. Beshay, A. Abdel hamid, H. Moaward and S. Zaki. 2003. Effects of nitrogen sources on biodegradation of phenol by immobilized *Acinetobacter* sp. strain w-17. *African Journal of Biotechnology*, 2: 8 - 12.
3) Adinarayana K., B. Jyothi and P. Ellaiah. 2005. Production of Alkaline Protease with Immobilized Cells of *Bacillus subtilis. AAPS Pharmaceutical Science and Technology*, 6 (3): 48.
4) Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, W. Zhang, W. Miller and D. J. Lipman. 1997. Gapped BLAST and PSI - BLAST: A new generation of protein database search programs. *Nucleic Acids Research,* 25: 3389 – 3402.
5) Beg Q. K., R. Gupta and P. Lorenz. 2002. Bacterial Alkaline Proteases. Molecular Approaches and Industrial Applications. *Applied Microbiology and Biotechnology,* 59: 15 - 32.
6) Beshay U., D. Abd-El-Haleem, H. Moaward and S. Zaki. 2002. Phenol biodegradation by free and immobilized *Acinetobacter. Biotechnology Letter*, 24: 1295 - 1297.
7) Deo S. K., J. C. Lewis, S. Dauhert. 1985. Bioluminescence detection of proteolytic bond of site specific proteases. *Proceedings in Natural Academy of Science*, 95: 2828 - 2833.
8) Godfrey, T. 1996. Textiles. In: Godfrey T., West S., editors Industrial enzymology. 2nd ed. London: Macmillan Press, pp. 360.
9) Johnsen, A. and J. M. Flink. 1986. Influence of alginate properties and gel reinforcement on fermentation characteristics of immobilized yeast cells. *Enzymology and Microbial Technology*, 8: 737 - 748.

10) Kumar R. and R. Vats. 2010. Protease production by *Bacillus subtilis* immobilized on different matrices. New York. *Science Journal*, 3: 20 - 24.
11) Longo, M. A., I. S. Novella., L. A. Gracia and M. Diaz. 1992. Diffusion of proteases in calcium alginate beads. *Enzymology and Microbial Technology*, 14: 586 - 590.
12) Ramakrishna, S. V and R. S. Prakasham. 1999. Microbial fermentation with immobilized cells. *Current Science*, 77: 87 - 100.
13) Rao, M. B., Aparna, M., Tanksale, M., Ghatge S. and Deshpande, V. V. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, 62: 597 - 635.
14) Romo, S. and C. Perezmartinez. 1997. The use of immobilization in alginate beads for long-term storage of *Pseudoanabaena galeata* (Cyanobacteria) in the laboratory. *Journal of Phycology*, 33: 1073 - 1076.
15) Sankaralingam. S., T. Shankar., K. Sendeshkannan, R. Ramasubburayan and S. Prakash. 2012. *European Journal of Applied Sciences*, 4 (4): 146 – 156. 2012.
16) Sen, S and T. Satyanarayana. 1993. Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40., *Indian Journal of Microbiology*, 33: 43 - 47.

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