Comparative studies of free and immobilized phytase, produced by *Penicillium purpurogenu* GE1, using grafted alginate/carrageenan beads

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Received 22 March 2015
Accepted 24 April 2015

Egyptian Pharmaceutical Journal
2015, 14:87–93

Aim
The aim of the study was to immobilize the phytase enzyme produced by *Penicillium purpurogenu* GE1 on grafted alginate/carrageenan beads and study the properties of the immobilized enzyme in comparison with free ones.

Materials and methods
The immobilization conditions were first optimized and then the optimum conditions of temperature and pH for the maximum activity of the immobilized and free enzyme were studied and compared. The stabilities of both immobilized and free phytase at moderate and low temperatures of 50 and 4°C, as well as their stability at the acidic pH of 4, were also studied. Finally, the activity of the immobilized enzyme was monitored over 20 successive repeated batches.

Results
The maximum loading capacity was obtained after 20 h at the enzyme/acetate buffer dilution ratio of 1 : 2. The optimum temperature and pH of the immobilized enzyme, as compared with free state, were found to have shifted from 37 to 45°C and from pH 5.5 to 4, respectively. Moreover, the results also proved that when phytase in both immobilized and free states was subjected to an acidic pH of 4 for 45 min, or to a moderately high temperature of 50°C for 60 min, the activity of the former remained stable, whereas that of the latter showed substantial losses. In contrast, at the refrigerator shelf temperature of 4°C, dry and wet immobilized forms retained 100% activity for 12 weeks, whereas that of the free enzyme was completely lost within a shorter period of 4 weeks. Furthermore, the activity of the phytase enzyme immobilized on gel beads was maintained at the 100% level for more than 12 repeated batch utilizations of the beads.

Conclusion
The results revealed that the physiological parameters of the immobilized enzyme were greatly improved compared with the free state. Further, the activity of the phytase enzyme immobilized on gel beads was maintained at the 100% level for more than 12 repeated batch cultivations of the beads.

Keywords:
alginate, carrageenan, *Penicillium purpurogenu* GE1, phytase, phytate

Egypt Pharm J 14:87–93

1687-4315

Introduction

The main role of the phytase enzyme is to catalyze the hydrolysis of inositol hexakisphosphate (phytate), releasing inorganic phosphate, thereby improving the bioavailability of the phosphate, originating from the phytate present in food [1–3]. According to this, the addition of phytase to the feed stock improves the utilization of the phosphorus content of phytate by simple stomached animals, such as swine and poultry, as such animals have a small percentage of phytase activity in their gastrointestinal tracts and are hence unable to digest phytate on their own. Moreover, phytase was found to have a potential effect in the reduction of blood clots, cholesterol, and triglycerides, thereby preventing heart diseases [4]. Also, several studies have shown that phytase greatly helps in the prevention of renal stone development [5,6]. Therefore, it could be stated that, generally, the addition of phytase enzyme to plant-based food increases their nutritional value. However, it was found that the immobilization of biocatalysts, including phytase, is an essential factor for their stabilization. For example, the immobilization of enzymes by covalent binding enables their reuse several times, which results in the reduction of their total cost as well as that of their products [7,8]. It also has the advantage of keeping the enzymes well bound to the carrier and hence avoids their diffusion into the fermentation medium. This is why this technique is widely preferred on the industrial scale. Consequently, the search for efficient and economic carriers is considered one of the most important problems in enzyme immobilization. Over the past few years, some authors such as Elnashar and colleagues [9,10] have

successfully immobilized enzymes by their covalent binding to hydrogels.

The aim of this study was the optimization of the immobilization conditions of phytase enzyme, produced by *Penicillium purpurogenu* GE1, on grafted alginate carrageenan gel beads. Furthermore, a comparative study of the optimum cultivation conditions as well as the stability of free and immobilized phytase under different conditions was performed.

Materials and methods

Microorganism

The microorganism under study was isolated in a previous study [11] from the rhizosphere soil sample of bean root nodules. In the latter study, the isolated strain was identified using the 18s rDNA sequence analysis method and deposited at the National Research Center culture collection as *P. purpurogenu* GE1.

Solid-state fermentation

The fermentation medium used for the enzyme production process was optimized in a previous study [11] and consists of (%) 7.5 g glucose, 3.75 g peptone, and 0.1 ml Tween 80. The experiments were performed in 250 ml Erlenmeyer flasks, each containing 10 ml of the above-mentioned medium supplemented with 5 g of a dry substrate composed of equal amounts of corn cob and wheat bran. The pH of the final suspension was adjusted at 8. After their sterilization at 121°C and 1.5 atm for 20 min, the flasks were inoculated with an inoculum size of 1.0 ml/flask, containing 5×10^6 spores of *P. purpurogenu* GE1. The contents of the flasks were well mixed and then incubated for 120 h at 27°C as reported by Awad *et al*. [11].

Analytical method for enzyme assay

At the end of the incubation period, the phytase enzyme was extracted by adding distilled water to the solid state fermentation (SSF) samples at a concentration of 20:01 (ml/g). The obtained suspensions were shaken at 200 rpm for 1 h and centrifuged at 5000 rpm for 20 min. The supernatants were then individually collected and subjected to enzyme assay. The reaction mixture consisted of 0.9 ml of acetate buffer (0.1 mol/l, pH 5.5), 1 mmol/l phytate, and 0.1 ml of the supernatant containing the produced phytase enzyme. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. The aliquot was subsequently analyzed for liberated inorganic phosphate, as described by Fiske and Subbarow [12] and Singh and Satyanarayana [13]. One unit of phytase is defined as the amount of enzyme releasing 1 μmol of inorganic phosphorus per ml per minute, under the assay conditions. The activity of the enzyme was expressed as units per gram dry substrate (U/g ds).

Protein determination

The protein concentration of free enzyme was determined, as described by Lowry *et al.* [14], using BSA as a standard protein. The protein concentration of the immobilized enzyme Pg (mg/g) was calculated according to the protein concentration in the initial solution as well as that of the unbound protein, using the following equation:

$$\mathrm{Pg} = \frac{C_o V_o C_f V_f}{w}$$

where C_o is the initial protein concentration (mg/ml), C_f is the protein concentration of the filtrate (mg/ml), V_o is the initial volume of the enzyme solution (ml), V_f is the volume of filtrate (ml), and w is the weight of gel carrier used (g).

Preparation of grafted alginate/carrageenan

Uniform alginate/carrageenan beads were prepared as per the two-step method [15] as follows: in the first step, which represents the hardening step, equal amounts of alginate and carrageenan were dissolved in distilled water to a final concentration of 2% (w/v), and then dropped through the 300 μm nozzle of an encapsulator (Innotech encapsulator IR50; Switzerland, Muhleweiher 4, 8606 Greifensee) (Fig. 1) into two successive hardening solutions of pH 8. The first was an aqueous 2% (w/v) $CaCl_2$ solution, whereas the second was prepared by dissolving 2% (w/v) $CaCl_2$ in

Figure 1

Innotech encapsulator IR50 Switzerland: for gel bead preparation.

4% (v/v) polyethylenimine. The latter solution provides the beads with amino groups. The beads were incubated in each solution for 2 h. In the second step, the treated gels were soaked in an aqueous solution of 2.5% (v/v) glutaraldehyde for a further incubation period of 2 h to incorporate aldehyde groups into the treated beads.

Immobilization of phytase on grafted alginate/carrageenan beads

The immobilization process was performed by soaking 0.5 g of the gel beads for 24 h in a 5 ml solution composed of 0.1 mol/l acetate buffer (pH 5.5), 3150 U phytase enzyme, and 99 mg protein. The gel beads were then soaked for 30 min in acetate buffer of the previously mentioned molarity and pH to eliminate any unbound enzyme. The beads containing the immobilized enzyme were stored at 4°C for further use. To determine the activity of the immobilized enzyme, 0.5 g of gel beads was added to 5 ml of 0.1 mol/l acetate buffer (pH 5.5) solution containing 1 mmol/l sodium phytate and incubated for 30 min at 37°C. The reaction was then stopped by the addition of 1 ml of 10% trichloroacetic acid. An aliquot of the reaction mixture was assayed for the amount of liberated inorganic phosphate as mentioned before in the Materials and methods section.

Optimization of the enzyme-loading capacity

To determine the optimum enzyme concentration for the maximum enzyme-loading capacity (ELC) of the gel, each 0.5 g of beads was incubated for 24 h in 5 ml of 0.1 mol/l acetate buffer solution (pH 5.5) containing different concentrations of the enzyme. The final enzyme/acetate buffer dilution ratios from which these 5 ml solutions were taken ranged between 01:20 and 01:01. Also, determination of the optimum incubation time for the maximum ELC was performed by incubating the same mass of beads in the above-mentioned buffer solution containing the selected optimum enzyme concentration for different periods of time ranging from 2 to 24 h.

Optimum pH

The optimum pH for the activity of both free and immobilized phytase was determined by incubating the enzyme in the two states, each in 5 ml of an appropriate buffer solution containing 1 mmol/l of sodium phytate, at 37°C for 30 min. The buffer solutions used were either phosphate or acetate buffer adjusted at different pHs ranging between 3 and 8. The highest value of enzyme activity, recorded at the most optimum pH, was considered to represent 100% activity. Moreover, percentage activities of the enzyme recorded at each pH were expressed as percentages of that obtained at the selected optimum pH.

Optimum temperature

The optimum temperatures for the activity of both free and immobilized phytase were determined by incubating the enzyme in the two states, each in 5 ml of the above-mentioned reaction mixture, for 30 min at different temperatures ranging from 30 to 65°C. The highest value of enzyme activity recorded at the most optimum temperature was considered to represent 100% activity. Moreover, percentage activities of the enzyme, recorded at each temperature, were expressed as percentages of that obtained at the selected optimum temperature.

pH stability

The stability of the enzyme in both free and immobilized states at the acidic pH of 4 as an approximated representative of the stomach pH was tested by their incubation in 0.1 mol/l acetate buffer solution, at pH 4, for a period of 2 h. The enzyme activities were then determined, as mentioned above, and expressed as percentages of those recorded at the optimum pH conditions for the enzyme in each state.

Thermal stability

The stability of the enzyme in both free and immobilized states at a relatively high temperature of 50°C was tested by their incubation in 0.1 mol/l acetate buffer solution, at pH 5.5, for a period of 120 min. The enzyme activities were then assayed, as mentioned above, and expressed as percentages of those recorded at the optimum temperature conditions for the enzyme in each state.

Shelf stability

The shelf stability at 4°C of the phytase enzyme in different forms was studied. Therefore, three different phytase samples were prepared as follows:

Sample 1: 50 ml of cell-free culture filtrate, containing crude phytase enzyme.
Sample 2: 10 g of gel beads, in wet state, loaded with immobilized phytase enzyme. The beads were soaked in 100 ml of 0.1 mol/l acetate buffer solution at pH 4 to stay moist.
Sample 3: 0.2 g of lyophilized dry gel beads (originating from 10 g of wet beads) loaded with immobilized phytase enzyme.

The shelf stability of the three samples was tested by monitoring their activity over a period of 12 weeks at 4°C. These activities were tested weekly in 0.1 ml clear culture broth from sample 1, in 0.5 g gel beads from sample 2,

and in 0.01 g dry beads from sample 3, as previously mentioned. The initial activities, recorded for the three samples at the beginning of the experiment, were considered as 100% activity levels. However, the different activities determined during the course of the experiment were expressed as percentages of those initial activities.

Operational stability

The reusability of phytase enzyme, immobilized in alginate beads, was studied by incubating 0.5 g of loaded gel beads in 5 ml of the previously mentioned reaction mixture for 30 min. The enzyme activity was then assayed, as previously mentioned, and considered as the 100% activity. After that, the gel beads were carefully recovered from the reaction mixture, washed thoroughly with acetate buffer (pH 5.5), and reincubated in 5 ml of a freshly prepared reaction mixture for a subsequent incubation period of 30 min. This step was repeated 20 times and the enzyme activity, recorded at the end of each incubation time, was expressed as a percentage of that obtained after the first batch.

Results and discussion

Enzyme-specific activity and protein content determination

The activity and protein content of the crude enzyme were assayed and found to be 444 U/g dry substrate and 14 mg/g dry substrate, respectively. Hence, the specific activity was calculated as 31.71 U/mg protein. The immobilization efficiency for grafted alginate/carrageenan gels was calculated to be 3000 U/g gel beads. According to the above equation, the amount of immobilized enzyme protein was found to be 35 mg. The specific activity was calculated to be 85.71 U/mg protein.

These results indicated that the specific activity of the immobilized phytase enzyme increased, resulting in an approximately three-fold purification. This could be attributed to the fact that most enzyme protein successfully bound to the carrier leaving proteins, of any other origin, in the solution [16].

Optimization of the enzyme-loading capacity

Optimization of the enzyme concentration

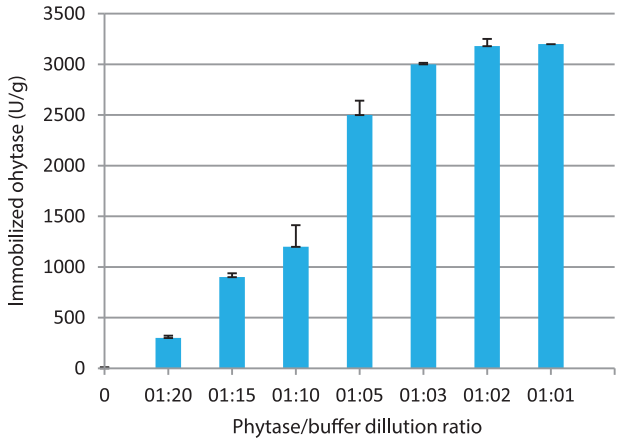
The optimum concentration of the crude enzyme for the maximum ELC of the beads was studied. Accordingly, each batch of 0.5 g of gel beads was soaked in 5 ml of 0.1 mol/l acetate buffer (pH 4) solution containing different concentrations of the enzyme, as mentioned in the Materials and methods section. The results showed that ELC increased gradually by increasing the enzyme concentration, reaching 3200 U/g gel beads when the 5 ml enzyme was taken from the buffer solution in which the enzyme/acetate buffer dilution ratio was 01:02, after which any increase in the concentration of the enzyme was found to have an insignificant effect on the ELC, as shown in Fig. 2.

The explanation for this result can be found in the study conducted by Elnashar *et al.* [9], who stated that, at a dilution ratio of 01:02, most of the aldehyde groups were engaged with the enzyme units; however, no more aldehyde groups were available for any excess enzyme units. This result is in agreement with those obtained by Elnashar *et al.* [10] and Danial *et al.* [15].

Optimization of the enzyme loading time

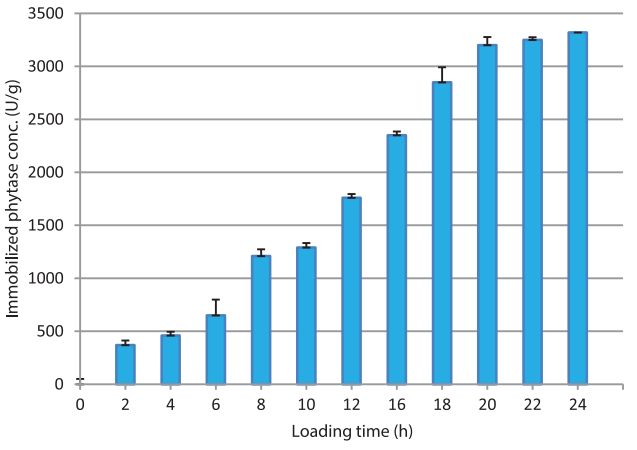
The results illustrated in Fig. 3 reveal that the ELC increased gradually by increasing the loading time, reaching ELC of 3359 U/g gel beads after 22 h of

Figure 2



Effect of different phytase concentrations on the enzyme-loading capacity of grafted alginate/carrageenan beads.

Figure 3



Effect of different loading times on the enzyme-loading capacity of grafted alginate/carrageenan beads.

incubation, after which any increase in the loading time had only a slight effect on the amount of the loaded enzyme.

Comparative studies of some physiological parameters of phytase enzyme in free and immobilized states

Optimum pH

The results in Fig. 4 indicate that the optimum pH value of the immobilized enzyme was more acidic than that of the free phytase, as the optimum recorded pH values were 4 and 5.5, respectively.

This shift in the optimum pH value was favorable as this will result in an increased enzyme toleration to digestion conditions, as the average pH of the stomach ranges between 2.25 and 4. This low pH is optimal for phytase to hydrolyze the phytate present in fodder, as well as for decreasing the ability of phytic acid to chelate with metal ions, thus improving the phosphorus bioavailability and reducing the phosphorus excretion in the environment and eventually decreasing the environmental pollution [17].

Optimum temperatures

As shown in Fig. 5, the operational temperature of the immobilized enzyme exceeded that of the free state as the optimum temperature of the free enzyme was seen to have shifted from 37 to 45°C after immobilization.

The shift of the optimum temperature toward higher temperature values when the biocatalyst was immobilized indicated that the enzyme structure was strengthened by the immobilization process. This could be due to the formation of a molecular cage around the protein molecule (enzyme) to protect the enzyme from the high temperature of the bulk solution [18,19].

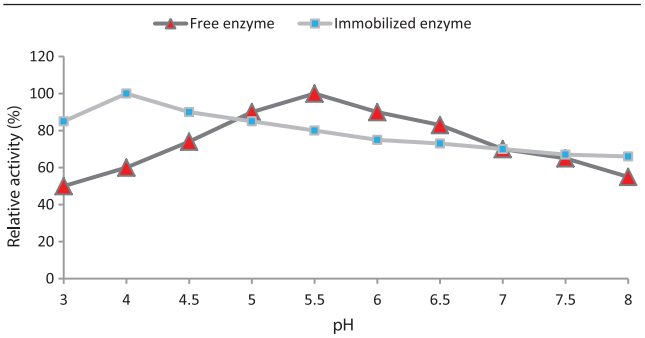
pH stability

The results presented in Fig. 6 reveal that, when the loaded beads were subjected to a relatively low pH of 4 for 45 min, the activity of immobilized phytase was maintained at 100%. However, the free enzyme activity dropped to 54% under the same conditions. On the other hand, the results showed that increasing the exposure time to 120 min affected the phytase enzyme in both states to different extents, as the activities of the immobilized enzyme decreased to about 60%, whereas that of the free enzyme was completely lost by the end of the experiment.

Thermal stability

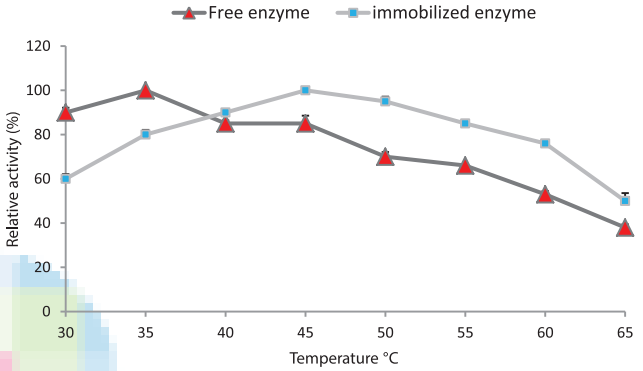
The results presented in Fig. 7 reveal that when the loaded beads were subjected to a relatively high temperature of 50°C for 60 min, the activity of the immobilized phytase was maintained at 100%. However, the free enzyme activity dropped to 54% under the same conditions. On the other hand, the results showed that increasing the exposure time at the same temperature up to 120 min greatly affected the phytase enzyme in both states, as the activities of phytase, in both free and immobilized forms, were

Figure 4



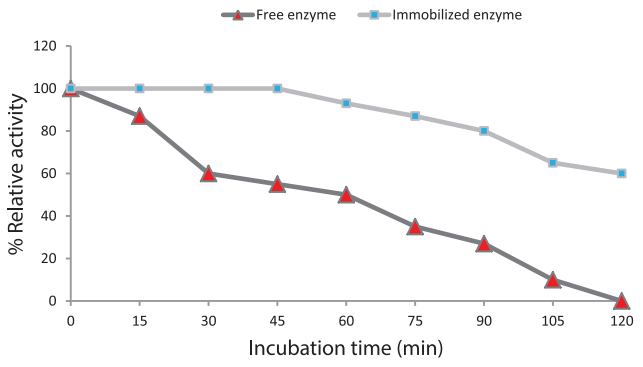
Effect of different pH values on phytase enzyme, free or immobilized, on grafted alginate/carrageenan beads.

Figure 5



Effect of different temperatures on phytase enzyme, free or immobilized, on grafted alginate/carrageenan beads.

Figure 6



pH stability profile of free and immobilized phytase at pH 4.

found to decrease to10 and 53%, respectively.

Bing *et al.* [20] reported that the immobilized phytase, produced by *Aspergillus ficuum*, resisted heat denaturation at 70°C in comparison with its free form.

Shelf stability

The data in Fig. 8 indicate that samples 2 and 3, representing immobilized phytase in both wet and dried beads, maintained over 100% activity for a shelf incubation period of 12 weeks at 4°C, compared with a complete loss of the activity of the free enzyme (sample 1) after only 4 weeks of incubation under the same condition.

Operational stability

The data in Fig. 9 indicate that the immobilized phytase maintained 100% activity for 12 successive batches and then gradually declined, reaching around 70% activity after 20 cycles.

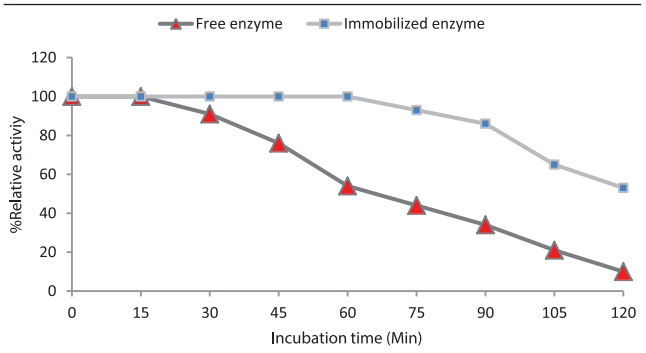
These results outweighed those of Tanaka *et al.* [21], who used polyethylene amine to immobilize glucoamylase enzyme on alginate beads, and stated that only 60% of the enzyme activity was recorded after eight successive repeated batches.

Moreover, the slight decrease in enzyme activity that was noticed after the 20th batch of the grafted gels utilization might be attributed to the inactivation of the enzyme due to continuous use, as explained by Nakane *et al.* [22], who studied the repeated batch utilization of the invertase enzyme immobilized in a gel fiber composite of cellulose acetate and zirconium alkoxide.

Conclusion

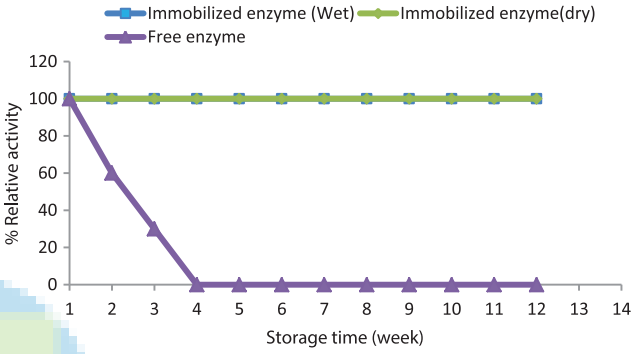
Improvement of phytase properties by an immobilization process is considered one of the most important developments in the fodder industry. The acidity and thermal stability, as well as the reusability, of the loaded beads are considered fundamental properties, encouraging food supplementation with immobilized phytase and eventually solving the phytate digestion problem of poultry and swine. The main aim of this study focused on two major tasks: first, the immobilization of phytase enzyme in alginate/carrageenan beads at a maximum loading capacity (ELC); and second, evaluation of the improved properties of the phytase enzyme in the immobilized state compared with those of the free state. The first task was successfully accomplished, as the maximum ELC was obtained by adjusting the enzyme protein concentration as well as the most proper loading time. The second task revealed that the optimum temperature and pH of the immobilized enzyme, as compared with the free state, were found to be favorably shifted from 37 to 45°C and from pH 5.5 to 4, respectively. Moreover, the results also proved that, unlike the free enzyme, the immobilized enzyme was able to sustain an acidic condition of 4 for 45 min,

Figure 7



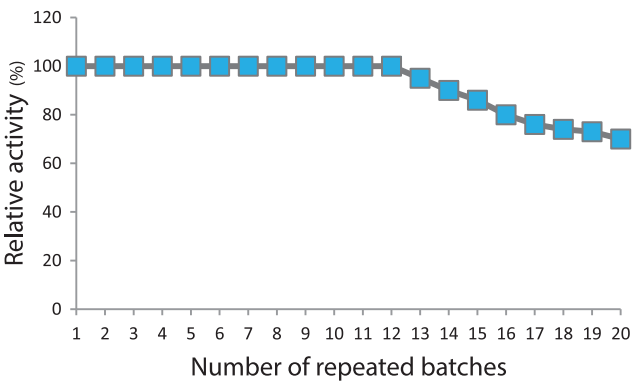
Temperature stability profile of free and immobilized phytase at 50°C.

Figure 8



Shelf stability at 4°C of phytase enzyme, free or immobilized, on grafted alginate/carrageenan beads in wet and dry states.

Figure 9



Effect of the reusability of phytase-loaded grafted alginate/carrageenan beads on the immobilized enzyme activity.

or a relatively high temperature of 50°C for 60 min, as well as the refrigerator shelf temperature of 4°C for an incubation period of 12 weeks. Furthermore, the activity of phytase enzyme immobilized in gel beads was maintained at the 100% level for more than 12 repeated batch cultivations of the beads.

Acknowledgements

The authors acknowledge the National Research Center, ID: 60014618, for providing financial support for this work.

Conflicts of interest

There are no conflicts of interest.

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