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Immobilization of Two Newly Isolated Proteases onto Mesoporous Silica Nanoparticles

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Running title: **Protease Immobilization**

Abstract
Proteases are versatile group of enzymes with indispensable function and diverse application. Because of their proteinous structure they are susceptible to inactivation and denaturation which limits their practical application. The immobilization on a suitable surface would enhance their stability in hostile environment and broader further their application. Two proteases, purified from actinomycete strain *Microbispora aerata* 11A were immobilized on mesoporous silica nanoparticles using APTES - glutaraldehyde method of immobilization. Temperature and pH optimum and stability of both free and immobilized enzymes were assessed as well as their operational stability. The resulted immobilized preparation kept similar biochemical characteristics of the free enzymes (t_{opt}= 70ºC and pH_{opt}=7.0) and at the same time significantly improved their stability. After being subjected to 10 operational cycles (each one with duration of 60 min at 50^0C) the immobilized preparations indicated a maximum activity (96%).

Practical applications
The immobilized proteases in this study can be used in self-cleaning surfaces with a prolonged activity thanks to the broader pH and temperature stability and higher operational stability as well.

Key words: immobilized proteolytic enzymes, nanoparticles, stability

Introduction

Immobilized enzymes are defined as "enzymes which are physically confined or localized in a certain defined region of space with retention of their catalytic activities and which can be used repeatedly and continuously" (Brena et al., 2013).
Basically, the methods of enzyme immobilization can be categorized into four major categories: adsorption, covalent bonding, entrapment, and cross- linking (Altinkaynak et al., 2016). Each of these methods results in structural changes in enzyme's molecule and its microenvironment (Krajewska, 2004). Therefore, the major components of an immobilized enzyme are the enzyme, the matrix, and the mode of interaction of the enzyme with the carrier (Mohamad et al., 2015). With the sole exception of the enzyme, the most important contributing component to the performance of an immobilized enzyme system is the carrier (Carpio et al., 2011). Although, there is no universal support material certain characteristics should be considered such as having high affinity for protein, availability of reactive functional groups, mechanical stability, rigidity, feasibility of regeneration, non-toxicity and biodegradability (Mohamad et al., 2015). A wisely chosen carrier can increase the operational stability of the immobilized enzyme.
Mesoporous silica nanoparticles have recently attracted attention as potential nanocontainers due to their high stability, biocompatibility, large surface area, controllable pore diameter and easy surface fictionalization (Falcón et al., 2016). It can be formed into different structures depending on the application. Mesoporous silica may be formed by using a template molecule which acts as a molecular spacer. The precursor molecules of tetraethylorthosilicate (TEOS) condense around the micelles formed by the template in solution effectively making a shell framework. When the template is removed by physical or chemical means, what remains is a mesoporous silica network with a low mass density. A common surfactant used for these preparations is cetyl trimethylammonium bromide which readily forms micelles in an alcohol solution (Gao et al., 2006).
Proteases (EC 3.4.21-24 and 99) are enzymes that break the peptide bond that links the amino acids in proteins. They are part of hydrolases group of enzymes that break a chemical bond by the addition of a water molecule. Unlike other proteins, proteases are susceptible to autolysis. These enzymes are found to have a wide range of applications, such as constituent in detergents, industrial processing of food, leather and pharmaceutical products.
The aim of the present study is to immobilize two newly isolated proteases (H1BT and H2BT) from *Microbispora aerata* 11A on mesoporous silica nanoparticles using APTES-glutaraldehyde method of immobilization. Thereafter, pH and temperature optimum and stabilities of immobilized protease were investigated and compared to free enzymes. The operational stability for 10 cycles was studied as well.

Materials and Methods

Materials

Mesoporous silica was provided by CREST (Centre for Research in Engineerign Surface Technology), Dublin, Ireland. (3-Aminopropyl) triethoxysilane – APTES, Glutaraldehyde, Casein were purchased from Sigma-Aldrich.

Enzyme immobilization

Prior to enzyme immobilization proteases H1BT and H2BT were mixed in 1:1 ration based on protein concentration and used for further analysis.
The immobilization was carried out by the method described by Nedelcheva and Krastanov (2012).
The degree of immobilization (%) was calculated as follows:

$$\eta = \frac{\text{effectively bound enzyme}}{\text{theoretically bound enzyme}} \times 100$$

$$\text{Effectively bound enzyme} = \frac{A}{B} \times 100$$

$$\text{Theoretically bound enzyme} = \frac{B - C}{B} \times 100$$

where: A - Effectively bound enzyme units; B – Enzyme units used in immobilization; C – unbound enzyme units.

Effect of temperature and pH on immobilized proteases activity

The proteolytic activity of immobilized enzymes was determined in the temperature range 30 - 80°C.
The proteolytic activity of immobilized enzymes was determined in pH range 5.5 -11.0 by measuring the proteolytic activity at the studied pH.

Effect of temperature and pH on free and immobilized proteases stabilities

The stability at 4, 25 and 50°C for 72 hour of free and immobilized was determined by measuring the

enzyme activity every one hour for the first 5 hours and then on the 48th and the 72th hour.
The stability of free and immobilized enzyme was determined by measuring the protease activity after incubation in pH range 7.5-11.0 for 60 min at 25°C.

Determination of operational stability of the immobilized enzymes
The operational stability of the immobilized enzyme is determined by measuring the proteolytic activity at 50°C. One cycle includes: casein hydrolysis for 60 min, recuperation of the immobilized enzyme by centrifugation at 10000xg, triple wash with distilled water and measuring the remaining proteolytic activity.

Proteolytic activity
The protease activity was measured according to the method reported by Jain et al. (2012) with slight modifications. The enzyme was added to 0.6% w/v casein buffered to pH 7.0. The reaction mixture was incubated at 60°C for 10 min thereafter 0.11 M trichloroacetic acid was added to terminate the reaction. The reaction mixture was kept at room temperature for 30 min followed by centrifugation at 4500xg for 5 min. An aliquot of the supernatant was added to 0.5 M sodium carbonate solution and 1N Folin-Ciocalteu reagent and incubated for 30 min at 37°C for color development and the absorbance at 660 nm was measured. The proteolytic activity was calculated in Units per cm^3 which is defined as the amount in micromoles of tyrosine equivalents released from casein per minute and per cubic centimeter of enzyme.
The proteolytic activity of immobilized enzymes was measured as follows: 100 mg of immobilized enzyme is mixed with 0.6 % w/v casein buffered to pH 7.0 and the mixture is homogenized for 10 min at 60°C in a shaker. One unit enzyme activity is defined as the quantity of immobilized enzyme that liberates 1 μM of tyrosine.

Statistical analysis
Experimental results are presented as mean value ± SD of three repetitions. All statistical analyses were conducted using Microsoft Excel.

Results

Proteases immobilization
In this study, both proteases (H1BT and H2BT) were produced simultaneously by the same microorganism (*Microbispora aerata* 11A). In order to take advantage of characteristics of both enzymes, they were co-immobilized on mesoporous silica nanoparticles. The molecular mechanism of their immobilization is presented in Figure 1. The unfunctionalized mesoporous silica particles contain hydroxyl groups on their surface. The reaction with (3-Aminopropyl)triethoxysilane imports amino groups which are further covalently linked with glutaraldehyde as a spacer arm. It was achieved 72 % of degree of immobilization.

Effect of temperature and pH on immobilized proteases activity
The effect of temperature on the activity of immobilized enzymes is presented in Figure 2. The maximal proteolytic activity was measured at 70°C and was maintained high at 75°C and then declined sharply. That temperature optimum is 10°C higher than the one measured for the free enzymes.
The effect of pH on the activity of immobilized enzymes is presented in Figure 3. Maximum activity was measured at pH 7.0.

Effect of temperature and pH on free and immobilized proteases stabilities
In Figures 4, 5 and 6 are presented the data about the effect of continuous temperature exposure at 4°C, 23°C and 50°C respectively on the free and immobilized enzymes activity. At 4°C (Figure 4) the free enzymes kept 80 % of their initial activity for the first 48h of treatment and lost another 10 % till the 72th hour, while the immobilized enzymes remained fully active during the whole duration of this experiment. The influence of incubation at room temperature (22-23°C) of immobilized and free enzymes on their stability is shown in Figure 5. The immobilized enzymes exhibit good stability and maintain their catalytic activity for 5 hours, after which a slight decrease to 89% of the initial activity was observed. According to the obtained results, free proteases are stable at the test temperature. Despite the slight decrease after the first hour, the enzyme retained 89% of its initial activity. After 48 hours, it fell below 80% and remained so until the 72th hour of the experiment. The exposure to 50°C of free and immobilized enzymes has led to rapid loss of activity in 5 hours. However, a lower rate of inactivation was recorded when immobilized enzymes were tested. After 10 min at 50°C, 88% of the initial activity of the immobilized enzyme and 78% of that of the free enzymes were recorded. On the thirtieth minute the immobilized enzymes retained over 60% activity, while free enzymes lost 70% of their activity. Also at the end of the test time almost 40% catalytic

activity of the immobilized and less than 20% of free enzymes was observed.

The stability of free and immobilized enzyme in alkaline pH was studied and the results are presented in Figure 7. During the experiment the pH was monitored and no deviation of the set value was observed. Both, the immobilized and free enzymes exhibited good stability in an alkaline environment. After 60 min at pH 11.0, however, the free enzymes lost 60 % of their activity, while the activity of immobilized preparation was maintained above 90%.

Determination of operational stability of the immobilized proteases

On Figure 8 presents the operational stability of the immobilized preparation. It was studied for 10 cycles at 50°C, each lasting 60 min. From the analysis of the results it is concluded that the immobilized preparation remained its maximum activity (96%) for the entire period of 10 cycles.

Discussion

Proteases constitute one of the most important groups of industrial enzymes. Their versatile applications have led to expansion in their production. In this aspect the enzyme stability in harsh environment is the most important property. For economical and environmental considerations, it is also very important that the enzyme could be removed and reused through a number of duty cycles (Kumari et al., 2015). Various methods for enzyme immobilization exist and their efficacy depends on both enzyme and carrier structure. Porous supports are generally preferred because their high surface area permits a higher enzyme loading and the immobilized enzyme receives better protection from the environment (Mohamad et al., 2015). Nowadays mesoporous silica materials have attracted significant attention for enzyme immobilization because of their better biocompatibility, stable mesoporous structures, larger surface areas, and tunable pore sizes and volumes (Hudson et al., 2008). APTES-modified silica produces terminal amine group (-NH_2) which are found to be useful for covalent coupling of protein to the surface of silica materials (Maria Chong and Zhao, 2003). An additional cross-linking with glutharaldehyde is used to introduce aldehyde groups, as well as to create the so called spacer arm, avoiding thereby steric hindrance and increasing enzyme activity (Zhang et al., 2013). The achieved degree of immobilization (in terms of expressed activity) in the present study (72 %) indicates that the bond between the functionalized support and enzymes does not involve groups from the catalytic center.

The kinetic behavior of a bound enzyme can differ significantly from that of the same enzyme in free solution. This is due to a change in enzymes microenvironment after immobilization. Therefore, the optimum temperature and pH for the immobilized enzymes action are generally shifted. In the present study the optimal temperature for immobilized proteases action was 10°C higher than the one of the unbound enzymes. An increase of the optimum temperature was observed for the other enzymes immobilized on the solid matrix (Aguiar-Oliveira and Maugeri, 2011; Ibrahim et al., 2016). With respect to the pH optimum of immobilized proteases it coincides with that of H1BT.

A main characteristic of immobilized enzymes is their improved stability to harsh environmental conditions. In the present study, the immobilized enzymes presented better thermal stability at all temperatures studied compared to free enzymes, probably due to the multipoint covalent attachment of protease molecules to the matrix that may stabilize the conformation of the enzyme and improve the resistance of the protein to thermal denaturation (Mateo et al., 2007). The improved thermal resistance at high temperatures as well as in alkaline environment makes the immobilized proteases a promising nanobiocatalyst for laundry detergent formulations.

The operational stability of immobilized enzyme is considered as one of the most important property that determines its application in various bioprocesses. It indicates how the activity of immobilized enzyme is affected by the number of times it was used. Although the carrier plays a protective role, in some cases enzyme inactivation occurs. Moreover, a common obstacle is the leakage of enzyme in repeated batch and continuous process (Corîci et al., 2011). Nevertheless, a wisely performed immobilization procedure broadens the enzyme application. Hu et al., (2015) immobilized alkaline protease onto amino-functionalized Fe3O4 nanoparticles yielding 54.2% that retains 50.1% of its initial activity after 10 cycles of successive reuse. The immobilized enzyme was capable of efficiently catalyzing hydrolysis of oat bran into oat polypeptides.

Conclusion

Two newly isolated proteases- H1BT and H2BT from *Microbispora aerata 11A* were covalently immobilized on mesoporous silica nanoparticles

using APTES-glutaraldehyde method of immobilization. The immobilized enzymes exhibited significant improvement of thermal and pH stability with respect to free enzymes. The temperature optimum of immobilized proteases was shifted to higher temperature by 10°C. In addition, the immobilized enzyme exhibited excellent operational stability for ten reaction cycles without losing catalytic activity.

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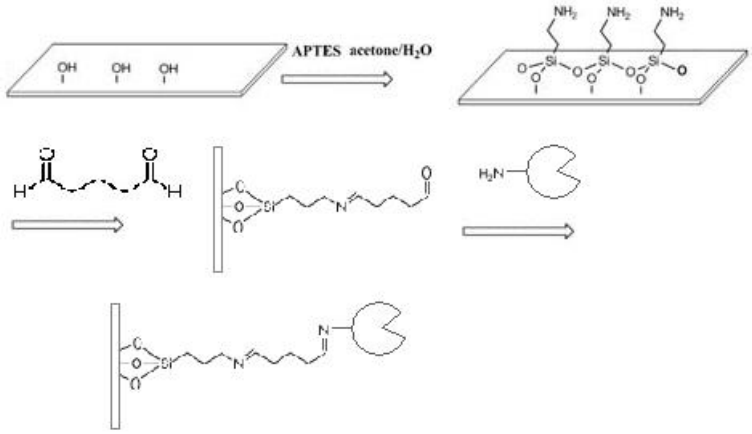

Figure 1.Molecular mechanism of enzyme immobilization

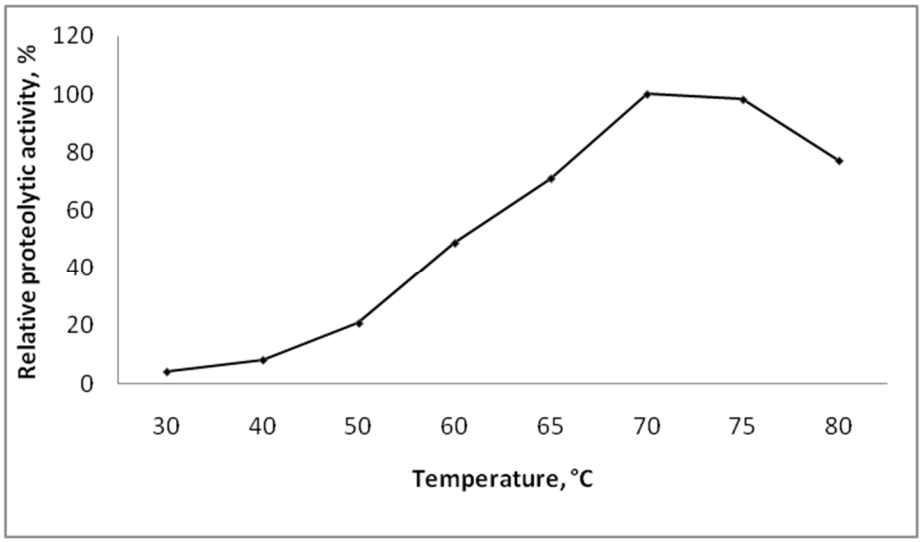

Figure 2. Effect of temperature on the activity of immobilized enzymes

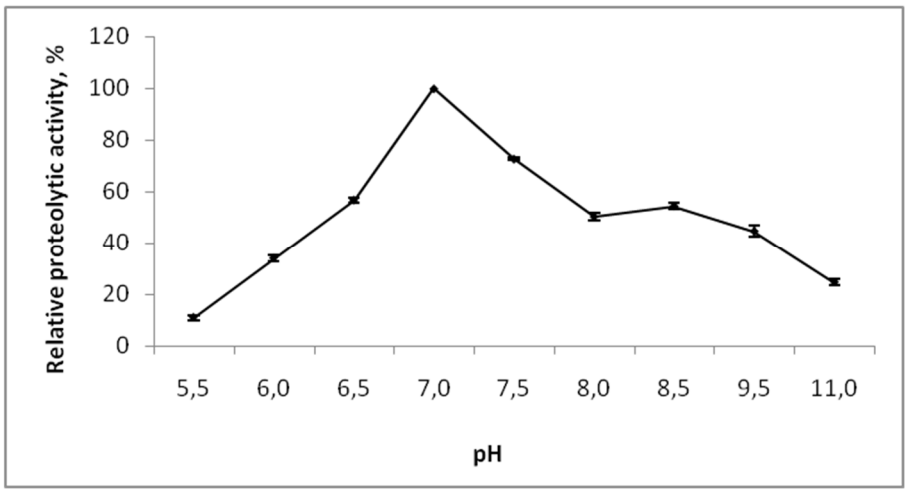

Figure 3. Effect of pH on the activity of immobilized enzymes

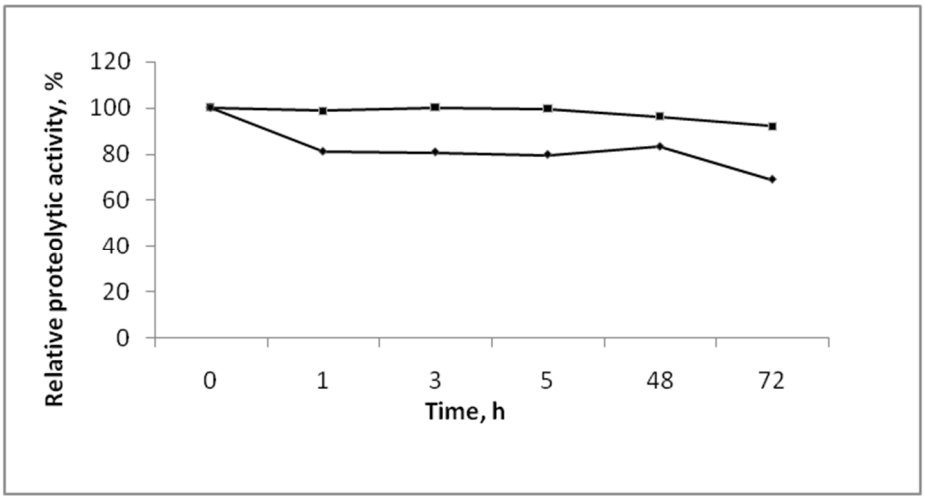

Figure 4. Temperature stability of free (—◆—) and immobilized enzymes (—■—) at 4°C

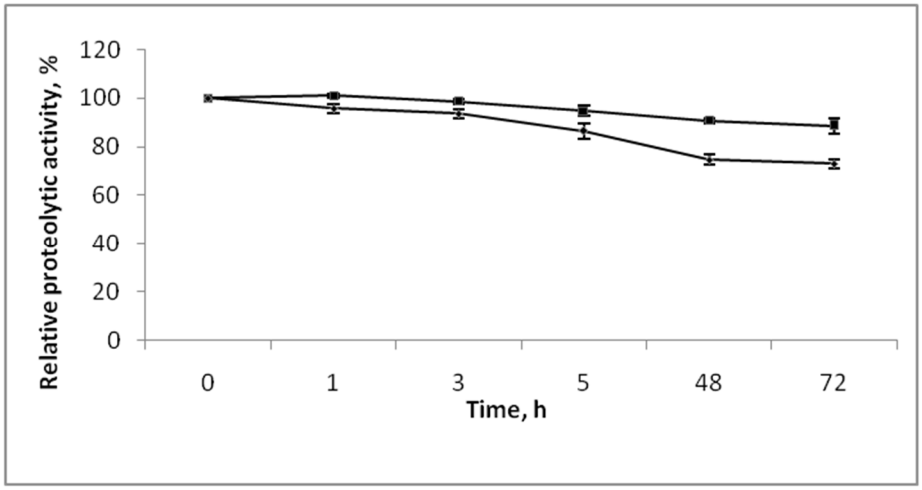

Figure 5. Temperature stability of free (—◆—) and immobilized enzymes (—■—) at 22°C

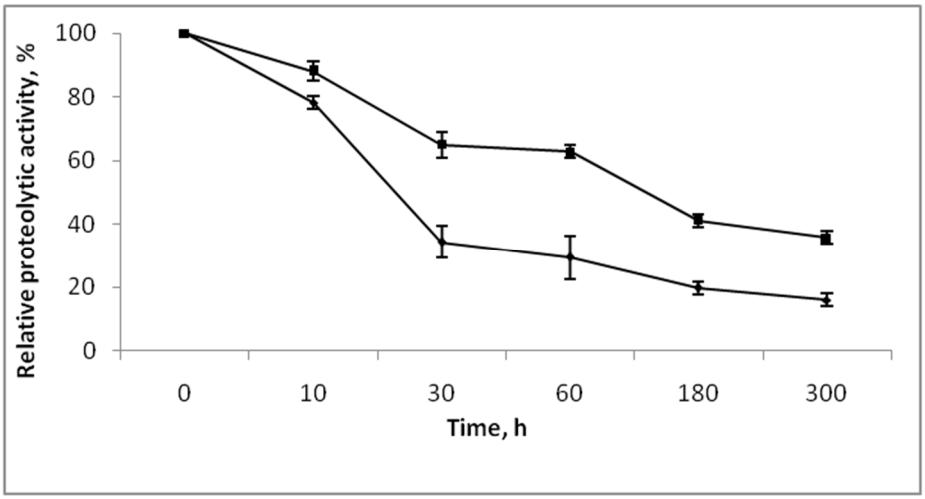

Figure 6. Temperature stability of free (—◆—) and immobilized proteases (—■—) at 50°C

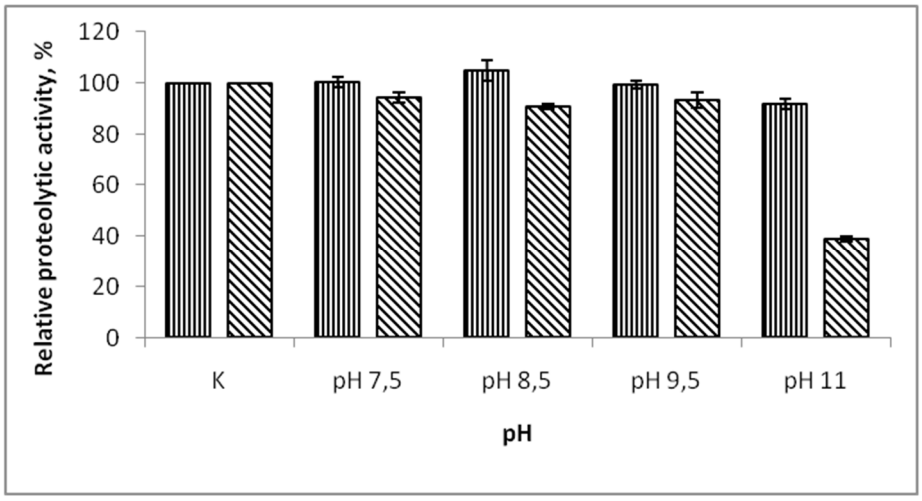

Figure 7. Effect of pH on the stability of free (▨) and immobilized proteases (▥)

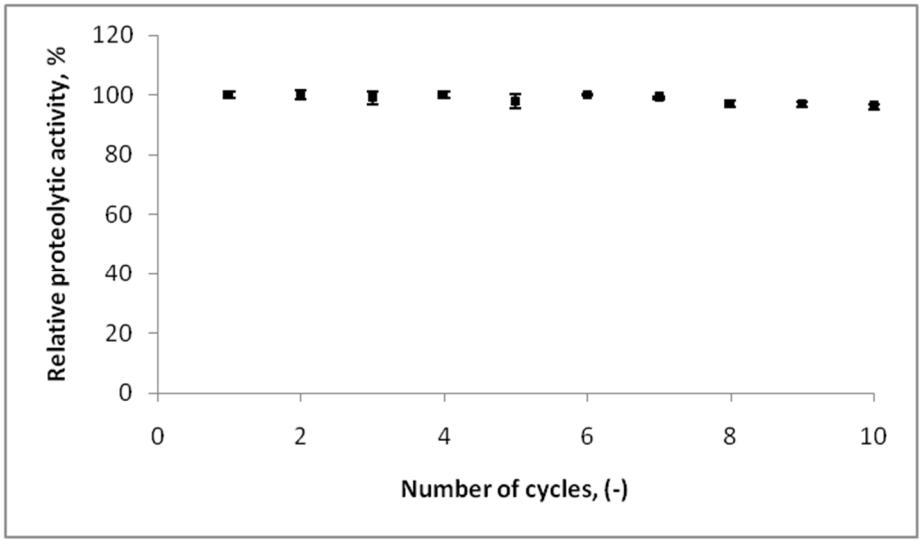

Figure 8. Operational stability of immobilized proteases