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ИММОБИЛИЗАЦИЯ ПЕНИЦИЛЛИНАЦИЛАЗЫ МИЦЕЛИАЛЬНЫХ ГРИБОВ НА *OPUNTIA IMBRICATA*

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Описана иммобилизация внеклеточных пенициллинацилаз из мицелиальных грибов *Aspergillus fumigatus* и *Mucor griceocianus* на *Opuntia imbricata*. *O. imbricata* – кактус (искусственно насажденное растение на севере Мексики). Для обеспечения химической пришивки фермента к носителю перед иммобилизацией проводили активацию носителя путем обработки периодатом в разных концентрациях. Результаты демонстрируют большие возможности *O. imbricata* для иммобилизации ферментов. В случае иммобилизации пенициллинацилаз из мицелиальных грибов наблюдали высокий уровень иммобилизации и высокую удельную активность, а также улучшение операционной стабильности и стабильности фермента при хранении. Предварительная активация носителя периодатом не оказывала заметного влияния на свойства иммобилизованного фермента, что объясняется иммобилизацией пенициллинацилазы на *O. imbricata* в основном за счет адсорбции, а не в результате химического связывания.

INTRODUCTION

Penicillin G acylase (PGA) is the enzyme used industrially to hydrolyze penicillin G to 6-aminopenicillanic acid (6-APA). It is the key intermediate for the production of semisynthetic antibiotics. Penicillin G acylases are produced by various microorganisms, bacteria as well as fungi and PGA from *Escherichia coli* is widely studied and commercially used [1]. However, we have recently described a novel and integrated evaluation of similar PGAs from *Aspergillus fumigatus* and *Mucor griseocyanus*, as a possible industrial alternative [2]. Evidently, the transformation of these potential prospects into a real industrial alternative requires the development of immobilization methods and further stabilization of this interesting enzyme. Enzyme lability is perhaps one of the main drawbacks for the implementation of enzyme derivatives as industrial catalysts. However, a number of papers related to the development of enzyme stabilization strategies are published [3–5]. One of these strategies is the use of immobilization on supports, which increases the stability and activity of the enzymes. Some studies described the use of polymeric organic materials such as resins, organic gels and fibers as the conventional support for the immobilization of enzymes [3–5]. However, these materials have a low reusability and created disposal problems [3].

In the present study we applied a support of natural origin called *Opuntia imbricata* (coyonoxtle). *O. imbricata* is an abundant cactus in the northern region of Mexico (Fig. 1). Recently it was demonstrated that the dry coyonoxtle trunk is composed of 28.68+/−6.27% hemicellulose, 34.02+/−5.04% of cellulose and the most abundant fraction is lignin (37.64+/− 6.31%) [6].

This support was recently used for the development of biofilm reactor systems and applied for treatment of different wastewater [7]. The operational and chemical stability, resistance to hydraulic pressure as well as to microbial attacks have been demonstrated. These characters were considered as important to test this natural

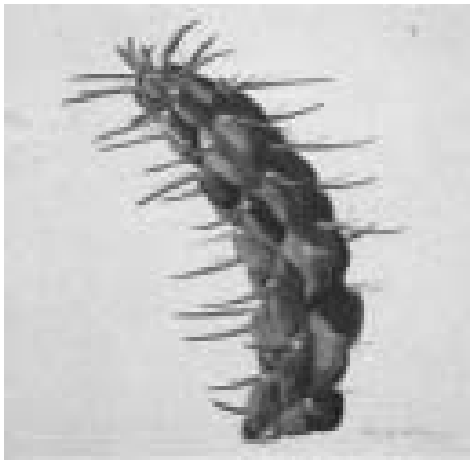

Fig 1. Trunk of *Opuntia imbricata* in natural state

material as a matrix for penicillin G acylase immobilization.

The goal of this study was to demonstrate that *O. imbricata* could be employed as a support for enzyme immobilization by means of adsorption and chemical bonding. Results on the first trial immobilization of the PGA on the pellets of this support material, treated and untreated with periodate and the operational and storage stability of the immobilized enzyme is presented in this paper.

MATERIAL AND METHODS

The reagents 6-APA, *p*-dimethylaminobenzaldehyde (PAB), penicillin G, sodium periodate were purchased from Sigma-Aldrich Chemical Company (USA). The Skim Milk medium was from ifco (USA). Methanol and acetic acid were purchased from Merck (USA). All reagents used in this study were of analytical grade.

Microorganisms

Aspergillus fumigatus H/6.17.3 and *Mucor griseocyanus* H/55.1.1 were gifted by r. ustet from the Institute of Sugar Cane erivatives (ICICA), Havana, Cuba. Strains were transferred on malt agar plates, incubated for 7 days at 30°C and then stored at 4°C for further use in this study.

Penicillin acylase production [8]

Both filamentous fungi were grown aerobically under submerged conditions in 100 ml reactors, containing 20 ml of 10% Skim Milk as medium. Penicillin G was employed as inducer at 2.5 mg/l. It was added aseptically after 24 h of fermentation. Fermentations were carried out for 144 h at 30°C in thermoregulated shaker at 150 rpm. Cells were removed by filtration. Penicillin G acylase activity was determined in the liquid extract.

Support treatment and activation

The coyonoxtle trunk was cut in to pieces of approximate 1 cmЧ1cmЧ1cm dimensions. Five g of coyonoxtle pieces were hydrolyzed with 250 ml of 2% HCl under reflux condition for 3 h to eliminate hemicellulose fraction [9]. Then the support was washed with distilled water to neutral pH and dried at 60°C for 24 h. Each 2 g of coyonoxtle was activated with $NaIO_4$ using 20 ml of 0, 0.05, 0.10, 0.15 and 0.2 M periodate solution [10]. The activation was carried out under agitation at 250 rpm for 1 h in the dark. Finally, the activated support was washed three times with 20 ml of distilled water.

Penicillin acylase immobilization on Opuntia imbricata pieces

PG acylases from *Aspergillus fumigatus* and *Mucor griseocyanus* were immobilized on support with and without $NaIO_4$ activation. Immediately after activation, 2 g of activated or non-activated support was added to 5 ml of enzymatic extract. The reactors were maintained under agitation condition at 100 rpm for 24 h at 4°C. The immobilized enzyme was recovered after extract separation and washed with 10 ml of 0.1 M phosphates buffer (PBS) at pH 8.0. All assays were done in triplicate.The concentration of proteins was determined by Bradford method [11] in the residual solution and the wash-out after washing with PBS and was deduced from the initial protein concentration of enzyme extract,

Activity measurement

Penicillin G acylase activity was determined according to the previous report technique [8] at 37°C and in 0.05 M PBS at pH 8.0. The technique is based on spectrophotometrical detection of reaction product of 6-APA, formed after penicillin G hydrolysis, with PAB [8].

The activity of the immobilized enzyme was measured under the same conditions. The pieces of *Opuntia imbricata* containing the enzyme were added to reaction mixture containing 10 ml of 0.1 M PBS at pH 8 and 1 ml of 0.05 M penicillin solution. The reaction mixture was maintained under agitation at 150 rpm for 30 min.

Then 300 ml of reaction mixture was transferred to a glass tube on an ice bath, which contained 2 ml of 20% acetic acid. The tubes were centrifuged at 3000 rpm for 10 min. Finally, 1.8 ml of supernatant was added to 600 ml of 5% PAB. After 5 min of reaction absorbance at 415 nm was measured.

The activity was calculated using calibration plot obtained for 6-APA under same conditions. The activity was expressed on International Units (U). One Unit was considered as the quantity of enzyme required for the formation of 1 mmol of reaction product in 1 min. The % of relative activity was calculated from specific activity (U/mg of protein), considering the free enzyme activity or the initial activity of immobilized preparation as 100 %, according to the case.

Storage stability evaluation

Penicillin acylase of A. fumigatus and M. griseocyanus immobilized on *O. imbricata* were stored at 4°C. The enzyme stability was evaluated by means of activity measurement as described above. The activity was deter-

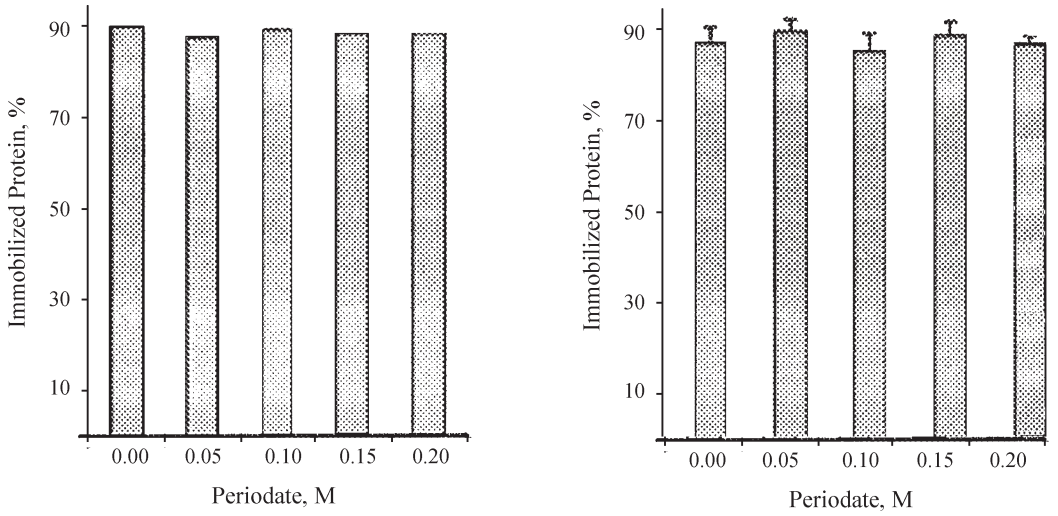

Fig. 2. Protein immobilized (%) on *Opuntia imbricata* in the presence and absence of periodate at different concentrations: left, – assay performed with penicillin acylase extract from *M.* griseocyanus; right, – from *A. fumigatus*

mined one time per week for one month. Moreover, the immobilized preparations were applied on an assay to define the enzyme activity after their repeated application in penicillin G hydrolysis. The activity was determined as described above. Between each cycle, the immobilized preparation was washed with 10 ml of PBS.

Statistical analysis

All assays were carried out in triplicate and the measurements were performed in duplicate. The results were analysed for ANOVA using Stat Graphics program.

RESULTS AND DISCUSSION

The penicillin acylase were obtained by fermentation of *M. griseocyanus* and *A. fumigatus* and the enzyme specific activity was 0.006 U/mg and 0.009 U/mg, respectively.

In the present study penicillin acylase from both fungi was immobilized on the pieces of *O. imbricata*. Two different immobilization methods were compared; adsorption using of non-activated support and covalent attachment of the enzyme on support activated by different concentrations of periodate. This activation technique was chosen considering that *O. imbricata* contains 34.02+/−5.04% cellulose. The mechanism of periodate activation suggested the transformation of cellulose alcohol groups to aldehydes, which are united chemically with amino groups of the enzyme forming −CH=N− bonds between enzyme and support [10].

No significant difference was observed between the two methods of immobilization, and as well as between treatments with different periodate concentrations. Fig. 2 showed that the percent of protein was immobilized on support without periodate treatment (see, 0 M concentration) was same as that present on *O. imbricata* treated with different concentrations of periodate. The immobilized protein was in the range of 88.31 to 89.76% and from 85.41 to 89.46% for *M. griseocyanus* and *A. fumigatus* penicillin acylase extract, respectively. The fact that only 85−89% of protein was immobilized signified that there was saturation of support with enzyme or the presence to steric hindrances during the immobilization process [12].

The results on high protein immobilization agreed with the data of high relative activity of the immobilized enzyme. Fig. 3 showed that practically 100% of initial activity was present on the immobilized preparation of enzyme from both fungi. The activity of residual solutions of enzyme extract obtained after immobilization demonstrated practically zero activity. In some cases the activity of immobilized preparations was higher than that of free enzyme. It could be due to the presence of enzymatic activity inhibitors in the crude penicillin acylase extract. It is known that the products of penicillin G hydrolysis (6-APA and phenyl acetic acid) are the inhibitors of acylase activity [2]. These and other probable inhibitors present on crude enzyme extract might have decreased the measured activity of free enzyme. The immobilized preparations are free of enzyme inhibitors present in the crude extract.

Similarly, the enzyme activity was not significantly different in the immobilized preparations on the activated and no activated supports (Fig. 3). This indicated that

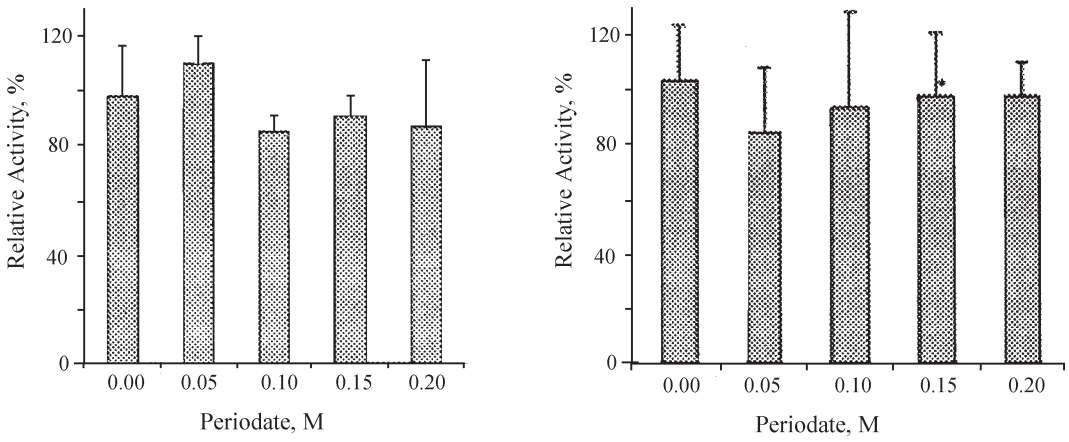

Fig. 3. Relative activity of penicillin acylase immobilized on *Opuntia imbricata* in the presence and absence of different periodate concentrations: left, – assay performed with penicillin acylase extract from *M*. griseocyanus; right, – from *A. fumigatus.* The specific activity (U/mg of protein) of free enzyme was used as 100 %

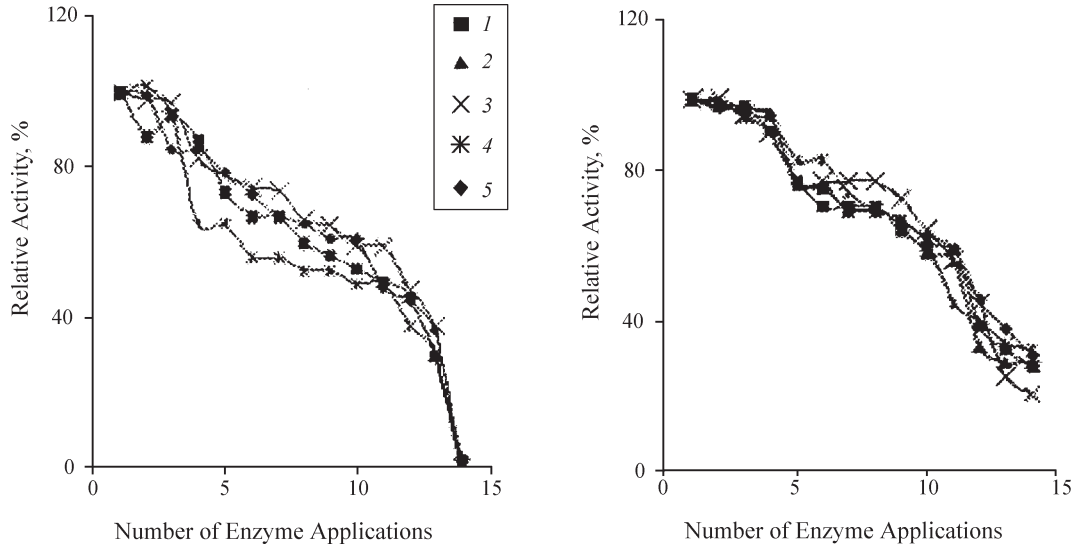

Fig. 4. Number of immobilized PGA applications in the penicillin G hydrolysis: left, – enzyme preparations from *M. griseocyanus*; right, – from *A. fumigatus.* Periodate concentrations applied for *O. imbricata* activation: 0.2 M (*1*), 0.15 M (*2*), 0.1 M (*3*) and 0.05 M (*4*) as well as 0 M corresponded to non-activated support (*5*).The activity of first measurement was used as 100%

there was no difference between the immobilization by adsorption and chemical attachment due to the treatment with different concentrations of periodate.

The obtained results demonstrated that the *O. imbricata* contained considerable amount of the active sites, which are appropriated for enzyme immobilization. The activation by periodate probably increased the number of active sites but it was smaller than the amount of naturally present groups and the interaction of enzyme-support seem to represent an adsorption equilibrium. This could be the reason for the no difference in activity between adsorption and chemical immobilization. We assumed that the subtle differences may be exhibited during the repeated used of the immobilized preparations, if the adsorption of the enzyme is of weak interactions. The enzyme might be desorbed, if the interactions were weak. The results of the activity of immobilized preparations on various cycles are presented in Fig. 4.

It was observed that all preparations immobilized by using of periodate were characterized by the same behavior than the preparation obtained without periodate treatment (Fig. 4). The activity decreased with increasing number of cycle. The decrease in activity was not dependent on the presence or absence of previous support

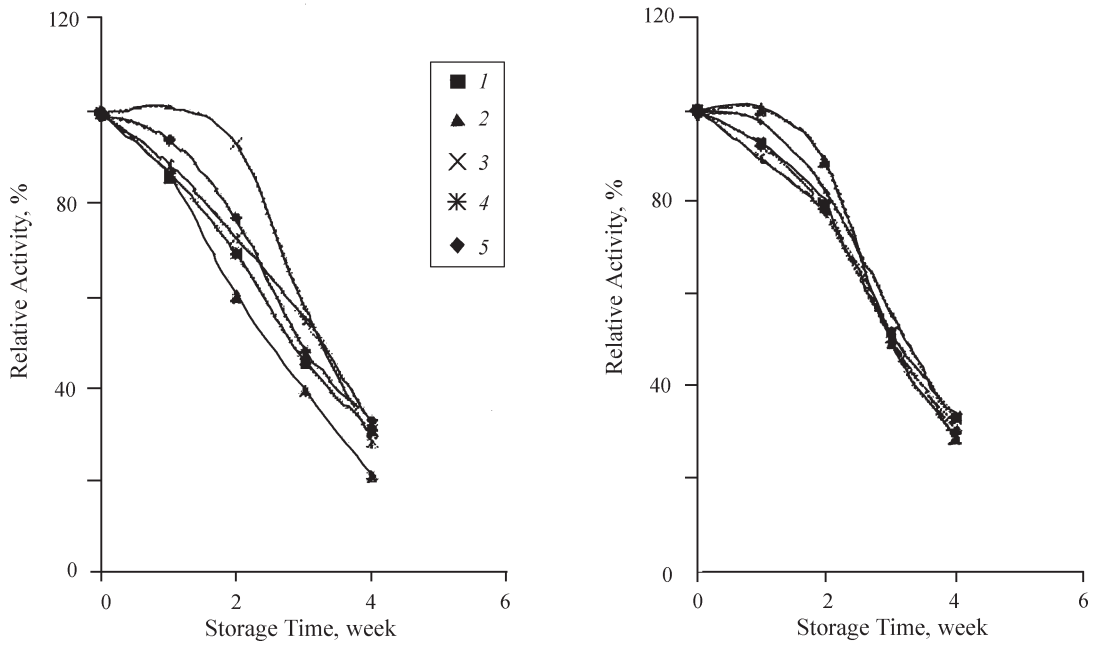

Fig. 5. Storage stability of immobilized PGA at 4°C: left, – enzyme preparations from *M. griseocyanus*; right, – from *A. fumigatus*. Periodate concentrations applied for *O. imbricata* activation: 0.2 M (*1*), 0.15 M (*2*), 0.1 M (*3*) and 0.05 M (*4*) as well as 0 M corresponded to non-activated support (*5*).The activity immediately after immobilization was used as 100%

activation as well as the activator concentration. This meant that under the applied conditions, the penicillin acylase immobilization on *O. imbricata* was more represented by adsorption equilibrium than by the chemical attachment.

There was difference in the performance of *M. griseocyanus* and *A. fumigatus* PGA immobilized preparations (Fig. 4). The immobilized enzyme from *M. griseocyanus* practically lost all its activity on 14th cycle (Fig. 4, left), but the major decrease in activity was detected after 10 cycles. The observed difference between different preparations of *M. griseocyanus* PGA (Fig. 4, left) was not statistically significant.

The immobilized preparation of PGA from *A. fumigatus* was more stable in comparison with *M. griseocyanus*. There was no significant change in activity for 4 cycles (Fig. 4, right). uring the 14th cycle, the immobilized enzyme demonstrated nearly 30–35 % of its initial activity.

The results demonstrated that the PGA from *A. fumigatus* provided better conditions for the support-enzyme interaction and facilitated increased cycle number of application than for *M. griseocyanus* PGA.

There was no correlation between type of immobilization and storage activity of immobilized enzyme (Fig. 5). All immobilized preparations lost their activity and this phenomenon was independent of activator application or its concentration. This is an evidence that adsorption prevailed the chemical attachment mechanism during immobilization of PGA on *O. imbricata*. Generally the enzyme maintained nearly 80% of its activity for the initial two weeks. The activity decreased rapidly to 27–32% during the next two weeks (Fig. 5). There was no considerable difference in stability of immobilized PGA from *M. griseocyanus* and *A. fumigatus*. The major variability in kinetics was observed for *M. griseocyanus*, however, these differences did not show statistical validity.

Inactivation constant (k_{in}) calculated from storage stability kinetics at 4°C for the immobilized PGA obtained from *A. fumigatus* and *M. griseocyanus* between14th and 28th days

Periodate concentration applied for support activation, M	k_{in}, day^{-1}	
	PGA from *M. griseocyanus*	PGA from *A. fumigatus*
Free enzyme*	0.05	0.13
0	0.06	0.07
0.05	0.06	0.06
0.10	0.08	0.08
0.15	0.08	0.07
0.2	0.07	0.07

* The data corresponded to free enzyme was reported by Martınez Hernőndez J.L. [2].

The comparison of immobilized and free enzyme stability was performed using the literature data [2], which reported the values of inactivation constant for free enzyme during its storage (Table). The inactivation kinetics was described by the exponential function that corresponded to first order mechanism. The inactivation constant (k_{in}) was calculated for both enzymes as a slope obtained in semi-logarithmic coordinates. The free enzymes lost its activity immediately upon storage. But in the case of immobilized PGA preparations, the inactivation of enzyme, which was described by exponential function, was observed only after 2 weeks of storage. Thus, for two weeks the immobilized enzyme from both fungi was more stable than the free enzyme.

Application of the semi-logarithmic coordinate to kinetic data obtained during next 2 weeks provided the values of inactivation constant corresponding to this storage period. Table showed that the k_{in} calculated for immobilized *M. griseocyanus* was greater than the free enzyme. It indicated that the immobilization of this enzyme on *O. imbricata* increased the enzyme stability only for initial 2 weeks. After this period a rapid enzyme inactivation occurred.

However, the enzyme from *A. fumigatus* was stable by means of immobilization. The effect was observed for all period of storage. The k_{in} corresponded to the last 2 week of storage was approximately two times less than the values reported for free enzyme (Table). Although the k_{in} values were similar for PGA from both fungi (Table), the obtained results demonstrated the different effect of immobilization on enzyme storage stability in relation to the stability of the free enzyme. As mentioned earlier, immobilization provided better conditions for increasing the staibility of PGA from *A. fumigatus* than from *M. griseocyanus*.

The results obtained in the present study showed that *O. imbricata* offers great possibilities for enzyme immobilization. In the case of PGA from filamentous fungi, high levels of protein immobilization and an activity relative to protein concentration was demonstrated. Likewise an improvement in operational and storage stability of the enzyme was observed. However, the support activation by means of periodate did not show appreciable effect on the properties of immobilized enzyme. This could be attributed to the reason that penicillin acylase immobilization on *O. imbricata* was more of adsorption equilibrium than chemical attachment. It can be concluded that *O. imbricata* has appropriate physical and chemical structure for the immobilization of enzymes.

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IMMOBILIZATION OF PENICILLIN ACYLASE OF FILAMENTOUS FUNGI ON THE PELLETS *OPUNTIA IMBRICATA* (COYONOXTLE)

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The immobilization of the extracellular penicillin acylases (PGA) from filamentous fungi (*Aspergillus fumigatus* and *Mucor griceocianus*) on the *Opuntia imbricata* is reported in this paper. The results obtained in the present study showed that *O. imbricata* offered great possibilities for enzyme immobilization. In the case of PGA from filamentous fungi, high levels of protein immobilization and relative activity were demonstrated. Likewise an improvement in the operational and storage stability of the enzyme was observed. However, the activation of support by means of periodate did not demonstrate any appreciable effect on the proprieties of immobilized enzyme.