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Operational Stability and Reusability of *Halobacterium* sp. JS_1 Cells Immobilized in Various Matrices for Haloalkaliphilic Protease Production

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Abstract: Immobilization techniques provide a special microenvironment in which cells always have different behaviors compared with free cells. The present study reveals the operational stability of *Halobacterium* sp.JS_1 cells immobilized in various matrices for the production of Haloalkaliphilic protease. Ca-alginate immobilized *Halobacterium* sp. JS_1 cells showed remarkable operational stability and high levels of protease production for 24 days (8 cycles). Repeated batch fermentation showed specific volumetric productivity of 0.78, 0.69, 0.56 and 0.42 U/ml/h with Ca-alginate, k carrageenan, polyacrylamide and gelatin respectively. Gelatin and polyacrylamide immobilized *Halobacterium* sp. JS_1 cells showed the highest average cell leakage of 0.85 and 0.81 mg/ml respectively, where as, Ca-alginate exhibited minimum cell leakage. Thus, the alginate-immobilized cells of *Halobacterium* sp.JS_1 proved to be an effective biocatalyst for long-term usage and maximum enzyme production.

Key words: Calcium Alginate · Haloalkaliphilic Protease · K-Carrageenan · Polyacrylamide · Gelatin

INTRODUCTION

Majority of the enzymes used to date, have been obtained from mesophilic organisms and, despite their many advantages; the application of these enzymes is restricted because of their limited stability to extremes of temperature, pH, ionic strength, salinity etc. Extremophi,es are a source of enzymes (extremozymes) with extreme stability and the application of these enzymes as biotransformation catalysts is attractive because they are stable and active under extreme environmental conditions that were previously regarded as incompatible with biological materials [1,2]. Both natural and artificial hypersaline environments harbor remarkably high and diverse microbial cell densities. Extremozymes from halophilic organisms have adapted to remain stable and active at high ionic strength, an essential requirement since the intracellular environment in extreme halophiles is isotonic with the growth medium [3,4]. While most research performed on hypersaline environments has focused on the microbial diversity and ecology of these environments, there is a growing interest in the extracellular hydrolytic enzymes from halophilic bacteria [5]. Halophilic proteases are one of the most extensively s4udied extremozymes and they can be widely exploited in various biotechnological and industrial processes [6,7].

Modification of biotechnology and processes, using immobilized biocatalysts, has recently gained the attention of many biotechnologists [8]. Microbial products are usually produced either by free or immobilized cells. The use of immobilized cells as industrial catalysts can be advantageous compared to batch fermentation process. Whole cell immobilization has been a better choice over enzyme immobilization [9,10]. Immobilization of microbial cells for fermentation has been developed to eliminate inhibition caused by high concentration of substrate and product, also to enhance the yield and productivity. If intact microbial cells are directly immobilized, the removal of microorganisms from the downstream product can be omitted and the loss of intracellular enzyme activity can be kept to a minimum level [11]. Immobilization has shown promising role as most the problems such as cell-retaining capacity, reduced susceptibility to contamination and reuse of the biocatalyst with higher product conversion capability over free-cell fermentations could be solved without much alteration in the fermentation conditions [12,13].

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The most extensively studied method in whole cell immobilization is the entrapment of microbial cells in a polymer matrix [14]. However the major limitations which may need to be addressed while using such cells are dispersion of cells, flow of nutrients away from cells, diffusion of substrate and products through the cell wall and unwanted side reactions due to the presence of other enzymes [15]. Among the various immobilization matrices employed, sodium alginate, polyacrylamide and k-carrageenan are widely employed for immobilization [16]. The purpose of the present investigation was to study the operational stability / reusability of immobilized *Halobacterium* sp. JS_1 cells for haloalkaliphilic protease production under repeated batch fermentation conditions.

MATERIALS AND METHODS

Halophilic Bacterial Strain: The bacterial strain (*Halobacterium* sp. JS_1) used in this study was isolated from solar evaporated ponds at Tuticorin, Tamilnadu, India and cultivated aerobically at 40°C for 96 h in a basal salt medium containing (g l^{-1}): NaCl: 200; $MgSO_4.7\ H_2O$: 20; KCl: 2; Yeast extract: 10; Trisodium citrate: 3; and Casaminoacid: 7.5, pH was adjusted to 7.0 [2].

Inoculum Preparation: 1 ml of a week old culture of *Halobacterium* sp. JS_1 was inoculated into a sterilized 250 ml Erlenmeyer flask containing 50 ml of the above growth medium and incubated at 40°C in an incubator shaker at 200 rpm for 96 h. The flask was removed after incubation and the content was centrifuged at 10,000 rpm at REMI Centrifuge for 10 min. and the supernatant was decanted.

Immobilization of *Halobacterium* Sp. JS_1 Cells in Various Matrices: The cell pellets were washed thoroughly with sterile 2% KCl solution, followed by 20% NaCl solution. Finally the cell pellets were suspended in 50mM tris-HCl buffer (pH 9.0) containing 3.3 M NaCl. These cell suspensions were immobilized in various matrices such as calcium alginate [17], k-carrageenan [18], polyacrylamide [8] and gelatin [19].

Production of Halophilic Protease by Batch Process with Immobilized *Halobacterium* Sp. JS_1 Cells: The immobilized beads/blocks with entrapped *Halobacterium* sp. JS_1 cells, prepared by using different matrices such as sodium alginate, polyacrylamide, k-carrageenan and gelatin were transferred into sterilized 250 ml Erlenmeyer flasks containing 50 ml of the above mentioned growth medium and incubated at 40°C in an incubator shaker at 200 rpm for 96 h. The flasks were removed at regular intervals; the contents were centrifuged at 10,000 g for 10 min at 40°C to remove cells and insoluble materials (sediment). Then, the cell free supernatant was filtered through a 45µm pore size membrane filter and was used as the source of crude enzyme.

Operational Stability of Biocatalysts and Halophilic Protease Production by Repeated Batch Fermentation: The reusability of *Halobacterium* sp. JS_1 cells immobilized in various matrices was examined. After achieving the maximum production of halophilic proteases (72 h), the spent media was replaced with fresh production media (100 ml) and at the end of each cycle, the biocatalysts were filtered, washed with 25 ml of 0.05 M $CaCl_2$ and distilled water, the process was repeated for several batches until the beads / blocks started disintegrating. The enzyme titers and cell leakage of each cycle were determined [8, 20].

Analytical Methods

Qualitative and Quantitative Protease Assay: Extracellular protease activity of the isolates JS_1 were qualitatively assayed by gelatin cup method [21], the diameter of the clear zone was used as a measure of protease activity. Enzyme activities were quantitatively measured by the modified method of Anson [22]. One unit (U) of enzyme is defined as the amount of enzyme that liberates 1µg of tyrosine per min from casein under standard assay conditions.

Cell Leakage (Leaching): Cell leakage from the gel matrix was determined as cell dry weight by measuring the optical density at 600 nm. One absorbance unit was equivalent to 0.23g/l (cell dry weight) [8].

RESULTS AND DISCUSSION

Protease Production by Free and Immobilized *Halobacterium* Sp. JS_1 Cells: Extracellular halophilic protease production by *Halobacterium* sp. JS_1 cells encapsulated in various matrices started after 48 h of incubation under shaking conditions and it reached maximum level after 72 h of incubation. On further incubation, enzyme production was found to be gradually decreased. However, enzyme production by free cells of *Halobacterium* sp. JS_1 was found to be maximized only after 96 h of incubation (Data not shown).

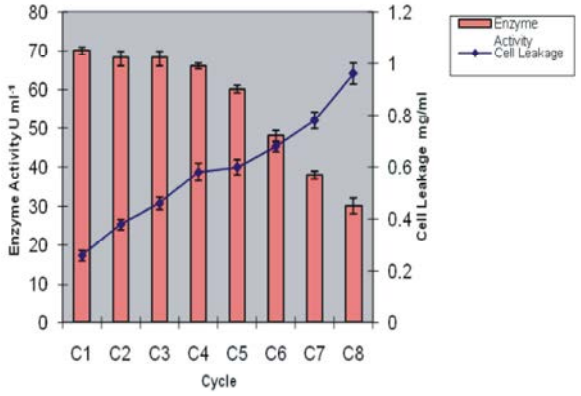

Fig. 1: Protease production by Ca alginate immobilized *Halobacterium* sp. JS_1 cells by repeated batch fermentation.

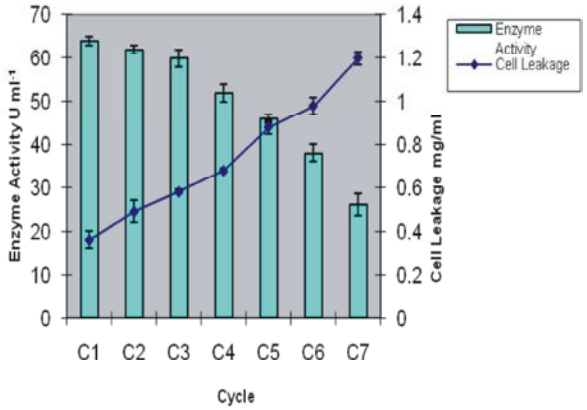

Fig. 2: Protease production by K-carrageenan immobilized *Halobacterium* sp. JS_1 cells by repeated batch fermentation.

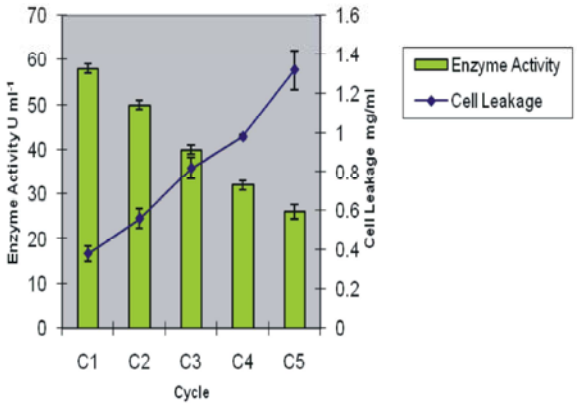

Fig. 3: Protease production by polyacrylamide immobilized *Halobacterium* sp. JS_1 cells by repeated batch fermentation.

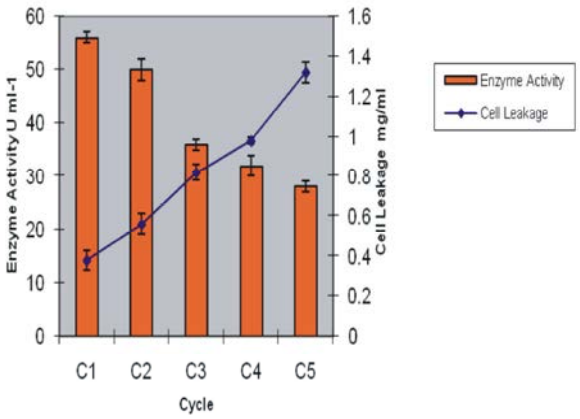

Fig. 4: Protease production by gelatin immobilized *Halobacterium* sp. JS_1 cells by repeated batch fermentation.

Operational Stability and Reusability of *Halobacterium* Sp. JS_1 Biocatalysts: The results revealed that the protease production by *Halobacterium* sp. JS_1 cells immobilized in various matrices gradually decreased from 1st cycle onwards and in contrast the cell leakage gradually increased from the 1st cycle.

Operational Stability of Calcium Alginate Immobilized JS_1 Cells: Ca-alginate immobilized *Haloba#terium* sp. JS_1 cells showed remarkable operational stability and high levels of protease production for a period of 24 days (8 cycles) (Fig.1). Similarly, protease production by alginate immobilized *Bacillus circulans* MTCC 6811 cells [23] and *Bacillus subtilis* KIBGE-HAS [24] were successfully reused for 9 cycles and 4 cycles respectively. Alginate entrapped *Streptomyces erythreus* cells [25] and *Streptomyces rimosus* cells [26] showed good levels of erythromycin and oxytetracycline production for 30days (12 batches) and 28 days (7 batches) respectively.

Operational Stability of K-Carrageenan Immobilized JS_1 Cells: *Halobacterium* sp. JS_1 cells immobilized in k-carrageenan showed stability and good levels of enzyme production for 21 days (7 cycles) (Fig.2). On the other hand, alkaline protease production by carrageenan immobilized *Bacillus subtilis* PE-11 cells showed stability for 6 days (6 batches) [8]. Audet *et al.* [27] suggested a cell entrapment process using k-carrageenan and locust bean gum, which significantly modified the mechanical properties of the gel. The k-carrageenan locust-bean gum mixed gel matrix showed significant

Table 1: Specific volumetric productivity of *Halobacterium* sp. JS_1 cells encapsulated in various matrices by repeated batch fermentation.

Matrix	Fermentation Period (Hours)	No. of Batches	Total Fermentation time (Hours)	Total Enzyme titre (U/ml)	Average Enzyme activity/Batch (U/ml)	Specific Volumetric Productivity (U/ml/h)
Free cells (Control)	96	01	62	62	62	0.645
Calcium alginate	72	08	576	448	56	0.78
K. carrageenan	72	07	504	348	49.71	0.69
Polyacrylamide	72	05	360	202	40.4	0.56
Gelatin	72	05	360	152	30.4	0.42

Table 2: Average cell leaching of *Halobacterium* sp. JS_1 cells from various matrices during repeated batch fermentation.

Matrix	No. of Batches	Total Cell Leakage mg/ml	Average Cell Leakage/Batch mg/ml
Calcium alginate	08	4.70	0.58
K. carrageenan	07	5.17	0.74
Polyacrylamide	05	4.04	0.81
Gelatin	05	4.24	0.85

stability for 3 months in continuous fermentation in a stirred tank reactor, involving lactic acid-producing bacterial strains [28,29].

Operational Stability of Polyacrylamide Immobilized Js_1 Cells

Polyacrylamide Entrapped *Halobacterium* Sp. Js_1 Cells: showed operational stability with protease production for 5 repeated cycles (15 days) (Fig.3). However, polyacrylamide entrapped *Bacillus subtilis* PE-11 cells [8] and *Bacillus subtilis* MTCC 441 [19] showed stability with enzyme production for 6 batches (6 days) and 6 batches (12 days) respectively.

Operational Stability of Gelatin Immobilized JS_1 Cells: Operational stability and enzyme production by *Halobacterium* sp. JS_1 cells immobilized in gelatin was found to be greatly reduced compared with the above mentioned matrices studied (Fig.4) Gelatin entrapped *Bacillus subtilis* MTCC 441 cells [19] and *Kluyveromyces marxianus* cells [30] showed significant stability with good levels of enzyme production for 7 batch cycles (14 days) and 10 batch cycles (10 days) respectively.

Specific Volumetric Productivity of JS_1 Protease: Specific volumetric productivity of extracellular haloalkaliphilic protease from *Halobacterium* sp. JS_1 cells immobilized in ca-alginate, k-carrageenan, polyacrylamide and gelatin was found to be 0.78, 0.69, 0.56 and 0.42 U/ml/h respectively (Table 1).

Average Cell Leakage (Leaching) of JS_1 Biocatalysts: Cell leakage (leaching) from various biocatalysts was found to be inversely proportional to the protease production by *Halobacterium* sp. JS_1 cells. Average cell leakage per batch from *Halobacterium* sp. JS_1 cells entrapped in ca-alginate, k-carrageenan, polyacrylamide and gelatin was 0.58, 0.74, 0.81 and 0.85 mg/ml respectively (Table 2).

CONCLUSIONS

Haloalkaliphilic protease production by immobilized *Halobacterium* sp. JS_1 cells proved to be superior to free cells. Specific advantages of this technique such as long term stability, reusability and possibility of regeneration to be adaptable also to scale-up the data. Maximized specific volumetric productivity was recorded in calcium alginate immobilized cells and in contrast, cell leakage was found to be minimum with the same matrix indicating its commercial importance.

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