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Enrichment and Immobilizaiton of *Enterococcus hirae* In Various Matrices to Study their Protease Production Efficiency

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Proteases are the most useful industrial enzymes. The protease has versatile applications including extensive uses in confectionary, fermentation process, leathering, detergent industry,etc. In this research *Enterococcushirae,* a gram positive bacteriawas enriched and immobilized in various matrices to study their protease producing efficiency. The immobilization was carried out by three different matrices under two different concentrations of 3% and 4%. Repeated batch fermentation using the immobilized cells were carried out in boiling tube with Skim Milk as the substrate and their protease producing efficiency were studied. The results showed that the 4% calcium alginate had the maximum enzyme activity of 283.2 (U/ml) and also maintained a good level of enzyme production throughout the entire 10 days cycle. The 4% calcium alginate also showed consistent protease production with the enzyme activity consistently maintained above 150 (U/ml). The average protease production by the microorganism is 176.64 (U/ml). The peak activities for other methods areas follows: 3% calcium alginate - 249.6 (U/ml), 3% agar-agar - 235.2 (U/ml), 4% agar-agar - 230.4 (U/ml), 3% Gelatin - 235.2 (U/ml) and 4% Gelatin - 174.4 (U/ml). The entrapment of cells in 4% calcium alginate was found to be the optimal method for immobilizing *Enterococcus hirae.*

Keywords :*Enterococcus hirae*, Immobilization , Enrichment, Alkaline- protease.

The genus *Enterococcus* consists of Gram-positive, non-spore-forming, facultative anaerobic bacteriathat can occur both as single cocci and in chains[1]. Enterococci arecommonly found in the environment and in various objects ranging from food to human[2]. They produce a group of hydrolytic enzymes hyaluronidases, gelatinase and serine protease[3]. The main role of these enzymes in Enterococcus is thought to be in providing nutrients to the bacteria by degrading host tissues[4]. Other potential protease producing bacteria includes genera Clostridium, Bacillus and Pseudomonas and fungi such as genera *Aspergillus, Mucor* and *Rhizopus*[5-6]. The protease produced by the microbes has numerous industrial applications such as food, complementary of beasts and poultries, confectionary, bakery, biotransformation, detergent industries, waste water refinementetc[7-8].The advantage of immobilizedcells over conventional free cell cultures for protease production is their continuous operation with retention of high cell density in the reactor, even beyond wash-out conditions[9].The Immobilization of microbes for protease production has been extensively carried out in the *Pseudomonas* and *Bacillus* species, but the *Enterococcus* species has not yet been immobilized and studied. This research concentrates on enriching *Enterococcus hirae*to increase their

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protease producing capability and then immobilize the cell in matrices such as Calcium Alginate, Agar-Agar, and Gelatin to study their protease producing efficiency.

MATERIALS AND METHODS

Microbial culture media and main instruments

Allthe chemical materials used in this research were purchased from Hi Media Laboratories, Mumbai. The main materials includes Nutrient Agar medium, Nutrient Broth medium, Skim milk medium, Sodium alginate, Calcium Chloride, Gelatin, Agar – Agar, Agar powder (Bacteriology), Formaldehyde, Ethanol, Milk , Casein powder for enzyme assay and biochemistry (Merck), L-Tyrosine (Merck), Folin-ciocaltus phenol reagent (Merck), and Tricholoro acetic acid (Merck).

The major instrument that we used in this research includes UV-VIS Spectrophotometer, Hot air oven, pH meter and Research Centrifuge (Remi).

Culture selection

An alkaline-protease producing strain of*Enterococcus hirae* was isolated in our lab from a dairy effluent, and was used as the study microorganism. All the microbial selection and enrichment process were carried out under sterile environmental conditions.

Enrichment

The micro-organisms showed low protease activity in casein hydrolysis test. These microorganisms were then enriched to improve protease production by growing them in a custom media comprisingNutrient broth supplemented with skimmed milk as protein source in the ratio (10:1). The microbes were grown in the medium for a period of three days. After the stationary phase has been reached they were sub cultured in freshly prepared custom media. This procedure was repeated for a period of 4 weeks. The resultant protease activity of the enriched microorganism was measured by allowing them to act in production medium and then subject the resultant product to enzyme assay.

Inoculum preparation

A sterile loop was used to transfer the culture aseptically fromcustom media to 50ml inoculum medium in a 150ml Erlenmeyer flask. The composition of the inoculum medium is $NaCl_2$ - 5[g/L], Beef extract - 1.5 [g/L], Yeast extract - 1.5 [g/L], glucose - 2 [g/L], KH_2PO_4 - 5 [g/L] and $MgSO_4.7H_2O$ - 5 [g/L]with a final pH of 7.0. The culture was kept in orbital shaker incubator at 37° C with speed of 90 RPM for a period of 24 hours. After incubation period the content of the flask was centrifuged at 5000 RPM for 10 minutes and the supernatant was decanted. The Pellet having the enriched microorganism was then washed with sterile sodium chloride solution [9.0 g/L] and then with sterile distilled water subsequently. It was suspended in sodium chloride solution [9 g/L] and was used as the inoculum for immobilization.

Immobilization

The immobilization of the enriched microorganism was carried out by three different matrices under two different concentrations of 3% and 4%. All operation were carried out aseptically under laminar flow chamber.

Whole cell immobilization by Calcium Alginate

The cell entrapment by calcium alginate was carried out as per the method device by Johnsen et al., (1986)[10]. The sodium alginate solution was prepared by dissolving 3g of alginate powder in 100ml of distilled water for 3% solution and 4g in 100ml of distilled water for 4% solution. The solution was autoclaved at 121°C for a period of 15 minutes. The solution was cooled down to room temperature and the inoculum was mixed with it. This mixture was stirred continuously for 10 minutes to bring consistent distribution of cell mass in the solution. The mixture was taken in Pasteur pipette and added drop wise into 0.2M of $CaCl_2$solution from a height of 15 cm. The beads formed were then allowed to cure in the $CaCl_2$ for a period of 2 hours at room temperature. Then the beads were washed with distilled water and suspended in sodium chloride solution.

Whole cell immobilization by Agar – Agar

3g / 4g of Agar-Agar was dissolved in 100 ml distilled water to prepare 3% and 4% of solution respectively. This solution was then autoclaved at 121°C for 15 minutes to bring complete dissolution of the Agar-Agar. After the solution has been cooled down the cell suspension was added to it and mixed till uniform distribution is achieved. The mixture was then poured into sterile 4 inchdiameter Petri dish and allowed to solidify. Once solidified the block was cut into 3mm cubes by using sterile scalpel. The cubes were washed with distilled water and allowed to cure in phosphate buffer for 2 hours.

Whole cell immobilization by Gelatin

3g / 4g of Gelatin was dissolved in 100 ml of distilled water to prepare 3% and 4% of solution respectively. The solution was then autoclaved at 121°C for 15 minutes. After it has been brought down to room temperature,inoculum culture was added to it and mixed until uniform distribution of cells is achieved. The mixture was poured into petri dish and they were overlaid with 10 ml hardening solution [20% formaldehyde in 50% ethanol (V/V)]. This mixture was then kept in deep freezer at -20°C for 48 hours. The resultant gel was cut using a sterile scalpel into cubes of 3mm sides. They were washed with distilled water and were cured by suspending them in phosphate buffer for 2 hours.

Alkaline protease production

Skim Milk was used as the production medium. Standard number of immobilized cells was introduced into 20 ml of production medium taken in sterile boiling tube. After 24 hours the medium was replaced with freshly prepared production medium. Batch fermentation of the medium was carried out till the supporting matrices showed disintegration. All spent medium were collected and stored in sterile test tubes at 4°C until further analysis. The enzyme assay and pH measurement was carried out for each cycle.

Analysis

Casein Hydrolysis test

1.26 g of Skim Milk agar was dissolved in 25 ml of distilled water and it was sterilized by autoclaving at 121°C for 15 minutes. The medium was poured into sterile petri dish and allowed to solidify. The test microorganism is streaked over the solidified Skim Milk agar. The plate is sealed with parafilm tape and then kept in the incubator for a period of 24 hours at 37°C. After the incubation period the plates are observed for the Zone of Clearance. The clear zone indicates that the protein in that area has been degraded by the proteolytic enzymes secreted by the microorganisms. The larger the cleared zone the greater is its protease production.

Protease Assay

Protease assaywas carried out forspent medium of respective immobilized cellsto determinetheir proteaseactivityusing a modified method of Universal protease assay[11]. The spent medium containing the enzyme and the unreacted proteins were centrifuged at 5000 RPM for 15 minutes. The supernatant was taken in a test tube and 5 ml of Casein buffer [1% casein solution (Casein with 0.2 M glycineand 0.2 Msodium hydroxide) was prepared and 5 mlof this casein solution was added with glycine -NaOH buffer solution] was added to the sample. It was then incubated at 60°C for 10 minutes. After the incubation time, 4 ml of Trichloro Acetic acid was added followed by 5 ml of Na_2Co_3 solution. They were mixed using a vortex shaker.1 ml of Folin-Ciocalteu reagent (1:1 dilution with distilled water) was added. The tube containing the mixture was kept in the water bath for 30 minutes at 37°C. Then the optical density of the solution was measure using UV VIS spectrophotometer at 660nm. By applying the OD values in standard L-Tyrosin graph the corresponding Tyrosine equivalents will be calculated. The Tyrosine equivalents are then applied in the formula for determining the Enzyme activity.

$$\text{Enzyme activity (Units/ml)} = \frac{(\mu \text{ moles tyrosine equivalents released}) * (\text{Total volume of assay})}{(\text{Volume of enzyme}) * (\text{Time of assay}) * (\text{Volume used in calorimetric determination})}$$

One unit of Enzyme activity is defined as the amount of enzyme that released 1 µg of tyrosine/ml/min.

RESULTS

Enrichment process

The microorganism showed tremendous improvement in the production of protease.The effect of the enrichment process was measure through casein hydrolysis test [Figure 2] and enzyme assay [Figure 1]. Pretreatment microorganism showed an average enzyme activity of 26.8 (U/ml) while the post treatment microorganism showed an average activity of 104.5 (U/ml), a 290 % increase in the protease production. This enrichment process proved to be effective in increasing protease production efficiency of the microorganism.

Alkaline Protease production by cell entrapped in 3% Calcium Alginate

The cells during the first four days, showed gradual increase in the amount of protease

produced till it reached its peak value. The highest amount of protease production occurred during the day 5 of the batch fermentation process, with enzyme activity of 249.6 (U/ml). The average protease production by *Enterococcus hirae* immobilized in 3% Calcium Alginate beads during the 10 days cycle was 163.14 (U/ml). After the peak period of the protease production occurred, the efficiency of the cells gradually decreased till it reached 104 (U/ml) [Fig. 3]. The pH of the reacted substrate proved that the produced protease was alkaline protease. The pH value peaked to a value of 7.76 during the day 5 of the 10 day cycle [Fig. 4].

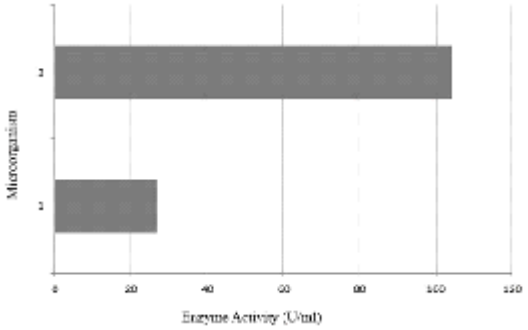

Fig. 1. Comparison of Protease production by conventional free cell method of 1. Non-Enriched microorganism and 2.Enriched microorganism

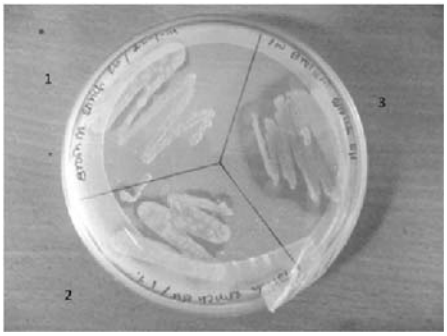

Fig. 2. Comparative analysis throughCasein Hydrolysis test of the 1. Non-Enriched *Enterococcus hirae,* 2. 1 Week enriched *Enterococcus hirae,* 3. 1 month enriched *Enterococcus hirae*

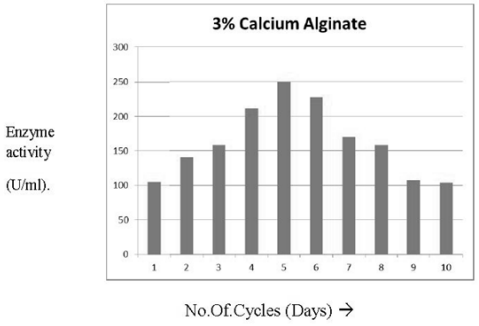

Fig. 3. Profile of Alkaline Protease production by *Enterococcus hirae* immobilized in 3% Calcium Alginate

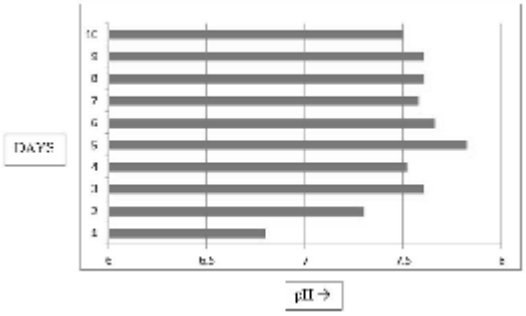

Fig. 4. Profile of pH variation of the production medium by *E. hirae* immobilized in 3% Calcium Alginate

Table 1. Comparison of Alkaline Protease Production with *Enterococcus hirae* immobilized in various matrices by Batch fermentation process

Matrix	Fermentation time for 1 cycle(Hours)	Total number of cycles	Total fermentation hours (Hours)	Total alkaline protease production(U/ml)	Avg. Protease production per cycle(U/ml)
Free cell (non-enriched)	24	3	72	80.4	26.8
Free cell (Enriched)	24	3	72	313.5	104.5
Calcium Alginate 3%	24	10	240	1631.4	163.14
Calcium Alginate 4%	24	10	240	1766.4	176.64
Agar-Agar 3%	24	10	240	1278.4	127.84
Agar-Agar 4%	24	10	240	1113.6	111.36
Gelatin 3%	24	10	240	1504	150.4
Gelatin 4%	24	10	240	1250.4	125.04

Alkaline Protease production by cell entrapped in 4% Calcium Alginate

During the first four days, moderate increase in the amount of protease production was recorded. The production reached its peak value during day 5. The highest amount of protease production occurred during the day 5 of the batch fermentation process, with enzyme activity of 283.2 (U/ml). The average protease production by *Enterococcus hirae* immobilized in 4% Calcium Alginate beads during the 10 days cycle was 176.64 (U/ml). After the peak period of the protease production occurred, the efficiency of the cells gradually decreased till it reached 168 (U/ml)[Figure 5]. The pH of the reacted substrate proved that the produced protease was alkaline protease. The pH value peaked to a value of 7.62 during the day 8 of the 10 day cycle [Figure 6].

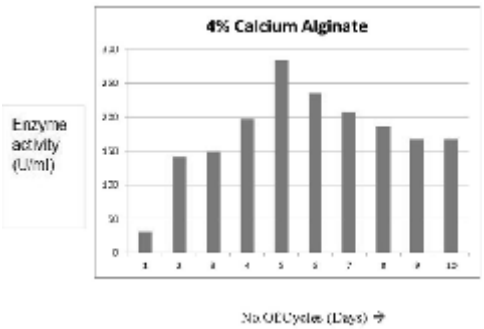

Fig. 5. Profile of Alkaline Protease production by *Enterococcus hirae* immobilized in 4% Calcium Alginate

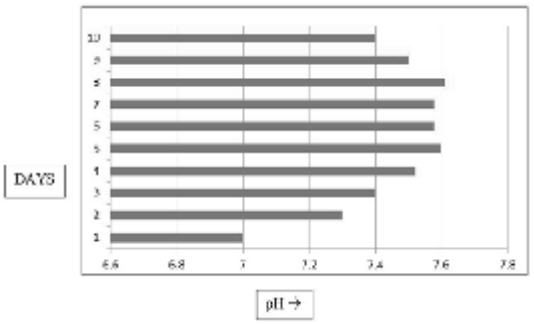

Fig. 6. Profile of pH Variation of the production medium by *Enterococcus hirae* immobilized in 4% Calcium Alginate

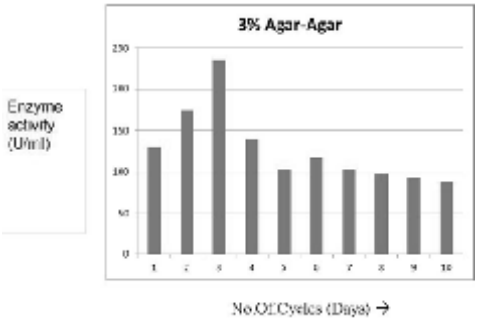

Fig. 7. Profile of Alkaline Protease production by *Enterococcus hirae* immobilized in 3% Agar-Agar

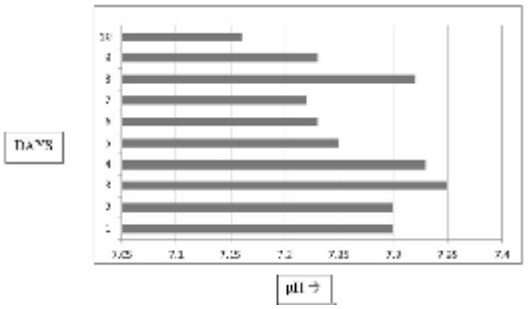

Fig. 8. Profile of pH Variation of the production medium by *Enterococcus hirae* immobilized in 3% Agar-Agar

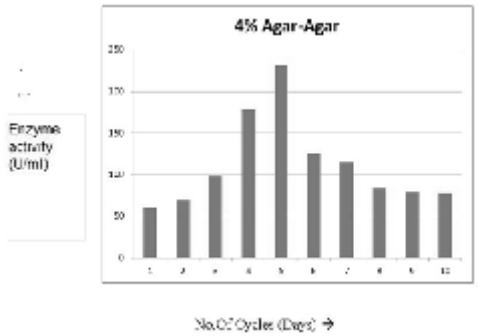

Fig. 9. Profile of Alkaline Protease production by *Enterococcus hirae* immobilized in 4% Agar-Agar

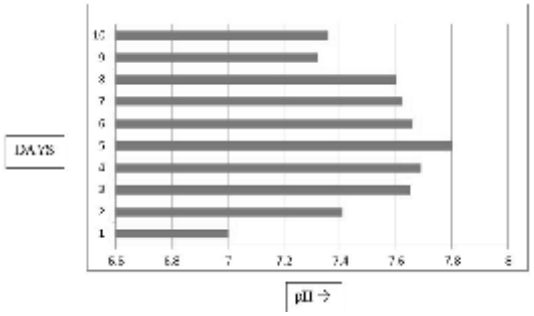

Fig. 10. Profile of pH Variation of the production medium by *E. hirae* immobilized in 4% Agar-Agar

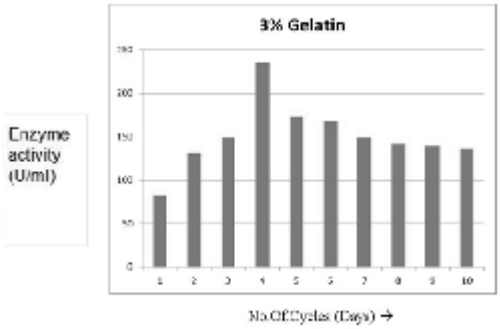

Fig. 11. Profile of Alkaline Protease production by *Enterococcus hirae* immobilized in 3% Gelatin

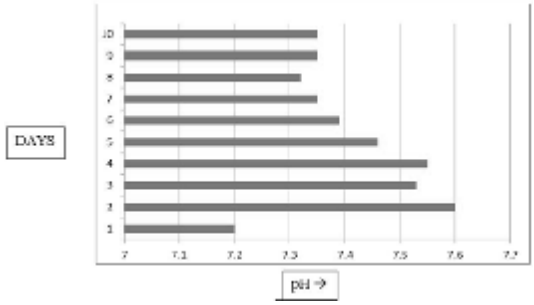

Fig. 12. Profile of pH Variation of the production medium by *Enterococcus hirae* immobilized in 3% Gelatin

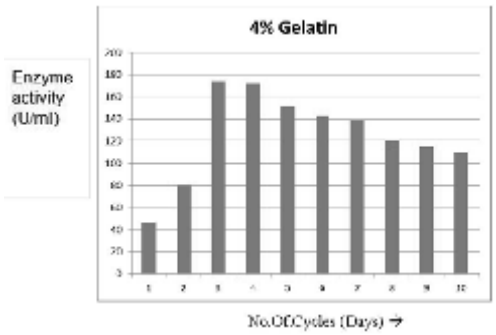

Fig. 13. Profile of Alkaline Protease production by *Enterococcus hirae* immobilized in 4% Gelatin.

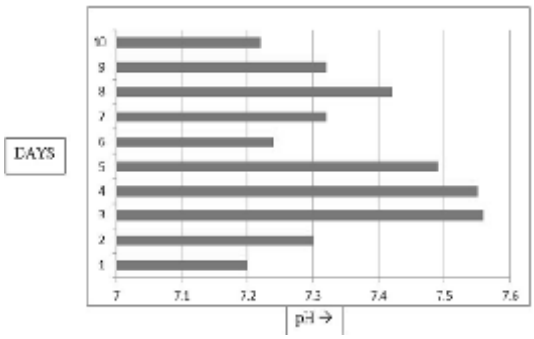

Fig. 14. Profile of pH Variation of the production medium by *Enterococcus hirae* immobilized in 4% Gelatin

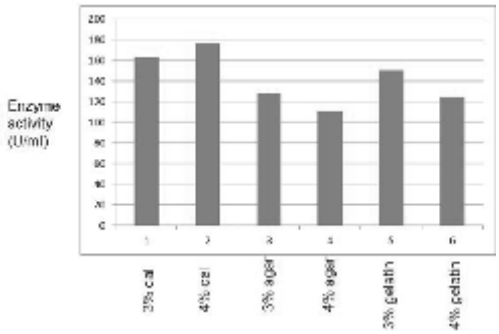

Fig. 15. Comparison of average protease produced by *Enterococcus hirae,* Immobilized in various naturally derived matrices thorough out the entire 10 day cycle of Batch Fermentation
1. 3% Calcium Alginate; 2. 4% Calcium Alginate;
3. 3% Agar-Agar; 4. 4% Agar-Agar
5. 3% Gelatin; 6. 4% Gelatin

Alkaline Protease production by cell entrapped in 3% Agar-Agar

During the first two days,tremendous increase in the amount of protease produced was recorded. The production reached its peak value by day 3. The highest amount of protease production occurred during the day 3 of the batch fermentation process, with enzyme activity of 235.2 (U/ml). The average protease production by *Enterococcus hirae* immobilized in 3% Agar-Agar cubes during the 10 days cycle was 127.84 (U/ml). After the peak period of the protease production occurred, the efficiency of the cells drastically decreased till it reached 88 (U/ml) by day 10 [Fig. 7]. The pH of the reacted substrate proved that the produced protease was alkaline protease. The pH value peaked to a value of 7.35 during the day 3 of the 10 day cycle [Fig. 8].

Alkaline Protease production by cell entrapped in 4% Agar-Agar

During the first two days, mild amount of protease production occurred. In the consecutive day 3 and day 4 the protease production showed tremendous improvement before reaching its peak value at day 5. The highest amount of protease production occurred during this day 5 of the batch fermentation process was 230.4 (U/ml). The average protease production by *Enterococcus hirae* immobilized in 4% Agar-Agar cubes during the 10

days cycle was 111.36 (U/ml). After the peak period, protease production occurred. But the efficiency of the cells drastically decreased till it reached 76.8 (U/ml) by day 10 [Figure 9]. The pH of the reacted substrate proved that the produced protease was alkaline protease. The pH value peaked to a value of 7.8 during the day 5 of the 10 day cycle [Figure 10].

Alkaline Protease production by cell entrapped in 3% Gelatin

The amount of protease produced by the cells during the first two days was very low. This might be due to the time spent the microbes in the deep freezer for the substrate hardening. During the day 3 and day 4 the protease production showed tremendous improvement and reached its peak value at day 4. The amount of protease production that occurred during this day 4 of the batch fermentation process was 235.2 (U/ml). The average protease production by *Enterococcus hirae* immobilized in 3% Gelatin cubes during the 10 days cycle was 150.4 (U/ml). After the peak period, protease production ceased drastically till it reached a value of 136 (U/ml) by day 10 [Figure 11]. The pH of the reacted substrate proved that the produced protease was alkaline protease. The pH value peaked to a value of 7.6 during the day 2 of the 10 day cycle [Figure 12].

Alkaline Protease production by cell entrapped in 4% Gelatin

The amount of protease produced by the cells during the first two days was very low. This might be due to the time spent the microbes in the deep freezer for the substrate hardening. The amount of protease production reached its peak value at day 3 with an enzyme activity of 174.4 (U/ml). The average protease production by *Enterococcus hirae* immobilized in 4% Gelatin cubes during the 10 days cycle was 125.04 (U/ml). After the peak period, protease production ceased drastically till it reached a value of 110.4 (U/ml) by day 10 [Figure 13]. The pH of the reacted substrate proved that the produced protease was alkaline protease. The pH value peaked to a value of 7.56 during the day 3 of the 10 day cycle [Figure 14].

DISCUSSION

On repeated Batch fermentation of the immobilized *Enterococcus hirae* by using Skim Milk as the production medium, it has been observed that the protease production occurred without any delay. The initial days of the cycle showed low production profile. This might be due to the changes in the biochemical efficiency of the cell in immobilized state compared to the free cell state.Diffusional limitation could also have affectedthe physiological activity of cells entrapped in matrix, resulting in low production rate for the first few days[12]. The peak values were reached as time progressed, after which the efficiency came down to normal range. A comparative study has been performed between the free cell fermentation and immobilized cell fermentation, and the results are tabulated [Table I]. The average protease production range for the entire ten day cycle is as follows: 3% Calcium Alginate – 163.14 (U/ml); 4% Calcium Alginate – 176.64 (U/ml); 3% Agar-Agar – 127.84 (U/ml); 4% Agar-Agar – 111.36 (U/ml); 3% Gelatin – 150.4 (U/ml) and 4% Gelatin – 125.04 (U/ml). From these results it has been inferred that, among the naturally derived matrices used for immobilizing cells, the 4% calcium alginate is sought to be the better method of immobilizing *Enterococcus hirae*as they showed higher average protease production rate during the entire 10 days cycle [Figure 15]. They reached their production peak during day 5 with enzyme activity of 283.2 (U/ml). This immobilized cells lasted for 10 days and they produce good amount of protease with a 69% increase in the overall production efficiencycompared to the free cell system of the enriched microorganism.

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