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A Process to Produce Penicillin G Acylase by Surface-Adhesion Fermentation Using *Mucor griseocyanus* to Obtain 6-Aminopenicillanic Acid by Penicillin G Hydrolysis

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Abstract The production of extracellular and mycelia-associated penicillin G acylase (maPGA) with *Mucor griseocyanus* H/55.1.1 by surface-adhesion fermentation using *Opuntia imbricata*, a cactus, as a natural immobilization support was studied. Enzyme activity to form 6-aminopencillanic acid (6-APA) from penicillin G was assayed spectrophotometrically. The penicillin G hydrolysis to 6-APA was evaluated at six different times using PGA samples recovered from the skim milk medium at five different incubation times. Additionally, the effect of varying the penicillin G substrate concentration level on the PGA enzyme activity was also studied. The maximum reaction rate, V_{max}, and the Michaelis constant, K_M, were determined using the Michaelis–Menten model. The maximum levels for maPGA and extracellular activity were found to be 2,126.50 international unit per liter (IU/l; equal to 997.83 IU/g of support) at 48 h and 755.33 IU/l at 60 h, respectively. Kinetics of biomass production for total biomass showed a maximum growth at 60 h of 3.36 and 2.55 g/l (equal to 0.012 g of biomass per gram of support) for the immobilized *M. griseocyanus* biomass. The maPGA was employed for the hydrolysis of penicillin G to obtain 6-APA in a batch reactor. The highest quantity of 6-APA obtained was 226.16 mg/l after 40-min reaction. The effect of substrate concentration on maPGA activity was evaluated at different concentrations of penicillin G (0–10 mM). K_M and V_{max} were determined to be 3.0×10^{-3} M and 4.4×10^{-3} mM/min, respectively.

Keywords Penicillin G acylase (PGA) · Surface-adhesion fermentation (SAF) · *Opuntia imbricata* · *Mucor griseocyanus*

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Introduction

Commercially, β-lactams constitute the greatest market of antibiotics in the world. They have been used extensively for therapeutic treatment of various bacterial infections for more than half a century. To date, the penicillin antibiotic production exceeds 50,000 to 60,000 tons per year [1]. Due to the great demand for β-lactams, the world production of 6-aminopencillanic acid (6-APA) has increased considerably, more than 7,000 tons in 2008 alone [2]. This penicillin nucleus, 6-APA, could be used to produce a wide range of semisynthetic penicillins by simple chemical acylation. Continued efforts are being made to improve the availability of 6-APA due to the impact and high demand for the production of semisynthetic penicillins. Reduced cost of immobilized enzyme, improved specificity, activity, and stability are essential for the production of 6-APA and semisynthetic penicillins.

Penicillin G acylase (PGA, amidase or amidohydrolase, E.C. 3.5.1.11) is a critical enzyme that is extensively used by the pharmaceutical industry in the hydrolysis reaction of penicillin G to produce two important intermediates: phenylacetic acid and 6-APA. The latter that is of most interest is an intermediate for the production of semisynthetic penicillins, such as amoxycillin and ampicillin [3], and is mainly produced by enzymatic or chemical deacylation [4] of the natural benzyl penicillin. Penicillin acylases or amidases are used in soluble or immobilized form under acidic or alkaline conditions for the enzymatic conversion of penicillins to 6-APA and semisynthetic penicillins [3].

PGA is produced by several microorganisms such as bacteria, actinomyces, fungi, and yeasts. *Escherichia coli*, *Bacillus megaterium*, *Streptomyces lavendulae*, *Achromobacter* sp., *Proteus rettgeri*, *Actinoplanes* sp., *Bovista plumbea*, *Kluyvera atrophila*, *Pseudomonas melanogenum*, and *Penicillium chrysogenum* [4–6] are known for PGA production, but *E. coli* and *B. megaterium* are the two preferred strains used by the industry [7]. Acylase from *E. coli* is one of the best studied enzymes [4]. The fungus *Mucor griseocyanus* has been reported to produce PGA with kinetic characteristics and reaction mechanism similar to the enzyme obtained from *E. coli* or *B. megaterium* [8]. 6-APA is mainly produced by chemical or enzymatic route [4, 9] in the industry of which the chemical methods are harmful to the environment because they require the use of hazardous chemicals such as pyridine, phosphorous pentachloride, and nitrosyl chloride [7, 10]. On the other hand, the enzymatic hydrolysis method is more specific; the reaction conditions are milder, and it is much cheaper than the chemical method. However, the enzymatic method requires various preproduction steps that include production, extraction, and purification of the acylase enzyme, before its application for the 6-APA production. Although free cells in suspension, immobilized whole cells, or immobilized enzymes can be used, the latter method is considered the most superior to all other methods [2, 11]. The various methods used for the immobilization of penicillin acylase include adsorption, fiber entrapment, microencapsulation, cross-linking, copolymerization, and covalent attachment. Various studies [4, 11] gave preference to using immobilized PGA for its higher activity per unit volume, better operational stability, and easier synthetic applications. Additionally, immobilized enzymatic systems have several advantages over the free enzyme systems in that they can be easily recovered, be reused repeatedly, be free from toxicity problems, offer spaces for growing cells, and is much cheaper [12]. Different types of synthetic and biomaterials from diverse plant sources can be used for cell and enzyme entrapment [11, 12]. Very few reports are available in the literature on the adsorption of enzyme on natural support [13]. In our previous study, PGA was immobilized on *Opuntia imbricata*, a cactus [13], also known as coyonoxtle and is considered an invasive plant in the north of Mexico (Fig. 1). The dry coyonoxtle trunk is composed of (wt.%): hemicelluloses (28.7±6.3), cellulose (34.0±5.0), and (the most abundant fraction) lignin (37.6±6.3) [14].

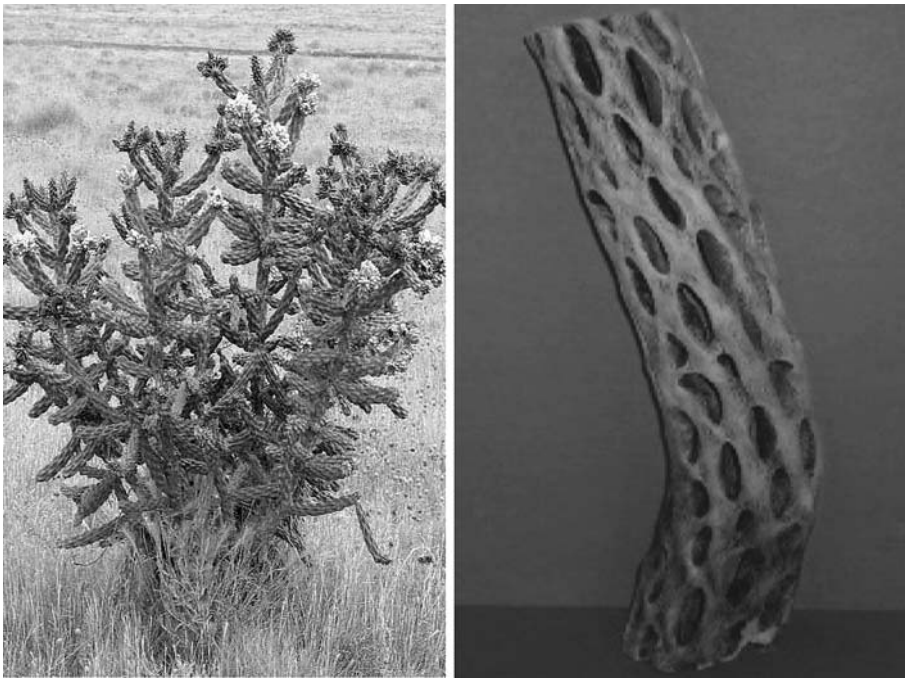

Fig. 1 *O. imbricata* in its natural habitat (*left*) and dry trunk (*right*) before its pretreatment for use as a natural support in the surface-adhesion fermentation

Filamentous fungi are naturally adapted to growing on surfaces; they demonstrate a physiological behavior which is different from that of submerged culture. The advantages of this form of growth have been industrially exploited in both solid-state fermentation (SSF) and immobilized systems, although there is a lack of knowledge on the molecular basis of growth on surfaces [15]. All solid supports have a common feature: their basic macromolecular structure. In general, these substrates for SSF are composite and heterogeneous products from agriculture or by-products of agro-industry. This basic macromolecular structure (e.g., cellulose, starch, pectin, lignocellulose, fibers, etc.) confers the properties of a solid to the support. The structural macromolecule may simply provide an inert matrix (sugarcane bagasse, inert fibers, resins) within which the carbon and energy source (sugars, lipids, organic acids) are adsorbed. But, generally, the macromolecular matrix represents the substrate and provides also the carbon and energy source.

Fungi cannot transport macromolecular substrates, but the hyphal growth allows a close contact between hyphal and support surface. The fungal mycelium synthesizes and excretes high quantities of hydrolytic exoenzymes. The resulting contact catalysis is very efficient, and the simple products are in close contact to enter the mycelium across the cell membrane to promote biosynthesis and fungal metabolic activities [15, 16]. Several investigators have reported that thermophilic species of *Mucor* are noncellulolytic [17, 18]. On the other hand, *Mucor pusillus* as *Mucor miehei* was reported to produce cellulase [19]. There are no reports about cellulolytic activity of *M. griseocyanus*, which may be useful for its adaptation to growing on surfaces of cellulose-containing support; however, this fungus is a lipase and penicillin acylase producer [8, 13, 16].

Surface-adhesion fermentation (SAF) is a new fermentation category described by Gutierrez-Correa and Villena [20]. It is a process where the spores are adsorbed to the solid

support (natural or synthetic), and the cells grow evenly to form a biofilm. Natural adsorption on solid supports is an immobilization technique that has been used with filamentous fungi [20].

The aim of our work is to study the production of PGA by *M. griseocyanus* using a cactus, *O. imbricata*, as a natural support for the biofilm formation of fungus employing SAF and to produce 6-APA by the hydrolysis of penicillin G.

Materials and Methods

Microorganism and Culture Medium

M. griseocyanus H/55.1.1 strain was obtained from the Culture Collection Institute of Sugar Cane Derivatives, Havana, Cuba. Inoculum was prepared by culturing the spores on potato dextrose agar for 7 days at 30 °C and then were scraped into a 0.01% Tween 80 solution and counted in a Neubauer chamber. Skim milk (10% *w*/*v*; pH 6.3±0.2) was used as the culture medium (DIFCO) for the production of penicillin acylase.

Support Treatment

The *O. imbricata* trunk (Fig. 1) was cut into small irregular pieces of about 8 cm^3 each, washed in boiling distilled water for 30 min, and left for 1 h in distilled water. The water was changed three times to thoroughly clean the pieces. Washed pieces were oven-dried at 70 °C for 48 h, cooled, and kept in desiccators to get to a constant weight for subsequent use [14].

Production of Penicillin Acylase by SAF

Into each of the 18 (triplicate of 0, 12, 24, 36, 48, and 60 h of incubation) 250-ml Erlenmeyer flasks were placed two items: 20 ml of 10% (*w*/*v*) skim milk as the culture medium and preweighed (5 g) pieces of *O. imbricata* that were sterilized by autoclaving at 121 °C for 15 min and cooled to room temperature. Each flask was then inoculated with 10^6 spores per milliliter of culture medium, and all the flasks were placed on a shaking platform (120 rpm) at 30 °C for a period of 60 h. Samples were removed from each flask at 12-h intervals and assayed in duplicate. The cell-free culture broth was assayed for extracellular PGA activity. Free-grown fungal biomass was separated by filtration using a preweighed filter paper (Whatman no. 1041) and oven-dried at 60 °C to constant weight for biomass determination. The pieces containing immobilized mycelia-bound enzymes were recovered from the medium and washed with 20 ml of 0.85% (*w*/*v*) saline solution to remove the nonfixed free hyphae under sterile conditions and then stored in 20 ml of 0.05 M phosphate buffer (pH 6.5) at 4 °C for subsequent hydrolysis experiments. The assay was repeated three times.

Assay of Penicillin Acylase Activity

Assay for Extracellular PGA Activity

Extracellular penicillin acylase activity was assayed by following the methods described by Balasingham [21]. 6-APA released during the hydrolysis of penicillin G was measured by its reaction with *p*-dimethylaminobenzaldehyde (*p*-DAB), and the absorbance was read at

415 nm (Spectrophotometer, Varian Cary Bio 50). One international unit (IU) was defined as the enzyme quantity that catalyzes the formation of 1 μmol 6-APA per minute, under standard reaction conditions. Liter of reactor is defined as the volume of liquid medium and volume of immobilization support.

Assay for maPGA Activity

The mycelia-associated PGA (maPGA) activity was carried out using the cactus pieces with adhered fungi biomass obtained at different incubation times. The pieces were soaked in a jacketed-glass reactor (150 ml) containing 24 ml of 10 mM penicillin G prepared in phosphate buffer (1 M) at pH 8, and the reactor was shaken (160 rpm) at 40 °C by using thermostatically controlled water bath with recirculation pump (VWR Scientific Products). After 20 min of reaction, aliquots of 600 μl were transferred into test tubes containing 1.8 ml of cold acetic acid (20% *v*/*v*) to stop the reaction and then were centrifuged for 10 min at 3,000 rpm. 6-APA released from penicillin G hydrolysis was allowed to react with *p*-DAB and then estimated by measuring the absorbance at 415 nm (Spectrophotometer, Varian Cary Bio 50). One unit of penicillin acylase activity was defined as described in "Assay for Extracellular PGA Activity."

Penicillin G Hydrolysis to Produce 6-APA

Penicillin G hydrolysis was carried out using the maPGA produced at five different incubation times (12, 24, 36, 48, and 60 h). The kinetic production of 6-APA was evaluated at six different hydrolysis times (0, 5, 10, 20, 40, and 80 min) and under the same conditions as described in "Assay for maPGA Activity." To determine the adhered biomass on this support, the pieces of *O. imbricata* with mycelia were oven-dried at 60 °C to constant weight.

Effect of the Penicillin G Concentration on the Initial Rate of Penicillin Acylase Activity and Determination of K_M and V_{max}

The influence of the substrate concentration (penicillin G) on PGA activity was studied by determining the initial rate of reaction for different concentrations of 1.5–10 mM in phosphate buffer (1 M, pH 8) and at 40 °C. The reaction was carried out in a jacketed-glass reactor (150 ml) and under the same conditions as described in "Assay for maPGA Activity."

Kinetic reaction parameters were determined by adjusting the Michaelis–Menten model to initial rate points obtained during the penicillin G hydrolysis at different concentrations. The adjustment was carried out utilizing kinetic parameters estimated using the Lineweaver–Burk plot.

Results and Discussion

Production of Penicillin Acylase

Figure 2 depicts the PGA production by *M. griseocyanus* in SAF with skim milk for both the extracellular and the maPGA activities at each 12 h of fermentation. Extracellular PGA achieved its highest activity of 755.33 IU/l of reactor at 60 h of fermentation, or 755.33 μmol of

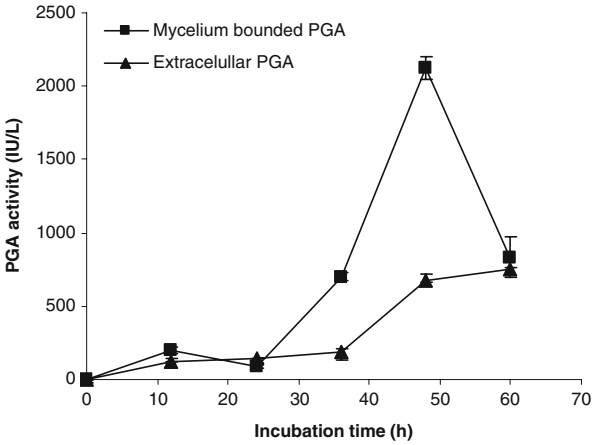

Fig. 2 PGA activity produced by *M. griseocyanus* H/55.1.1 in SAF at different incubation times; extracellular (*filled triangles*) and mycelia-associated (*filled squares*)

6-APA was produced. The maximum maPGA activity of 2,126.50 IU/l (997.83 IU/g DC) is observed at 48 h and subsequently declines. It is possible that this decline could be due to the limitations of mass transfer inside the mycelia mass.

Axelsson and Persson [22] reported that the mass transfer limitations existed inside the microbial pellets and could be related to the biofilm of fungus being formed over the support. Similarly, Réczey et al. [23] reported that the mycelia-associated β-galactosidase activity in the pellets of *Aspergillus* and *Penicillium* strains was affected due to mass transfer limitations. Our results on the PGA activity are very encouraging and promising as they confirm that penicillin acylase can be successfully produced by *M. griseocyanus* using SAF.

There are only a few reports on the production of enzymes using the SAF system. Gutiérrez-Correa and Villena [20] recently reported the production of cellulases from *Aspergillus niger* using SAF, and Réczey et al. [23] investigated the hydrolysis of lactose with mycelia-associated β-galactosidase activity in a batch process using pellets from five strains of *Aspergillus*. SAF presents several advantages over other fermentation systems in that the enzyme produced by the fungus can be self-immobilized in the support or remain mycelia-associated. Fermented material can act as support for the enzyme, without the need for a previous extraction step and its subsequent immobilization as shown by Nagy et al. [24]. Fernandez et al. [25] reported another system of fermentation similar to SAF for the production of lipases from *Burkholderia cepacia*, in which the enzyme was self-immobilized on the support and the fermented solids were used to catalyze esterification and transesterification reaction.

Biomass Production

Kinetics of both the total and the immobilized *M. griseocyanus* biomass formation is presented in Fig. 3. Though the fungus shows an exponential growth phase during the first 24 h, the maximum growth is attained at 60 h (3.36 g/l of reactor). On other hand, the maximum immobilized biomass is also recorded at 60 h (2.55 g/l equal to 0.012 g/g).

Hydrolysis of Penicillin G to Produce 6-APA in Batch Reactor

6-APA production was performed by hydrolysis of penicillin G with maPGA in batch reactors at six reaction times: 0, 5, 10, 20, 40, and 80 min. This production obtained from

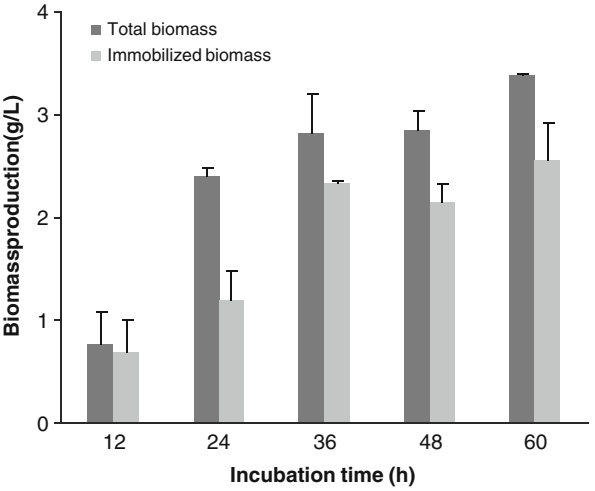

Fig. 3 Biomass production by *M. griseocyanus* H/55.1.1 in SAF: total (*dark bars*) and adhered to support (*gray bars*)

samples with different times of fermentation of 0, 12, 24, 36, 48, and 60 h is presented in Fig. 4.

The maPGA from the 12-h fermentation sees a slight production after 20 min of hydrolysis but levels off at about 25 mg/l thereafter. The hydrolysis results substantiate that the maximum 6-PGA production at 226.16 mg/l of reactor was achieved by the maPGA from the 48-h fermentation after 80 min of hydrolysis. These results correspond well with the maPGA enzyme activity results presented in Fig. 2.

Effect of the Penicillin G Concentration on Initial Rate of Penicillin Acylase Activity and Determination of K_M and V_{max}

Effect of different substrate concentrations on the maPGA activity of *M. griseocyanus* was evaluated. Kinetic parameters were determined adjusting the Michaelis–Menten model to experimental initial rate points obtained during penicillin G hydrolysis. Michaelis–Menten

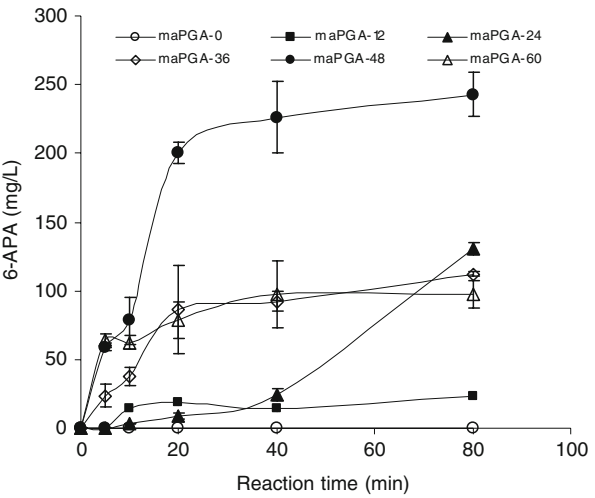

Fig. 4 Penicillin G hydrolysis using mycelium-associated PGA in batch reactor, maPGA-0 (*empty circles*), maPGA-12 (*filled squares*), maPGA-24 (*filled triangles*), maPGA-36 (*empty diamonds*), maPGA-48 (*filled circles*), maPGA-60 (*empty triangles*)

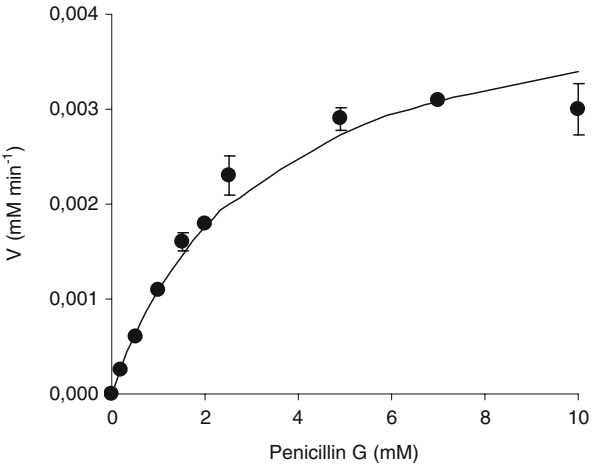

Fig. 5 Michaelis–Menten plot of mycelia-associated PGA from *M. griseocyanus* H/55.1.1 utilizing penicillin G (0–10 mM) as substrate at 40 °C and at pH 8, experimental (*filled circles*) and adjusted (*line*) data

plot of the PGA with penicillin G as substrate is shown in Fig. 5. According to the model, K_M and V_{max} were found to be 3 mM and 4.4×10^{-3} mM min^{-1}, respectively, suggesting that maPGA has a great affinity for penicillin G. The maximum rate of hydrolysis detected experimentally was 3×10^{-3} mM/min at 40 °C ±1 and pH 8.

As a comparison, Domínguez-Malfavón [26] had reported K_M and V_{max} values of 2.8 mM and 6.12 mM/min, respectively, for extracellular PGA of *M. griseocyanus*, which are in accordance with the results obtained in this study. Similarly, in the case of *B. megaterium* ATCC 14945, the K_M and V_{max} were determined to be 1.83 mM and 0.165×10^{-3} mM/min, respectively, at 37 °C and pH 8 [1]. However, Savidge and Cole [27] studied the PGA from *E. coli* and *B. megaterium* and reported a K_M value of 7.7 and 4.5 mM, respectively, at 37 °C and pH 8.7, which are higher than the values reported in this paper.

Conclusions

A new method to demonstrate the feasibility of producing PGA from *M. griseocyanus* by SAF in a skim-milk-based medium containing suspended pieces of a cactus, *O. imbricata*, was successfully achieved. The use of this enzyme to catalyze the penicillin G hydrolysis to produce the penicillin nucleus, 6-APA, in a batch reactor was successfully demonstrated. The extracellular PGA enzyme activity was achieved at a significant level of 755.33 IU/l of reactor after 60 h of incubation, and the immobilized PGA enzyme activity attained 2,126.50 IU/l (equal to 997.83 IU/g of support) at 48 h of incubation. The total biomass produced by the fungus was an impressive 3.36 g/l of reactor. Furthermore, the maPGA was found to have a strong affinity for penicillin G during hydrolysis.

Our new method of producing PGA from *M. griseocyanus* by SAF to produce 6-APA via the hydrolysis of penicillin G with the enzyme certainly would make a good and significant contribution to the literature on β-lactams antibiotics and semisynthetic penicillin derivatives.

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References

1. Thykaer, J., & Nielsen, J. (2003). *Metab Eng, 5*, 56–69.
2. Shewale, J. G., & Sivaraman, H. (1989). *Process Biochem, 24*, 146–154.
3. Souza, V. R., Silva, A. C. G., Pinotti, L. M., Selistre-Araújo, H. S., & Camargo-Giordano, R. L. J. (2005). *Braz Arch Biol Technol, 48*, 105–111.
4. Chisti, Y., & Moo-Young, M. (1991). In V. Moses & R. E. Cape (Eds.), *Biotechnology: The science and the business* (pp. 167–209). New York: Harwood.
5. Savidge, T. A. (1984). In E. J. Vandamme (Ed.), *Biotechnology of industrial antibiotics-drugs and pharmaceutical sciences* (Vol. 2, pp. 172–224). New York: Dekker.
6. Shewale, J. G., Deshpande, B. S., Sudhakaran, V. K., & Ambedkar, S. S. (1990). *Process Biochem, 25*, 97–103.
7. Matsumoto, K. (1993). In A. Tanaka, T. Tosa & T. Kobayashi (Eds.), *Production of 6-APA, 7-ACA and 7-ADCA by immobilized penicillin and cephalosporin amidases* (pp. 67–88). New York: Dekker.
8. Martínez Hernández, J. L., Ilyina, A., Domínguez Malfavón, L., & Dustet, M. J. C. (2003). *Vestn Mosk U KHIM, 44*, 53–56.
9. Huber, F. M., Chauvette, R. R., & Jackson, B. G. (1972). In E. H. Flynn (Ed.), *Preparative methods for 7-aminocephalosporanic acid and 6-aminopenicillanic acid, cephalosporins and penicillins* (pp. 27–33). New York: Academic.
10. Vandamme, E. J. (1988). In M. Moo-Young (Ed.), *Bioreactor immobilized enzymes and cells: fundamentals and applications* (pp. 261–268). New York: Elsevier.
11. Kallenberg, A. I., van Rantwijk, F., & Sheldon, R. A. (2005). *Adv Synth Catal, 347*, 905–926.
12. Iqbal, M., & Saeed, A. (2005). *Lett Appl Microbiol, 40*, 178–182.
13. Becerra-Jiménez, J. M. E., Martínez-Hernández, J. L., Rodríguez-Martínez, J., & Ilyina, A. (2006). *Mosc Univ Chem Bull, 61*, 36–43.
14. Ilyina, A., Huerta-Guel, P. J., Martínez-Hernández, J. L., Rodríguez-Martínez, J., & Gorokhovsky, A. (2008). *J Mol Catal B Enzym, 51*, 1–9.
15. Raimbault, M. (1998). *Elect J Biotech, 1*, 1–15. available from http://www.ejb.org. Accessed June 10, 2009.
16. Aguilar, C. N., Gutiérrez-Sánchez, G., Prado-Barragán, L. A., Rodríguez-Herrera, R., Martínez-Hernandez, J. L., & Contreras-Esquivel, J. C. (2008). *Am J Bioch Biotech, 4*, 354–366.
17. Fergucs, L. (1969). *Mycol, 61*, 120–129.
18. Tanseym, R. (1971). *Arcltiv Fiir Mikrobiol, 77*, 1–1I.
19. Somkuti, G. A. (1974). *J Gener Microbiol, 81*, 1–6.
20. Villena, G. K., & Gutiérrez-Correa, M. (2003). *Rev Perú Biol, 10*, 78–87.
21. Balasingham, J., Warburton, D., Dunhill, P., & Lilly, M. (1972). *Biochim Biophys Acta, 7*, 276–250.
22. Axelsson, A., & Persson, B. (1987). *Appl Biochem Biotechnol, 16*, 231–250.
23. Réczey, K., Stalbrand, H., Hahn-Hägerdal, B., & Tjerneld, F. (1992). *Appl Microbiol Biotechnol, 38*, 393–397.
24. Nagy, V., Toke, E. R., Keong, L. C., Szatzker, G., Ibrahim, D., Omar, I. C., et al. (2006). *J Mol Catal B Enzym, 39*, 141–148.
25. Fernandes, M. L. M., Saad, E. B., Meira, J. A., Ramos, L. P., Mitchell, D. A., & Krieger, N. (2007). *Mol Catal B Enzym, 44*, 8–13.
26. Domínguez-Malfavón, L. (2003). MS thesis, Universidad Autónoma de Coahuila, Saltillo, Coahuila, Mexico.
27. Savidge, T. A., & Cole, M. (1975). In J. H. Hash (Ed.), *Methods in enzymology: penicillin acylase (bacterial)* (Vol. 43, pp. 705–721). London: Academic.