Biosynthesis of alkaline protease by alkaliphilic *Bacillus* sp. NPST-AK15 cells immobilized in gel matrices

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Received 22 January 2019
Accepted 6 February 2019

Egyptian Pharmaceutical Journal 2019, 18:201–207

Background and objectives
Proteases are an important group of hydrolytic enzymes catalyzing the hydrolysis of various proteins by cleavage of the peptide bonds between the amino acids residues. Proteases have applications in several fields including medical and pharmaceuticals industries. Bacterial cell immobilization by entrapment techniques is one of the most effective approaches used in biotechnology at laboratory and industrial scale. Herein, we report the production of alkaline protease by immobilized halotolerant alkaliphilic *Bacillus* sp. strain NPST-AK15 cells in batch and repeated batch fermentation.
Materials and methods
Alkaline proteases-producing halotolerant alkaliphilic *Bacillus* sp. strain NPST-AK15 (accession no. KP295749) was previously isolated from hypersaline soda lakes, located at Wadi El- Natrun Valley (Egypt). Three different matrices were tested for immobilization of *Bacillus* sp. strain NPST-AK15 whole cells by entrapment technique including alginate, gelatin, and agar gel.
Results and discussion
Among various matrices tested for whole cell immobilization of *Bacillus* sp. NPST-AK15, alginate was found to be the best matrix for cell entrapment and alkaline protease production, showing the highest specific productivity (3214.34 U/g wet cells/h) and enzyme production (923.4 U/ml), followed by cells immobilized in agar and gelatin. Furthermore, the production of alkaline protease by *Bacillus* sp. NPST-AK15 immobilized in alginate gel was enhanced by investigation of the influence of various parameters on alginate beads preparation including alginate concentration, bead size, and biomass loading. Maximum enzyme production (1020.1 U/ml) and specific productivity (4086.9 U/g wet cells/h) were achieved using alginate concentration of 3.0% (w/v), bead diameter of 3.5 mm, and cell loading of 0.50 g wet weight of cell biomass per 0.3 g of sodium alginate. The immobilized *Bacillus* sp. NPST-AK15 cells exhibited operation stability in repeated batch fermentation, retaining 89.1 and 61.3% of its productivity after five (120 h) cycles and 10 (240 h) cycles, respectively.

Keywords:
alkaline protease, biosynthesis, cell immobilization, entrapment, repeated batch fermentation

Egypt Pharmaceut J 18:201–207
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1687-4315

Introduction

Proteases are an important group of hydrolytic enzymes that catalyze the hydrolysis of various proteins by cleavage of peptide bonds between the amino acid residues. The quantity of proteases produced annually for commercial applications is much greater than any other group of industrial enzymes [1]. Alkaline proteases are referring to proteolytic enzymes that work optimally in alkaline pH [2]. Proteases have diverse applications in various industries, such as detergents, leather, food, silk, diagnostics, and pharmaceuticals industries [3–5]. In addition, alkaline proteases are also used for developing of several products of medical importance. For instance, elastoterase prepared from *Bacillus subtilis* 316 M was investigated for the treatment of burns, purulent wounds, carbuncles, furuncles, and deep abscesses and as a thrombolytic agent having fibrinolytic activity. Recently, protease isolated from *Bacillus* sp. 158 was found to have a potential application in contact lens cleansing [6,7].

Biochemical processing using immobilized cells for production of extracellular metabolites offers a number of unique advantages over the traditional fermentation using free cells such as higher productivity, no wash-out, better control of the bioprocess, ease of cell mass separation, and less risk of contamination. In addition, immobilized cells can offer other advantages including higher catalysis efficiency and better operational stability and allow

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 DOI: 10.4103/epj.epj_2_19

repeated batch and continuous fermentation process [8–10]. Therefore, microbial production of extracellular metabolites using immobilized cells is gaining more attention and is considered as a very powerful approach for applications in various bioprocesses [10]. However, the catalytic efficiency, productivity, and stability of immobilized whole cell depend on the applied immobilization methods and matrices used for immobilization. Various methods have been applied for whole cell immobilization including adsorption, covalent binding, and gel entrapment. However, owing to its economic potential, microbial cell entrapment technique is one the most effective approach for microbial cell immobilization [11–13]. In this regard, several matrices have been investigated as carriers for cell immobilization including natural materials such as chitosan, agar, k-carrageenan, and alginate, or synthetic materials such as polyurethane and polyacrylamide [10,14].

We have previously isolated alkaline proteases-producing halotolerant alkaliphilic *Bacillus* sp. strain NPST-AK15 from hypersaline soda lakes (Egypt). The enzyme production was optimized [15] and purified and characterized [16]. The current study aimed to the production of alkaline protease by immobilized *Bacillus* sp. strain NPST-AK15 whole cell in batch and repeated batch fermentation.

Materials and methods

Microorganism

Halotolerant alkaliphilic alkaline proteases-producing *Bacillus* sp. strain NPST-AK15 (accession no. KP295749) was previously isolated from hypersaline soda lakes, located at Wadi El-Natrun Valley, Egypt. In addition, the enzyme production was optimized by the investigation of various fermentation parameters [15]. Furthermore, NPST-AK15 alkaline proteases were purified and characterized [16].

Preparation of *Bacillus* sp. NPST-AK15 cell biomass

A volume of 5 ml optimized alkaline liquid medium in 50 ml glass tube was inoculated with a loopful of *Bacillus* sp. strain NPST-AK15. The inoculated medium was kept overnight in a shaking incubator (150 rpm) at 40°C [15]. This culture was used to inoculate 50 ml of the production medium, incubated for 32 h at 40°C with shaking (150 rpm). The cell biomass was pelleted by centrifugation at 10 000 rpm for 10 min. Then, cell biomass was washed thoroughly with sterile distilled water and saline solution, respectively. The obtained cell biomass of *Bacillus* sp. strain NPST-AK15 was used as inoculum for cell immobilization as well as for free cell fermentations. The alkaline production medium (pH 11) contained [5]: fructose (20 g/l), yeast extract (20 g/l), K_2HPO_4 (1 g/l), $Mg_2SO_4.7H_2O$ (0.2 g/l), NaCl (50 g/l), $CaCl_2$ (5 mM), and Na_2CO_3 (10 g/l). Both fructose and Na_2CO_3 solutions were autoclaved separately and thereafter were added to the medium.

Cell immobilization

Three different matrices were tested for immobilization of *Bacillus* sp. strain NPST-AK15 whole cell including alginate, gelatin, and agar gel as previously reported [17,18]. For each immobilization process, 0.5 g of wet cell biomass was re-suspended in sterile saline solution and used for preparation of the immobilized bacterial cells. All the immobilization processes were performed under aseptic conditions.

Cell immobilization in alginate

Immobilization of *Bacillus* sp. strain NPST-AK15 whole cell by entrapment in Ca-alginate beads was carried out under aseptic conditions as previously described with some modification [8,19]. In brief, sodium alginate slurry was prepared by dissolving sodium alginate in hot distilled water and autoclaved at 121°C for 15 min. Thereafter, the cell suspension containing 0.5 g of wet weight cells was added to 10 ml alginate solution and stirred for 10 min to get a homogeneous slurry, with final alginate concentration of 3.0%. The mixture obtained was taken into a sterile syringe and extruded dropwise into sterile 0.2 M $CaCl_2$ as cross-linking agent. The obtained beads were kept in the $CaCl_2$ solution for further 2 h to be hardened. The beads were collected, washed three times with sterile distilled water, and used as inoculum for 50 ml production medium, which was then incubated at 40°C in a shaking incubator (100 rpm) for 32 h. After the incubation period the enzyme activity was measured as described later. The entire process was carried out under aseptic conditions.

Cell immobilization in agar

Cell immobilization in agar gel was carried out as previously reported [8,20] under aseptic conditions. In brief, cell immobilization in agar gel was carried out by adding *Bacillus* sp. strain NPST-AK15 cells suspension containing 0.5 g cell biomass to 10 ml of sterile molten agar solution, keeping the final agar concentration at 3%. The agar slurry was mixed and poured into sterile petri dishes. The plates were cooled and kept for 1 h in refrigerator for agar solidification. Thereafter, the solidified agar was cut into approximately 1-cm^3 fragments and washed twice

with sterile distilled water. The agar fragments was transferred to 50 ml of the production medium, which was then incubated at 40°C in shaking incubator (100 rpm) for 32 h. After the incubation period, the alkaline protease activity was measured as described later.

Cell immobilization in gelatin

Immobilization of *Bacillus* sp. strain NPST-AK15 whole cells in gelatin was carried out according to Naidu *et al.* [18]. In brief, cell suspension of 0.5 g of wet weight cells was added to 10 ml of 10% (w/v) sterile molten gelatin solution, giving a final gelatin concentration of 3.0%. Then, the gelatin/cells mixture was poured in petri dish, cooled, and left for solidification in a refrigerator for 1 h. Thereafter, the gel was over-layered with 5% (v/v) glutaraldehyde solution for covalent cross-linking and gel hardening and maintained for further 1 h at room temperature. The gel was washed twice and cut into approximately 1.0-cm^3 fragments, which were further washed three times with sterile distilled water. The gelatin fragments were transferred into 50 ml of the production medium, and the fermentation was conducted for 32 h at 40°C in a shaking incubator (100 rpm) for 32 h. All procedures were performed under aseptic conditions.

Optimization of the immobilization process

The effect of alginate concentration on the efficiency of cell immobilization was investigated by preparing the alginate beads using various concentration of sodium alginate solution ranging from 1 to 5% (w/v). The beads were prepared as described previously [15,16], washed twice with sterile distilled water, and transferred into 50 ml of the production medium. The fermentation was conducted at 40°C for 32 h with shaking (100 rpm), and the alkaline protease activity was measured.

The influence of the alginate beads size on cell immobilization efficiency was studied. Alginate beads with different diameters ranged from 1.5 to 5.4 mm were prepared using suitable syringe needles with different sizes (1–10 cm^3) for extrusion of the alginate-cells mixture into the calcium chloride solution. The beads were washed twice and transferred to the production medium for fermentation.

The effect of cells density per alginate beads on the efficiency of the immobilization process and alkaline protease production was studied. This process was carried out by preparing the alginate beads using different amounts of cell biomass ranged from 0.25 to 1.5 g wet weight using the same amount of alginate. The beads were washed twice and transferred into the production medium for fermentation.

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Biosynthesis of alkaline protease in batch and repeated batch process

Biosynthesis of alkaline protease in batch and repeated batch fermentation was carried out according to previously reported methods [8,19] with some modification. For batch culture, the free cells and cells immobilized in agar, gelatin, and calcium alginate beads and fragments (containing 0.5 g of wet weigh cells each) were transferred to 50 ml of the production medium. The cultures were incubated at 40°C (100 rpm) for 32 h with shaking. Thereafter, the alkaline protease activity was measured.

In the repeated batch fermentation, the beads were collected after 24-h incubation period, washed twice with sterile distilled water, and transferred to fresh medium. These procedures were repeated for 12 cycles. Alkaline protease activity was measured after the end of each cycle. All the experiments were carried out in triplicates under aseptic conditions.

Determination of the cell leakage

For determination of the bacterial count of the cells leaked from the beads, the cultures were serially diluted, plated on alkaline agar medium and incubated at 40°C for 24 h. For determination of bacterial count of bacterial cells entrapped in the beads, the gel beads were dissolved and homogenized in 1% (w/v) sodium pyrophosphate. Then, the samples were serially diluted, plated on agar alkaline medium, and incubated at for 24 h at 40°C. After the incubation period, the CFUs were counted and recorded. The bacterial cells leakage was estimated as percentage of leaked cells to the total cell count [14].

Assay of alkaline protease activity

Alkaline protease activity was measured as previously reported with some modifications [21]. In brief, 1.0 ml of 1% (w/v) casein solution prepared in 50 mM glycine buffer (pH 10.0) containing 10 mM $CaCl_2$ was preincubated for 5 min at 60°C. Then, 1.0 ml aliquot of suitably diluted culture supernatant was added to the substrate solution, and incubated for 20 min at 60°C.

Thereafter, the reaction was terminated by addition of 1.0 ml of 20% (w/v) trichloroacetic acid. The mixture was centrifuged at 10 000 rpm for 10 min to remove the precipitate, and the acid-soluble materials were estimated using Lowry method [22]. A standard curve was obtained using various tyrosine concentration ranged from 0 to 100 μg/ml. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the experimental conditions.

Statistical analysis

All experiments and assays were performed in triplicate, and the means and SDs were recorded and/or calculated using SPSS, version 14.0 [15].

Results and discussion

Immobilization of *Bacillus* sp. strain NPST-AK15 whole cells

Bacterial cell immobilization by entrapment techniques in various matrices is one of the most effective approaches used in biotechnology at laboratory and industrial scale. In comparison with free cell, cell immobilization can offer several advantages such as increasing cell concentration and productivity, easier product separation, protecting cells from shear forces, and ability of continuous and semicontinuous cultivation process [23].

Bacillus sp. NPST-AK15 cells were immobilized by entrapment technique in different gel matrices including alginate, gelatin, and agar gel. Batch fermentation was used for both free and immobilized cells using the same amount of the cell biomass under the same culture conditions. As shown in Table 1, among various supporting matrices tested for immobilization of *Bacillus* sp. NPST-AK15 whole cell, calcium alginate was found to be the best entrapment matrix. *Bacillus* sp. NPST-AK15 immobilized in alginate beads exhibited the highest protease activity (923.4 U/ml) and specific productivity (3214.34 U/g wet weight cells/h) followed by cells immobilized in agar and gelatin, respectively. The decrease of protease production by immobilized cells in comparison with the free cell is mostly owing to the diffusion barriers resulted from cells immobilization in the gel, affecting nutrients, oxygen, and metabolic product transfer and exchange with the external environment [8,18,23].

Table 1 Biosynthesis of alkaline protease by *Bacillus* sp. strain NPST-AK15

Carriers	Alkaline protease activity (U/ml)	Specific productivity (U/g wet weight cells/h)
Free cells*	1203.1	4820.11
Ca-alginate	923.4	3214.34
Agar	821.2	2008.01
Gelatin	655.2	2625.00

Cells immobilized in different carriers using batch fermentation. The same amount of cell biomass was used in both the free and immobilized cells. SDs (*n*=3) were 2.0–3.5%. *Inoculation was performed with 0.5 g of viable wet cell pellets.

Optimization of cell immobilization in alginate beads

Among the tested carriers for *Bacillus* sp. NPST-AK15 immobilization, Ca-alginate beads showed the highest specific productivity and protease activity. Consequently, it was used for further investigation. Alginates are a class of polysaccharides that consist of unbranched copolymer of D-mannuronic acid and α-l-guluronic acid with varying ratios depending on the organism from which they are isolated [24]. Alginate is nontoxic and cost-effective and can be prepared easily. In addition, whole cell entrapment in alginate beads is performed under mild condition with no effect on the cell growth and viability [14]. Therefore, alginate has been applied as a carrier for various enzymes. However, the efficiency of the immobilization by entrapment in alginate beads is based on various parameters. To enhance the productivity of *Bacillus* sp. NPST-AK15 immobilized in alginate gel, the effect of various factors on the beads preparation was studied including alginate concentration, bead size, and biomass loading.

Effect of various alginate concentrations

The optimum concentration of Na-alginate used for immobilization of *Bacillus* sp. NPST-AK15 cells was determined by preparation of the alginate gel using different Na-alginate concentrations. The results shown in Table 2 indicated that the sodium alginate concentration used for preparation of the immobilization *Bacillus* sp. NPST-AK15 cells had significant effect on the alkaline protease production. Maximum enzyme production (986.1 U/ml) and specific productivity (3950.7 U/g wet cells/h) were

Table 2 Effect of alginate concentration on alkaline protease production by immobilized *Bacillus* sp. strain NPST-AK15

Alginate concentration (%)	Alkaline protease activity (U/ml)	Specific enzyme productivity (U/g wet cells/h)	Leaked cells (%)*
1	931.2	3730.8	12.2
2	929.2	3722.8	8.9
3	**986.1**	**3950.7**	**3.9**
4	788	3157.1	2.2
5	515.3	2064.5	1.3

SDs (*n*=3) were in range of 2.1–4.0%. *This was calculated from the original concentration of cells in the beads prepared from 10 ml alginate gel (0.5 g wet cells).

seen using sodium alginate concentration of 3% for preparation of the alginate beads. Further increase of alginate concentration led to significant decrease in the enzyme yield, which can be attributed to limitation of nutrients and oxygen. In addition, accumulation of the secondary metabolites, owing to less porosity, can lead to change of pH of the microenvironment within the alginate beads [14,18]. On the contrary, preparation of the alginate beads using lower sodium alginate concentration (1.0–2.0%) resulted in high NPST-AK15 cells leakage, owing to the large pore size of the alginate beads prepared using such low concentration of alginate [8].

Effect of alginate bead size

The influence of alginate bead size on alkaline protease production by the immobilized *Bacillus* sp. strain NPST-AK15 cells was investigated. Alginate beads with different sizes (1.5–5.4 mm) were prepared using suitable needle for extrusion of the alginate into the $CaCl_2$ solution, with equal amount of *Bacillus* sp. NPST-AK15 cells biomass (Photo 1). The results presented in Table 3 revealed that the production of alkaline proteases by the immobilized cells is inversely proportional to the alginate bead size. This can be attributed to the enhancement of mass transfer owing to the increased surface area of the smaller beads [24]. However, it was found that the *Bacillus* sp. NPST-AK15 cell leakage was increased as the beads' size decreased. Therefore, alginate beads with 3.5-mm diameter were found to be the optimum alginate bead size for production of alkaline protease by immobilized NPST-AK15 cells and was used for further investigation. Similar observation was reported by Adinarayana *et al.* [19] and Potumarthi *et al.* [25].

Effect of cell biomass loading

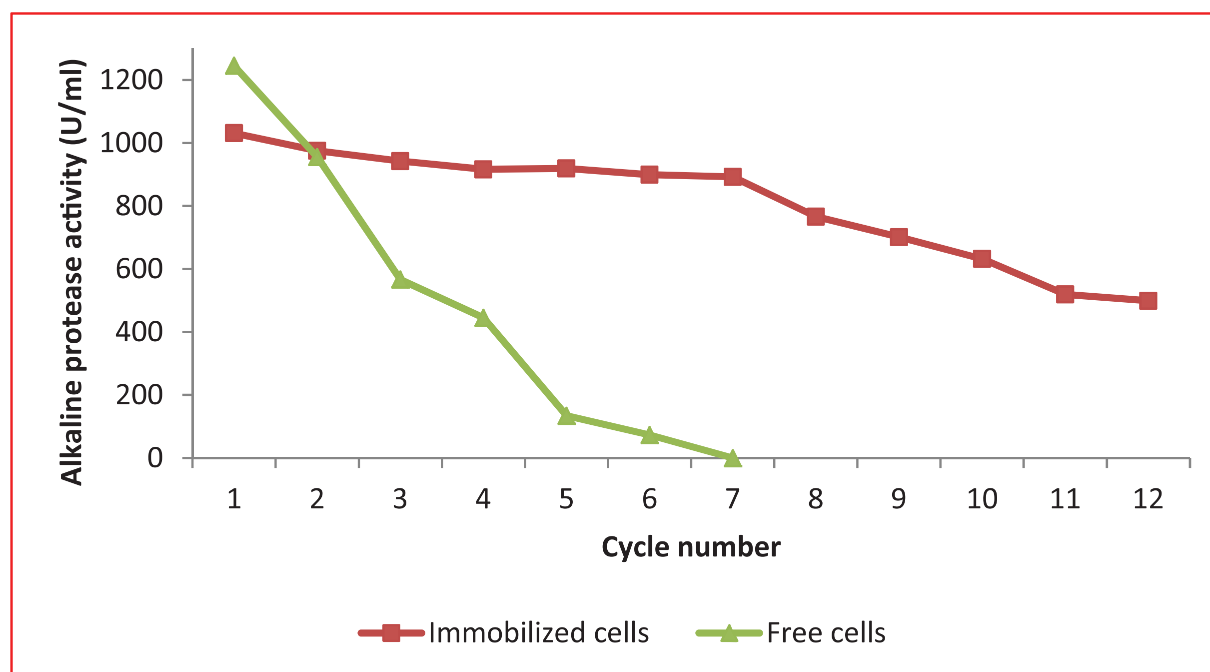
The effect of cell loading on alkaline protease production by immobilized *Bacillus* sp. NPST-AK15 was studied by immobilization of different amount of cell biomass ranged from 0.25 to 1.5 g (wet weight) using the same amount of 3.0% alginate. The beads were transferred into 50 ml of the production medium, and fermentation was conducted for 24 h. The results revealed that immobilization of *Bacillus* sp. NPST-AK15 using 0.50 wet weight cell biomass per 0.3 g Na-alginate showed the maximal alkaline protease production and productivity (1020.1 U/ml and 4086.9 U/g wet weight cell biomass per hour,

Table 3 Influence of bead size on alkaline protease production by immobilized *Bacillus* sp. strain NPST-AK15

Bead diameter (mm)	Alkaline protease activity (U/ml)	Specific enzyme productivity (U/g wet cells/h)	Leaked cells (%)
1.5	1111.2	4451.9	9.7
2.2	989.2	3963.1	4.1
3.5	892.7	3576.5	3.2
4.6	812.9	3256.8	3.0
5.4	702.3	2813.7	2.8

SDs (n=3) were in range of 2.0–3.5%.

Photo 1



Bacillus sp. NPST-AK15 immobilized in Ca-alginate beads with different sizes.

respectively) (Table 4). Further increase of the cell biomass caused slight increase of the enzyme production, but the specific alkaline protease productivity was significantly decreased. Similar pattern was reported for protease production by *Teredinibacter* turnerae and *B. subtilis* – K 30 [18,26,27].

Alkaline protease production in repeated batch fermentation

The efficiency of *Bacillus* sp. NPST-AK15 immobilized in alginate beads for production of alkaline protease in repeated batch fermentation was evaluated up to 12 cycles. The results shown in Fig. 1 indicated that the productivity of free NPST-AK15 cells was significantly decreased up to 45.5% of the initial productivity after three cycles of repeated batch fermentation, and completely lost after seven cycle. On the contrary, the productivity of *Bacillus* sp. NPST-AK15 was significantly improved upon cell immobilization in alginate beads, showing residual productivity of 89.1 and 61.3% after five (120 h) and ten cycles (24 h), respectively. The stability of the immobilized cells of *Bacillus* sp. NPST-AK15 in repeated batch fermentation was superior to that reported for the production of alkaline protease by *B. subtilis* PE-11 immobilized in alginate beads, retaining only 71% of its initial activity after the fifth cycle [19].

Table 4 Effect of cell biomass/alginate ratio on alkaline protease production by immobilized *Bacillus* sp. strain NPST-AK15

Cell biomass weight (g)	Alkaline protease activity (U/ml)	Specific enzyme productivity (U/g wet cells/h)
0.25	489.3	3920.7
0.50	**1020.1**	**4086.9**
0.75	1285.2	3570.0
1	1241	2585.4
1.5	1213.9	1686.0

Different weights of the cell biomass were added to the same amount of 3% Na-alginate. SDs (n=3) were in range of 3.9–4.6%.

Conclusion

Alkaline protease production by alkaliphilic *Bacillus* sp. strain NPST-AK15 immobilized in various matrices by cell entrapment approach was investigated. Alkaline protease production by *Bacillus* sp. strain NPST-AK15 immobilized in alginate gel exhibited the highest protease production and cell productivity in comparison with other tested matrices. The alkaline protease production and cells productivity were significantly improved by optimizing the alginate concentration, alginate beads size, and cell biomass per alginate gel ratio. The immobilized *Bacillus* sp. NPST-AK15 exhibited high operation stability up to 10 cycles of repeated batch fermentation.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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Figure 1

Alkaline protease production of free and immobilized (in alginate beads) cells of *Bacillus* sp. strain NPST-AK15 in repeated batch bioprocess.

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