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Production of Protease from *Pseudomonas* Sp. by Immobilization Approach on Different Matrices

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Abstract: The present investigation evaluated the suitability of different matrices such as sodium alginate, polyacrylamide, agar-agar, K-carrageenan and gelatin for production of alkaline protease from *Pseudomonas* sp. using immobilization approach. Sodium alginate was found to be an effective and suitable matrix for moderately halophilic protease productivity compared to other matrices studied. All the matrices were selected for repeated batch fermentation. The experiment on the effect of immobilization using agar-agar showed that the beads gave 4 to 5 folds enhanced production over the free cells. The optimized concentration for this matrix was 7% (272. 75 + 1.22 U/ml). In this study, the maximum protease production in polyacrylamide was observed at 48 hours in *Pseudomonas* sp. (348.14 ± 2.45 U/ml) and the optimized suitable concentration for this matrix was 12%. The effect of K-carrageenan on protease production revealed that the concentration of 2.5% was suitable and the production of protease was 339.15 ± 1.93 U/ml in *Pseudomonas* sp. Here, the protease production was also studied by using gelatin as a carrier material and the protease production with this carrier was very high compared with other matrixes such as agar-agar, K-carrageenan and polyacrylamide. The suitable optimized concentration for the protease production was 5%the tested bacterial species. Production of protease by immobilized cells in sodium alginate showed that the amount of cell mass entrapped in this matrix increased gradually up to 48 hours of incubation and reached maximum level in *Pseudomonas* sp. (550.13 ± 1.63 U/ml). On further incubation, the enzyme production gradually decreased.

Key words: Protease · *Pseudomonas* Sp. AP.MSU2 · Immobilization · Matrices 16S rRNA Sequence

INTRODUCTION

Cell immobilization technology is often studied for its potential to improve fermentation processes and bioremediation [1, 2]. Whole cell immobilization by entrapment is a widely used and simple technique. Romo and Perezmartinez [3] reported 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 use of immobilized cells in the production of metabolites by culture of microorganisms is one of the most interesting techniques proposed during decades for improvement of fermentation process [4]. 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 offers 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 [5]. The previous efforts for the multiple binding of enzymes have been conducted mostly with organic synthetic polymers because of the ease of fabrication of desirable structures and the availability of reactive functional groups. One of the most suitable methods for cell immobilization is entrapment in sodium alginate, because this technique is simple and cheap. Sodium alginate is readily available and it is a non-toxic biological material. Therefore, it is suitable as an immobilization matrix for bio-molecules and microorganisms [6]. Sadjadi *et al*. [7] reported that beads of calcium alginate are prepared under mild conditions and have been extensively used for

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microencapsulating and entrapping cells. Hence the present work was undertaken to investigate the protease production by the immobilized fish gut bacterial isolate *Pseudomonas* sp.

MATERIALS AND METHODS

Preparation of the Inoculum: The protease producing bacterial strain was isolated from the fish gut and it was maintained on nutrient agar slant at 4°C. Five ml of distilled water was added to a 24 hours old slant of the protease producing bacterial strain. The cells were scrapped from the slant with sterile distilled water and the resulted cell suspension at 10% level was transferred aseptically into 250ml Erlenmeyer flasks containing 45ml sterile inoculum's medium of beef extract - 0.3%; peptone - 0.5%; NaCl - 0.5% and glucose - 0.5% at pH 7.5 for 24 h and then 10% of enriched culture was inoculated in 250 ml flask containing 45 ml basal medium containing (g/l) - $(NH_4)_2SO_4$ - 2g; K_2HPO_4 - 1g; KH_2PO_4 - 1g; $MgSO_4.7H_2O$ - 0.4g; $MnSO_4.H_2O$ - 0.01g; $FeSO_4.7H_2O$ - 0.01g; yeast extract - 1g; and peptone - 10g at pH 7. The culture was then incubated for 2 days by shaking at 32°C. The cells were then harvested by centrifugation at 10000 rpm for 15 min and the supernatant was used for further protease assay [8]. This cell suspension was used as inoculums 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 2ml Zobell Marine Broth (HiMedia cat#) at 27°C for 24hrs. 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 1h at 37°C. To this tube, 100 µl of 0.5M 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°C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated [9]. 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 rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ 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 [10].

Protease Assay: The assay system consisted of the 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°C. Then 3 ml of 5% TCA was added to this mixture and placed at 4°C for 10 min to form the 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 [11].

Whole Cell Immobilization by Entrapment: The alginate entrapment of cells was performed according to the method of Johnsen and Flink [12]. Sodium alginate solution (3%) was prepared by dissolving sodium alginate in 100ml boiling water which was autoclaved at 121°C for 15 minutes. Both alginate slurry and cell suspension (equivalent to 0.03g dry cell weight) were mixed and stirred for 10 minutes to get a uniform mixture. The slurry was taken into a sterile syringe and added drop wise into 0.2M $CaCl_2$ solution from 5cm height and kept for curing at 4°C for 1hour. The cured beads were washed with sterile distilled water for 3 to 4 times. When the beads were not being used, they were preserved in 0.9% sodium chloride solution in the refrigerator. All operations were carried out aseptically under laminar flow unit.

Effect of Whole Cells Immobilization on Protease Production by the Candidate Species: To study the effect of whole cells 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 studied. The alginate entrapment of cells was performed according to the method described by Johnson and Flink [12]. 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 M $CaCl_2$ solution through a sterile ml pipette. Gel beads of approximately 2 mm diameter were obtained. The beads were hardened by resuspention into a fresh $CaCl_2$ solution for 24 h at 4°C with gentle agitation. Finally these beads were washed with distilled water to remove excess calcium ions and un-entrapped cells. Then the approximately 100 beads were transferred to 50 ml production medium and repeated batch cultivations were done. 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: To study the effect of whole cells immobilization in k-carrageenan (Sigma, Mumbai, India), different concentrations of Carrageenan were weighed and added to 18 ml of 0.9% sodium chloride. It was dissolved by gentle heating and sterilized by autoclave. The cell suspension (2 ml equivalent to 0.03 g DCW) was added to the molten k-carrageenan solution maintained at 40°C, mixed well and poured into sterile flat bottom 4-inch diameter Petri plates. After solidification, the k-carrageenan blocks were cut into equal size cubes (4 mm^3) 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.

Immobilization of Whole Cells in Agar-Agar: The various concentrations of agar–agar (Hi-media, Mumbai, India) were dissolved in 18 ml of 0.9% sodium chloride solution to get final concentration of 2% and sterilized by autoclaving. The cell suspension (2 ml equivalent to 0.03 g DCW) was added to the molten agar-agar maintained at 40°C. Then it was shaken well for few seconds (without forming foam) and poured into sterilized Petri plates 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 hour) for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water for 3 to 4 times.

Immobilization of Whole Cells in Polyacrylamide: A cell suspension 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 (Fluka, Buchs, Switzerland), 0.15g bisacrylamide (Fluka), 10 mg ammonium persulphate and 1 ml TEMED (NNN1N1 tetra methyl ethylene diamine). The cell suspension and the above phosphate buffer mixture was mixed well and poured into sterile flat bottom 10 cm-diameter Petri plates. After polymerization (solidification), the acrylamide gel was cut into equal size cubes (4 mm^3), 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 sterile distilled water at 4°C until use.

Immobilization of Whole Cells in Gelatin: Five milliliters (0.06% DCW) of cell suspension was added to 15 ml of 20% sterile gelatin (Hi-media), maintained at 45°C and poured into a sterile Petri dish. 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 mm^3) and the cubes were washed thoroughly with sterile distilled water for complete removal of excess glutaraldehyde.

Production of Protease by Batch Process with Immobilized Cells: The 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% ($MgSO_4.7H_2O$ - 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 incubated at 37°C for 48 hours. Samples were withdrawn at regular intervals of 6 hours and assayed for protease activity [13]. Production of protease by repeated batch process is one of the advantages of using immobilized biocatalysts that they can be used repeatedly and continuously [13]. Therefore, the reusability of bacterial strains immobilized in matrix was examined. After attaining the maximum production of alkaline protease (24 hours), 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 were determined.

RESULTS

Identification of Protease Positive Colony: Based on the morphological, physiological and biochemical characteristics the protease producing isolate was identified as *Pseudomonas* sp. AP.MSU2 by the following standard keys of Bergey's Manual of Determinative Bacteriology (Table 1,) and the isolated bacterial strain was screened for protease producing ability on skim milk agar. The zone formation around the bacterial colony

Table 1: Biochemical characteristics of the *Pseudomonas* sp.

Biochemical tests	*Pseudomonas* sp.
Gram's staining	Gram negative (-ve)
Endospore staining	Rod
Motility	+
Carbohydrate fermentation test	
a. D-glucose	+
b. Mannitol	+
c. Lactose	+
d. Sucrose	+
Indole production	-
Methyl red test	-
Voges-Proskauer test	+
Citrate utilization test	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Urease test	-
Catalase test	+
Oxidase	+
Nitrate utilization test	-

+: Positive Results; -: Negative Results

indicated that the strain was protease positive. Hence the strain was identified as a protease producer and it was taken for further experimental studies. Phylogenetic studies revealed that the 16S rRNA of the strain *Pseudomonas* sp. AP-MSU2 has 97% similarity with the nearest match in the (Accession Number - FJ752235) Genebank.

Effect of Whole Cell Immobilization

Production of Protease by Immobilized Cells Using Agar-Agar: The data indicated that protease production started from 24 hours and it reached maximal level at 48 hours. 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 matrix. The highest enzyme production recorded was 272.75±1.22 U/ml in *Pseudomonas* sp and the optimized concentrations for the protease production was 7% (Fig. 1a). Statistical analysis indicated that, the variation in protease production by the tested bacterial isolate was highly significant in *Pseudomonas* sp. (P<0.000).

Production of Protease by Immobilized Cells in Sodium Alginate: Figure 2a shows that the amount of cell mass entrapped in sodium alginate matrix was increased gradually up to 48 hours of incubation after which there was no appreciable change. The enzyme production reached maximum level in *Pseudomonas* sp. (550.13±1.63 U/ml) by 48 hours. On further incubation, enzyme production gradually decreased. Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates were highly significant in *Pseudomonas* sp. (P<0.000).

Production of Protease by Immobilized Cells in K-Carrageenan: Figure 3a shows that the maximum protease production was attained after 48 hours of incubation after which there was no extensive change. The highest enzyme production recorded in *Pseudomonas* sp. was 312.44±1.63 U/ml. The optimized concentration for the protease production was 2.5%. Statistical analysis indicated that, the variations in protease production by the tested bacterial isolates were highly significant in *Pseudomonas* sp. (P<0.000).

Production of Protease by Immobilized Cells in Polyacrylamide: Figure 4a shows a gradual increase in protease production was noticed from 24 hours. The maximum protease production was obtained in 48 hours of incubation after which there was no sizeable change and the highest enzyme production recorded in *Pseudomonas* sp. was 348.14±2.45 U/ml. The optimized concentration for the protease production was 12%. Statistical analysis indicated that, the variations in protease production by the tested bacterial isolates were highly significant in *Pseudomonas* sp. (P<0.000).

Production of Protease by Immobilized Cells in Gelatin Beads: A detectable protease production was observed initially at 6 hours and reached maximum level (496.22±1.63 U/ml) in *Pseudomonas* sp by 48 hours of incubation (Fig. 5a). Statistical analysis indicated that, the variations in protease production by the tested bacterial isolates were highly significant in *Pseudomonas* sp. (P<0.000).

Comparison of Protease Production by Immobilized Cells in Various Matrixes by Entrapment Technique: Protease production with the immobilized cells in alginate matrix was found the highest and it was followed by gelatin beads, whereas low level of protease production was observed in agar-agar immobilized cells. Glutaraldehyde was used as a cross linking agent.

Repeated Batch Fermentation with Free Cells 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

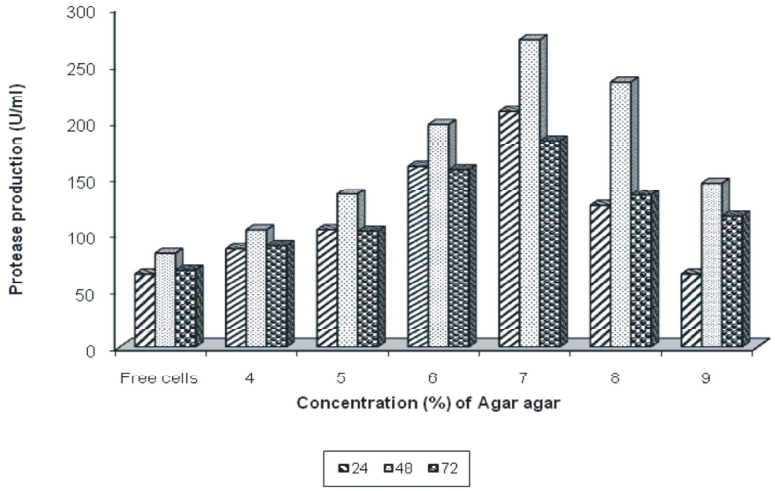

Fig. 1(a): Immobilization of protease producing *Pseudomonas* sp. using agar-agar (Batch – I)

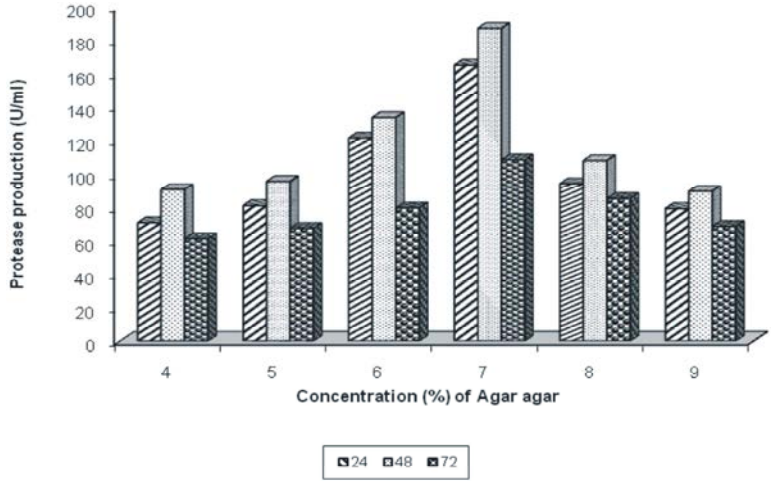

Fig. 1(b): Immobilization of protease producing *Pseudomonas* sp. using agar – agar (Batch – II)

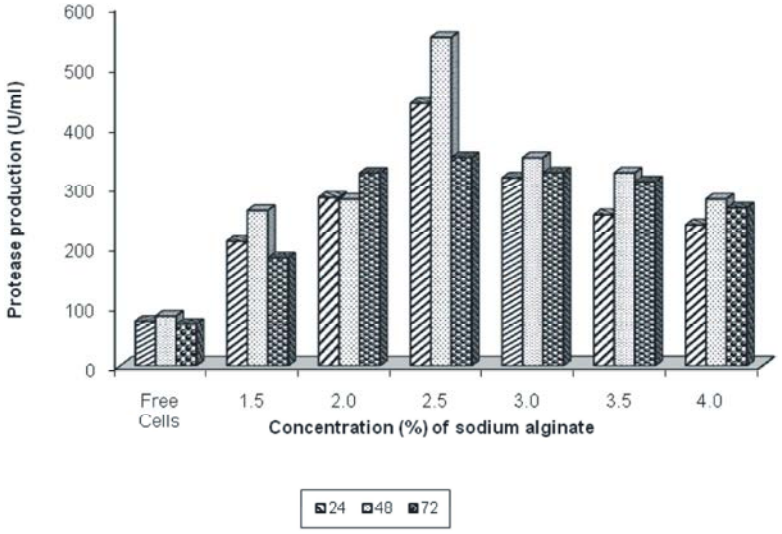

Fig. 2(a): Immobilization of protease producing *Pseudomonas* sp. using sodium alginate (Batch – I)

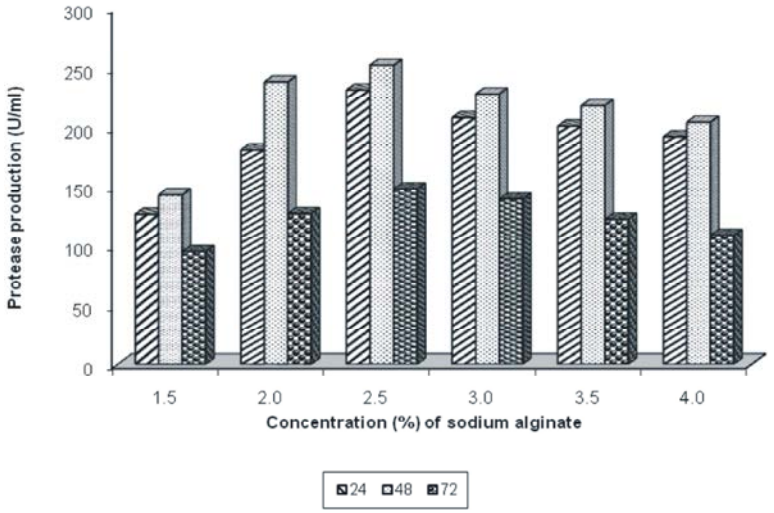

Fig. 2(b): Immobilization of protease producing Pseudomonas sp. using sodium alginate (Batch – II)

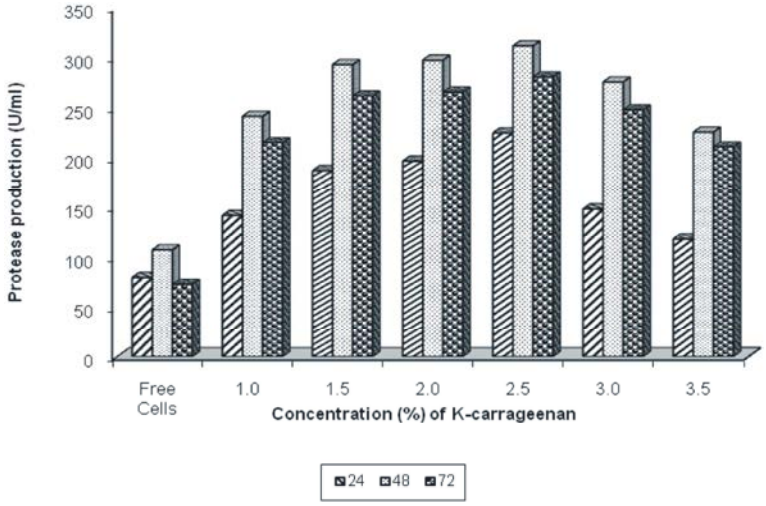

Fig. 3(a): Immobilization of protease producing *Pseudomonas* sp. using K-carrageenan (Batch – I)

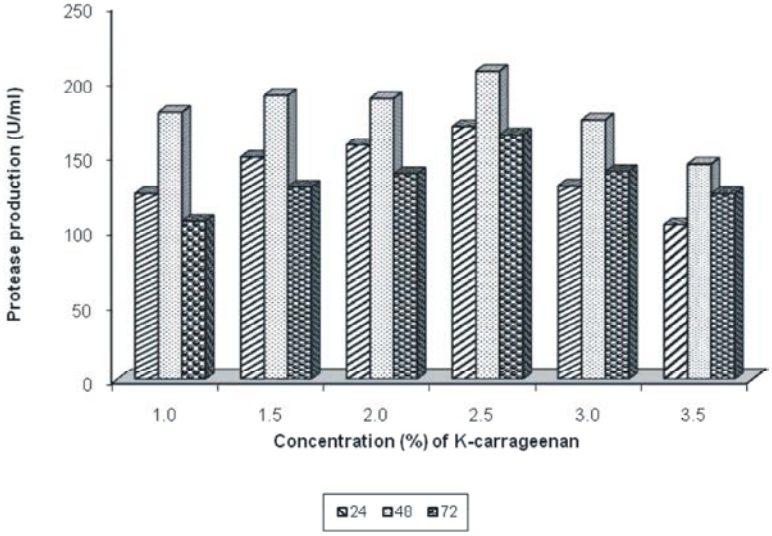

Fig. 3(b): Immobilization of protease producing *Pseudomonas* sp. using K-carrageenan (Batch – II)

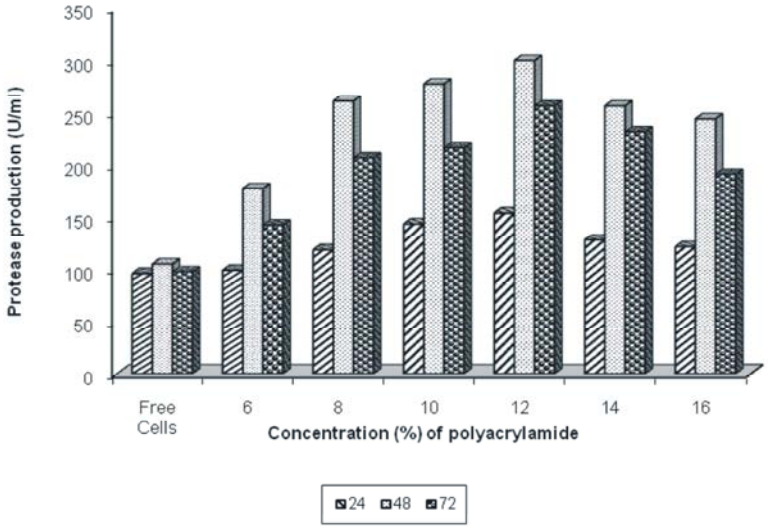

Fig. 4(a): Immobilization of protease producing *Pseudomonas* sp. using polyacrylamide (Batch – I)

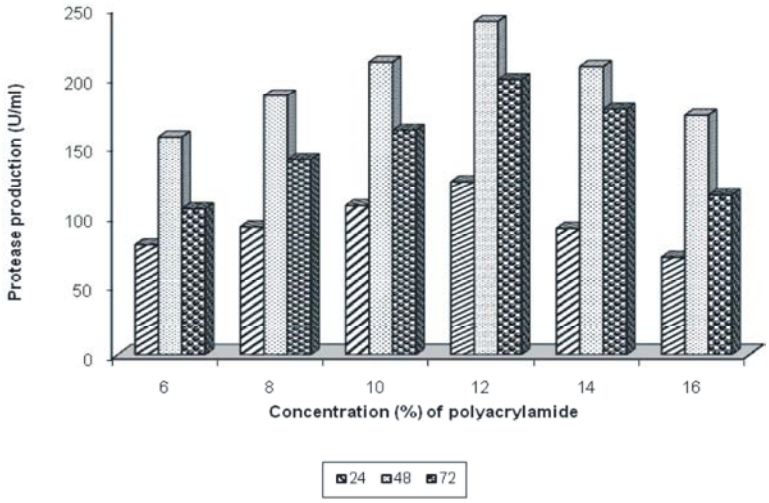

Fig. 4(b): Immobilization of protease producing *Pseudomonas* sp. using polyacrylamide (Batch – II)

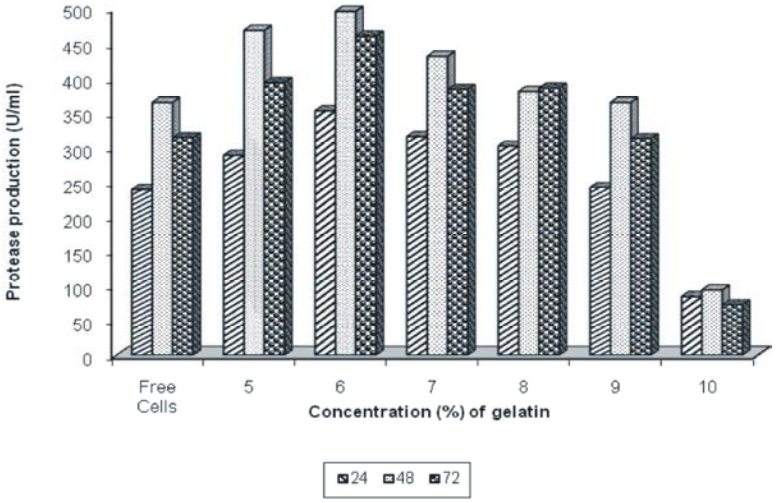

Fig. 5(a): Immobilization of protease producing *Pseudomonas* sp. using gelatin (Batch – I)

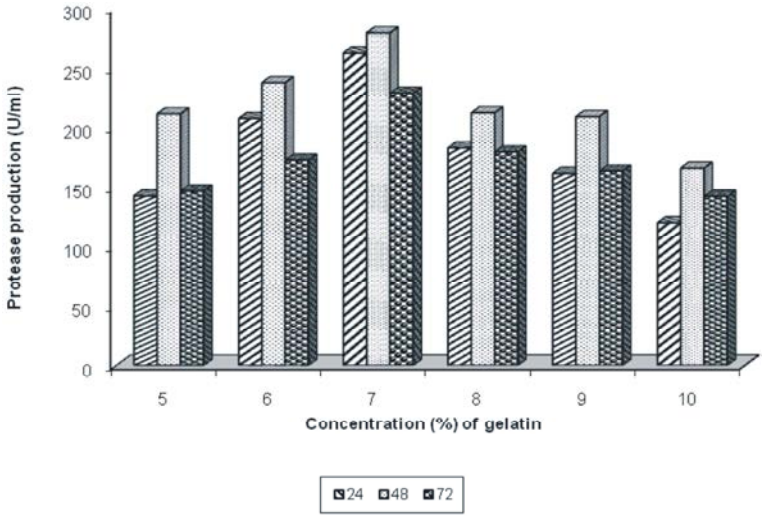

Fig. 5(b): Immobilization of protease producing *Pseudomonas* sp. using gelatin (Batch – II)

immobilized cells gradually decreased from the end of first batch. Thus the repeated batch fermentation performed well with all beads ran in two batches.

Repeated Batch Fermentation of Protease by Immobilized Cells in Agar-Agar: During the time of second batch, the protease production was reached optimal at 48 hours. The significant protease production was obtained at 48 hours of incubation after which there was no considerable change. The highest enzyme production in agar-agar matrix by *Pseudomonas* sp. was recorded as 272.75 ± 1.22 U/ml. The optimized concentration for the protease production was 7% (Fig. 5). Statistical analysis indicated that, the variations in protease production by the tested bacterial isolates were highly significant in *Pseudomonas* sp. (P<0.000) (Figure 1b).

Repeated Batch Fermentation of Protease by Immobilized Cells in Sodium Alginate: Figure 2b shows the amount of cell mass entrapped in sodium alginate matrix indicated that the protease production was gradually increased from 24 hours to 48 hours of incubation after which there was no large yield. The protease production at maximal level in *Pseudomonas* sp. recorded was 550.13±1.63 U/ml by 48 hours during the second batch. Statistical analysis indicated that, the variation in protease production by the tested bacterial isolates was highly significant in *Pseudomonas* sp. (P<0.000).

Repeated Batch Production of Protease by Immobilized Cells in K-Carrageenan: For the period of second batch, the effect of cell mass entrapped in K-carrageenan gel matrix on protease production was recorded in 48 hours of incubation (Fig. 3b). Afterwards there was no significant improvement in protease production at 72h of incubation. The highest enzyme production recorded was (312.44±1.63 U/ml). The optimized concentrations for the protease production were 2.5%. Statistical analysis indicated that, the variations in protease production by the tested bacterial isolates were highly significant in *Pseudomonas* sp. (P<0.000).

Repeated Batch Production of Protease by Immobilized Cells in Polyacrylamide: Figure 4b shows the amount of cell mass entrapped in polyacrylamide gel matrix on protease production. A gradual increase in protease production was noticed from 24 hours for the period of second batch. The enzyme production was studied from 24 hours up to 72 hours with immobilized cells and the highest enzyme yield was recorded (348.14±2.45 U/ml). The optimized concentration for the protease production was 12%. Statistical analysis indicated that, the variations in protease production by the tested bacterial isolates were highly significant in *Pseudomonas* sp. (P<0.000).

Repeated Batch Protease Production by Immobilized Cells in Gelatin Beads: Results on protease production in consecutive period revealed that the maximum level was (496.22±1.63 U/ml) in *Pseudomonas* sp. by 48 hours of incubation. Statistical analysis indicated that, the variations in protease production by the tested bacterial isolates were highly significant in *Pseudomonas* sp. (P<0.000) (Figure 5b).

DISCUSSION

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 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 [1]. Earlier researchers reported that the whole cell immobilization on *Bacillus mycoides* was considerably more stable than the free enzyme and could be stored for extended periods in both wet and dry forms before use [14].

Various reports confirm that the thermal and storage stability of immobilized protease depend on the application of immobilization methods [15, 16, 17]. The experiment on the effect of immobilization using agar-agar showed that the beads gave 4 to 5 folds higher productions over the free cells. The optimized concentration for this matrix was 7%. This report is in accordance with the earlier report of Anna *et al*. [18], 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 an enzyme activity (180 U/ml) after 48 hours of incubation.

Polyacrylamide was successfully used for immobilization of many enzyme systems [19]. In this study, the maximum protease production was observed at 48 hours in *Pseudomonas* sp. and the optimized suitable concentration for this matrix was 12%. The report is consistent with the positive influence of these matrixes on fermentative production of protease by *Bacillus subtilis* [13]. Low levels of alkaline protease production observed in cells with polyacrylamide carrier are due to the fact that polyacrylamide monomer is toxic to the cells [20].

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 [21]. In the present study, the effect of K-carrageenan on protease production revealed that the 2.5% was the suitable concentration for the protease production and the production of protease was registered 339.15±1.93 U/ml in *Pseudomonas* sp. Using K-carrageenan immobilized *Brevibacterium flavum* attained high stability against several denaturing chemicals [22].

The natural polymers such as agarose, pectin and gelatin were also employed for cell immobilization of the protease production [13]. In the present study, the protease production was studied by using gelatin as a carrier material. The suitable optimizing concentration for the protease production was 6%. Concurrent to this study, gelatin as a carrier material for the production of ß-galactosidase and Penicillin acylase by *E. coli* was carried out by Ramakrishna and Prakasam [22].

In this study, the enzyme production was studied from 24 to 72 hours with immobilized cells sodium alginate and reached maximum level in *Pseudomonas* sp. (550.13±1.63 U/ml) by 48 hours. Ramakrishna *et al*. [23] investigated the immobilization of *Bacillus cereus* in calcium alginate by using paused - bed reactors to continuously synthesize thermostable amylase. The suitable optimum concentrations for the protease productions were 2.5%. This report was supported by the earlier report of Adinarayanan *et al*. [20]. They have reported that the production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 by using alginate matrixes. The immobilizations of the alkaline protease by entrapment in calcium alginate, agar (or) agarose were done using different concentrations. The protease production decrease in higher concentration was due to decreased gel porosity with the increase in gel concentration and consequently diffusion limitation was developed [24]. Similar results were observed previously for other entrapped enzymes [25]. The specificity of the immobilized yield was about 71.1% of the free enzyme which was higher than that obtained by Mittal *et al*. [26].

CONCLUSION

Immobilization techniques provide a special microenvironment in which cells always have different behaviors compared with free cells. Whole cell immobilization technique is generally being 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. In the highest enzyme production was recorded in cells immobilized with sodium alginate matrix and it is followed by gelatin, agar-agar, K-carrageenan and polyacrylamide. The least amount of protease production was found in cells immobilized with agar agar gel matrix. Repeated batch fermentation with free cells and immobilized cells revealed that the 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.

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