Immobilization of Pseudomonas Aeruginosa in Different Matrices for the Production of Alkaline Protease

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Abstract: ***Numerous enzymes are used in various types of industries, and one such enzyme used in several of these industries is proteases. Aforementioned, industries such as dairy, detergent, leather, fermentation and several other industries are benefitted with protease enzyme. In the present study, the efficiency of protease production was studied by enriching and immobilizing several matrices by a gram negative bacteria known as Pseudomonas aeruginosa .The immobilization was carried out by four different matrices under two different concentrations of 3% and 4%.Yielded results revealed highest enzymatic activity of 240 (U/ml) in 3% calcium alginate. Still, second highest enzymatic activity of 230 (U/ml) was seen in 4% calcium alginate. On the contrary, free microbial cells showed an enzymatic activity of 100 (U/ml). The peak activities for other methods area as follows: 4% calcium alginate - 133 (U/ml), 3% agar-agar - 100 (U/ml), 4% agar-agar - 91 (U/ml), 3% Gelatin - 85 (U/ml), 4% Gelatin - 88 (U/ml) and Polyacrylamide – 104 (U/ml). The most optimum matrix for the cellular entrapment of Pseudomonas aeruginosa is seen in 3% calcium alginate for alkaline protease production.***

Keywords: ***Pseudomonas aeruginosa, Immobilization, Enrichment, Alkaline- protease.***

I. INTRODUCTION

The organism *Pseudomonas aeruginosa* is a gram negative, single cocci or chained cocci bacterium which is non-sporulating and is facultative anaerobic in nature [1]. They are Enterococci class of microorganisms and are common inhabitants of the environment, seen in various objects ranging from food to human [2]. Hyaluronidases, gelatinases and serine proteases are several type of hydrolytic enzymes produced exclusively by the organism [3]. Host tissue degradation with the help of these enzymes is how nutrients are up taken by the Enterococci [4]. Under alkaline conditions, peptides and amino acids are obtained as a result of protein hydrolysis which was catalyzed by these enzymes [9]. Food Processing [10], leather production [11], pharmaceutics [12], cosmetics [13], laundry industries [14-16] and also protein rich solid waste treatment industries [17] are several of the industries with high applications of alkaline proteases. Other potential protease producing bacteria includes genera Clostridium, Bacillus and Pseudomonas and fungi such as genera *Aspergillums*, *Macro* and *Rhizopus* [5]. 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 refinement etc [3].

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High cell density retention during prolonged or continuous operation even during extreme wash out circumstances in protease production by immobilized cell culture is the comparative advantage over conventional free cell culture [4].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 *Pseudomonas aeruginosa* to increase their protease producing capability and then immobilize the cells in Agar-agar, Gelatin, Polyacrylamide and Calcium Alginate matrices to study their protease producing efficiency [3].

II. MATERIALS AND METHODS

A. Immobilization:

Even though improved stability is achieved by immobilization of enzymes, it also affects and decreases the enzymatic activity during the process [18]. Inclusion of an activator preserves the activity of enzyme as well as delivers stability to enzymes [19, 20]. Agar-agar, Gelatin, Polyacrylamide and Calcium Alginate matrices were the four different matrices selected for immobilizing the AVL-3 Pseudomonas aeruginosa KC991295 strain.

The selected matrices were set in a concentration range of 3% to 4% and decreased concentration like 2% decreases stability of matrix encapsulation, whereas increased concentration above 4% affects substrate – product diffusion during encapsulation.

B. Culture Preparation:

Culturing in nutrient broth medium and centrifugation was carried out for the selected Pseudomonas aeruginosa strain. Nutrient broth of volume 50ml was steam sterilized for 15 minutes using autoclave which operates at a temperature of 121° C. A flame-sterilized loop was used to inoculate the strain when the broth attains room temperature. The organism was incubated at 35° C for 24hrs in a rotary incubator at 80RPM. Post-incubation, the cultured organism was centrifuged to discard supernatant while the pellet at the bottom is removed and washed continuously using distilled water and saline. Thus, immobilization process is advanced with the obtained pellets.

C. Calcium Alginate:

3 grams of sodium alginate and 4 grams of sodium alginate were added to two different 100ml distilled water and autoclaved for better solubility to obtain 3% and 4% solution of sodium alginate. A solution of calcium chloride with 0.25M molarity was also prepared. The microbial pellets were mixed with the calcium

alginate solution. With the help of pasture pipette, the sodium alginate – microbial pellets mixture were added gradually drop by drop to the prepared 0.25M solution of calcium chloride. When sodium alginate solution comes in contact calcium chloride, as a result of chemical displacement the sodium ions in sodium alginate and the ions of calcium in calcium chloride solution interchanges to obtain sodium chloride solution and calcium alginate solution, as the calcium alginate comparatively provides higher stability and better rigidity than sodium alginate. Thus, to obtain calcium alginate and by providing adequate curing time of 2 hours to obtain better stability and rigidity, the sodium alginate beads were soaked for 2 hours in the calcium chloride solution. Until further usage, the prepared beads were preserved in saline at 4°C [6].

D. Agar- Agar:

3 grams of Agar-agar and 4 grams of Agar-agar were added to two different 100ml distilled water and autoclaved for better solubility to obtain 3% and 4% solution of Agar-agar. The microbial pellets were added to the prepared solution and is added to a petri dish and allowed to solidify for 20minutes. After solidification, using a sterile blade the solidified agar with the pellets was cubically cut in 3mm in sides to obtain several cubes. Until further usage, the prepared agar cubes were preserved in saline at 4°C [6].

E. Gelatin:

3 grams of gelatin and 4 grams of gelatin were added to two different 100ml distilled water and were autoclaved for better solubility at 121°C for 15 minutes to obtain 3% and 4% solution of gelatin. The microbial pellets were added to the prepared solution with constant stirring to obtain clear soluble solution and the solution is added to a petri dish. Using 20% formaldehyde in 50% ethanol (v/v), a hardening solution was prepared and poured over the gelatin mixture in the petri dish and allowed to be solidified and hardened at -20°C for a period of 48 hours. After hardening, using a sterile blade the solidified gel with the pellets was cubically cut in 3mm in sides to obtain several cubes [7].

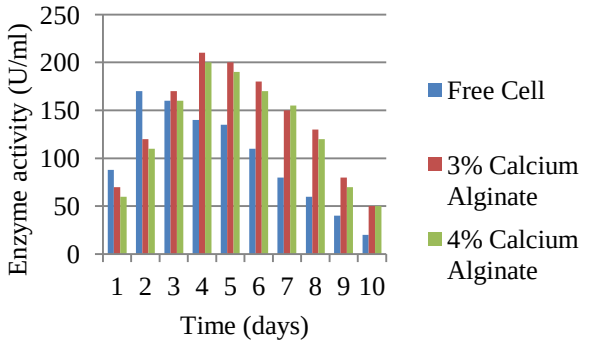
F. Polyacrylamide:

With the usage of acrylamide of 5.6grams, bis acrylamide of 0.3 grams, ammonium persulphate of 20mg and N,N ,N',N'-tetramethyl thylendiamine (TEMED) of 1ml to prepare 0.2M of phosphate buffer with pH 0.7. The microbial pellets were added to the prepared solution. Post-polymerization, the prepared gel was cubically cut of sides 3mm to obtain polyacrylamide cubes containing the microbial pellets. The same phosphate buffer was used to cure the cut polyacrylamide cubes by refrigerating the cubes for a period of 1 hour. After refrigeration, saline water was used two to three times to wash out the cubes and preserved until further usage [7]. All of the above described laboratory practices were performed under a laminar flow unit.

G. Batch Reaction:

Based on the concentrations of 3% and 4%, 250ml conical flasks were added with the prepared immobilized cells. Growth medium of 100ml was taken and added. Constant quantities of cubes were used in each flask. Room temperature incubation of the cells in diverse matrices present in the respective flasks was done for 24hours in a rotary shaker at 140RPM. The products obtained as a result of the reaction were collected post-incubation and until further usage they were preserved at 4°C. The flasks used for the reaction was cleaned, and using distilled water the remaining cubes were washed.

The cleaned flasks were used again and growth media was added and for the next 10 days the same above process was repeated. After 10 days, the samples of each day with several matrices were collected and evaluated for protease activity.



Graph 1. Comparison of enzymatic activity of free cells and calcium alginate (3% & 4%) for 10 days

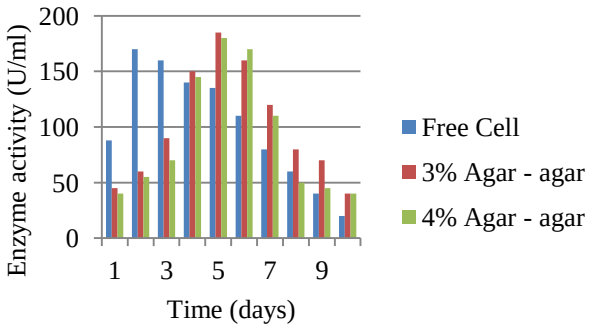
The tyrosine standard curve method of protease estimation and the BSA standard curve method of protein estimation by Folin and Ciocalteu (1927) were used to measure the enzyme activity.

From the reacted samples, the concentration of the unknown protease enzyme is estimated by plotting the optical density values of the samples obtained using UV-Visible Spectrometer against the standard graph, thus, the enzymatic activity was estimated.

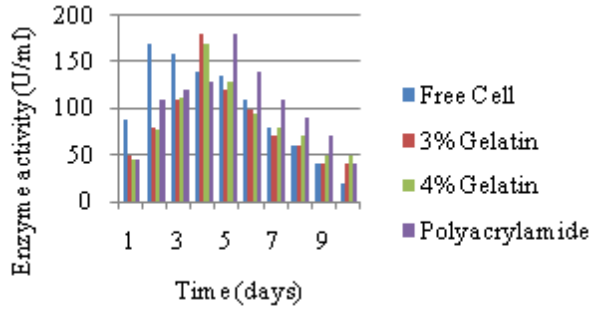
III. RESULT AND DISCUSSION

The present study shows that the activity of the protease enzyme was higher in 3% calcium alginate rather than 4% calcium alginate. The 4th day of the experiment showed the highest peak value in protease enzyme activity with 210 U/ml activity in 3% calcium alginate and 200 U/ml activity in calcium alginate of 4% (Graph 1). Previous experiments involving 3% calcium alginate matrix with immobilized cells showed 585 U/ml activity which is considered maximum value after 36 hours of incubation, however, prolonged incubation period gradually reduced enzyme production [8].

Similarly, immobilization of cells using Agar-agar as matrix for protease production showed that 3% agar solution has better enzyme activity of 185 U/ml rather than 4% agar solution which has an activity of 180 U/ml and this maximum activity was obtained on the 5th day of incubation (Graph 2).

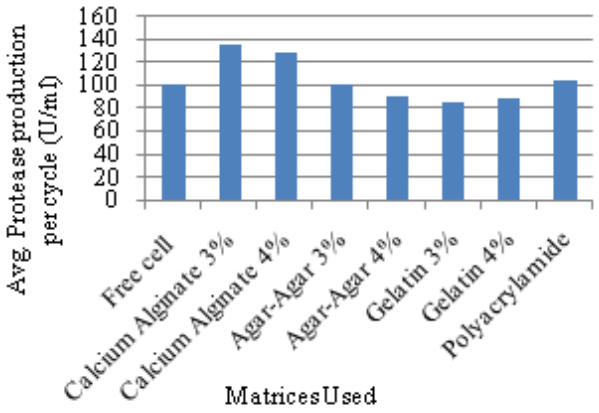


Graph 2. Comparison of enzymatic activity of free cells and Agar-agar (3% & 4%) for 10 days



Graph 3. Comparison of enzymatic activity of free cells, gelatin (3% & 4%) and polyacrylamide for 10 days

The usage of gelatin as matrix for cell immobilization for protease enzyme production showed that 3% gelatin showed better enzyme activity of 180 U/ml whereas, 4% gelatin showed reduced activity of 170 U/ml and these peak activity values were obtained on the 4th day of incubation (Graph 3). On the other hand, polyacryamide showed enzyme activity peak of 180 U/ml on the 5th day of incubation (Graph 3). Previous experiments performed by Naidu et al [9], reveals that immobilization of cells for alkaline proteases decline in titer when using polyacrylamide matrix, however, the titer value of alkaline protease after 24 hours was 295 U/ml.



Graph 4. Comparison of matrices for enzyme activity immobilization

From the present study, comparing calcium alginate (3% & 4%), agar-agar (3% & 4%), gelatin (3% & 4%) and polyacrylamide as optimal matrix for protease enzyme production by cell immobilization of Pseudomonas aeruginosa; 3% calcium alginate produces higher yield of protease of 137 U/ml (Graph 4). A Study on optimizing production parameters for alkaline protease by Beshay [10] for immobilizing T. turnirae reveals entrapment of cells by calcium alginate is a promising methodology. Comparative study between calcium alginate, κ-carrageenan and polyacrylamide as immobilization matrices for enzyme production by Adhinarayana [6] reveals calcium alginate to be a better entrapment matrix for immobilization.

Table 1. Estimation of cumulative protease activity of *P. aeruginosa* in different immobilized methods at different days

Matrices Used	Incubation Time (1 Cycle)	Cycles Taken	Total Time Taken	Alkaline Protease Produced (in 10 Cycles)	Average Alkaline Protease Produced
Free cell	24 hours	10	240 hours	1003 U/ml	100 U/ml
3% Calcium Alginate	24 hours	10	240 hours	1370 U/ml	137 U/ml
4% Calcium Alginate	24 hours	10	240 hours	1325 U/ml	133 U/ml
3 % Agar-Agar	24 hours	10	240 hours	1000 U/ml	100 U/ml
4% Agar-Agar	24 hours	10	240 hours	905 U/ml	91 U/ml
3% Gelatin	24 hours	10	240 hours	850 U/ml	85 U/ml

4 % Gelatin	24 hours	10	240 hours	880 U/ml	88 U/ml
Polyacrylamide	24 hours	10	240 hours	1035 U/ml	104 U/ml

IV. CONCLUSION

The present study involves the usage of several matrices which includes calcium alginate (3% & 4%), agar-agar (3% & 4%), gelatin (3% & 4%) and polyacrylamide for production of protease by immobilizing Pseudomonas aeruginosa cells. The results reveals that 3% calcium alginate produces the highest amount of protease of 137 u/ml whereas the second highest was seen calcium alginate of 4% with 133 U/ml and the third highest was seen in polyacrylamide of 104 U/ml (Table 1). Thus, the study helps in optimizing the growth of Pseudomonas aeruginosa in several distinct matrices for immobilizing of cells which can be further used for large-scale manufacturing or production of one of the commercially important enzyme, protease.

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