

Activation and Stabilization of Penicillin V Acylase from *Streptomyces lavendulae* in the Presence of Glycerol and Glycols

Miguel Arroyo,* Raquel Torres-Guzmán, Isabel de la Mata, M. Pilar Castellón, and Carmen Acebal

Departamento de Bioquímica y Biología Molecular I, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid, Spain

Penicillin V acylase (EC 3.5.1.11) from *Streptomyces lavendulae* showed both enhanced activity and stability in mixed water/glycerol and water/glycols solvents. The catalytic activity was increased up to a critical concentration of these cosolvents, but further addition of the latter led to a gradual protein deactivation. The highest stabilizing effect was achieved in the presence of glycerol. Thermal stability was increased proportionally to the concentration of glycerol and glycols in the reaction mixture only if the amount added is below the threshold concentration. Reaction conditions that allow simultaneously enhanced activity and stability in the hydrolysis of penicillin V catalyzed by penicillin V acylase from *S. lavendulae* could be established.

Introduction

Nonaqueous enzymology has greatly expanded the potential scope and economic impact of biocatalysis, opening a new field in the biotechnological applications of enzymes (Gupta, 1992). Enzymatic activity sometimes may be improved as a result of the incorporation of a small amount of a water-miscible organic solvent in the reaction mixture (Tan and Lovrien, 1972; Batra and Gupta, 1994). However, aqueous–organic monophasic systems have not become very popular because many water-miscible organic solvents can act as denaturing agents for proteins at moderate concentrations (Mozhaev et al., 1989). Despite this, there are some polyhydric cosolvents that may improve enzyme stability. These cosolvents such as glycerol and glycols, are well-known protective agents against thermal denaturation, and they are less deleterious at high concentrations in the reaction medium (Mozhaev et al., 1989). In particular, glycerol is known to be a strong stabilizing agent of biomolecules (Gekko and Timasheff, 1981a) and is widely employed for storing proteins and enzymes. In 1991, Khmelnitsky and co-workers introduced a new parameter called denaturation capacity (DC), which indicates the protein denaturing strength of an organic solvent on a scale from 0 to 100. In the DC scale of Khmelnitsky, glycerol and ethylene glycol have very low DC values (20.2 and 18.7, respectively), meaning that these solvents exert the least denaturing effect on proteins. In this work, we have systematically studied the effect of increasing the concentrations of glycerol, ethylene glycol, diethylene glycol, and triethylene glycol on the activity and stability of penicillin V acylase (PVA) from *Streptomyces lavendulae*. This enzyme catalyzes the hydrolysis of penicillin V to yield 6-amino penicillanic acid (6-APA), an important key intermediate of semisynthetic penicillins (Shewale and Sudakaran, 1997). The use of water/glycerol and water/glycol mixtures can enhance the hydrolysis rate of penicillin V catalyzed by PVA, in addition to improving its thermal stability. The present work describes a useful

medium in the manufacture of 6-APA employing PVA from *S. lavendulae*.

Experimental Section

Enzyme Production and Partial Purification. Penicillin V acylase (EC 3.5.1.11) was produced from *S. lavendulae* (ATCC 13664) by fermentation (Torres et al., 1999), and it was purified 30-fold with a yield of 26.4% as reported elsewhere (Torres et al., 1998).

Enzyme Activity. The potassium salt of phenoxy methyl penicillin (PVK) from Sigma (St. Louis, MO) was used as substrate in the enzyme assays. A 50 μ L sample of the purified PVA solution was mixed with 85 μ L of water, 15 μ L of 1 M potassium phosphate buffer (pH = 8.0), and 150 μ L of a PVK solution (45 mg/mL) in water. The reaction mixture was incubated for 30 min at 40 °C under gentle shaking. The reaction was stopped by addition of 0.9 mL of 20% (v/v) acetic acid solution. The reaction mixture was centrifuged, and a 0.9 mL aliquot was processed for estimation of 6-APA by addition of 300 μ L of a 0.5% (p/v) solution of *p*-dimethylamino benzaldehyde (Sigma Chemical Co., St. Louis, MO) in methanol (Shewale et al., 1987). One activity unit is defined as the amount of enzyme producing 1 μ mol/min of 6-APA under the assay conditions. Previous kinetic analyses were performed to make sure that the activity was linear in the assay conditions for 30 min (in buffer and in the presence of cosolvents) using the saturating concentration of penicillin V described above. We employed an enzyme solution with an activity of 1.0 unit/mg protein in all the assays.

Effect of Glycerol and Glycols on Enzyme Activity. The effect of glycerol, ethylene glycol, diethylene glycol, and triethylene glycol (Merck, Germany) on penicillin V acylase activity at different solvent concentrations was analyzed. The solvents were incorporated into 50 μ L of enzyme solution prior to the addition of penicillin V. The volume of water was corrected for the added solvent so that the volume remained 150 μ L. The enzyme activity, in contact with the solvent, was measured after the addition of 150 μ L of PVK solution, so

* Tel: (+34) 91-394 41 50. Fax: (+34) 91-394 46 72.

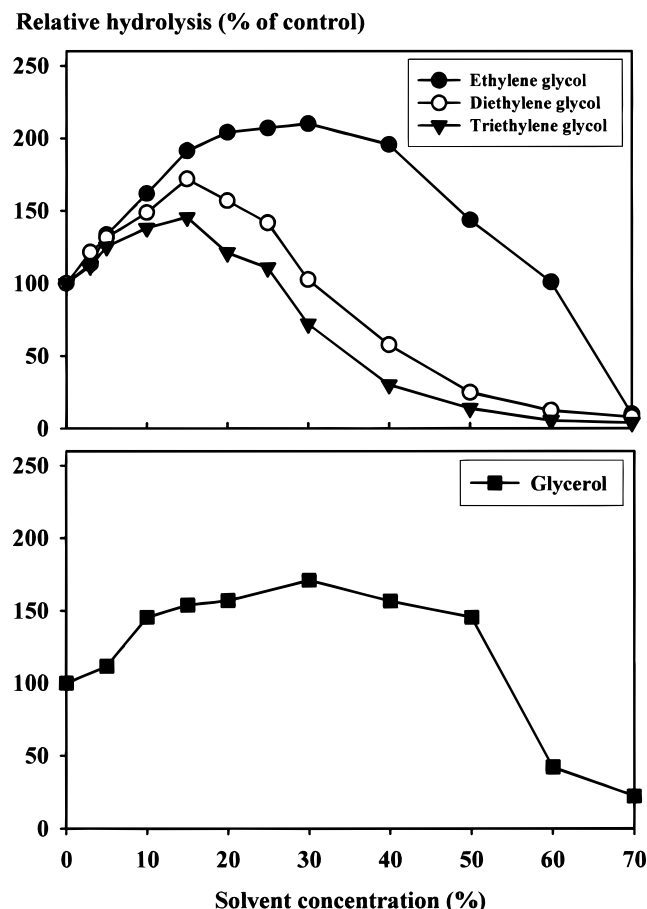


Figure 1. Effect of glycols and glycerol concentration on the hydrolysis of penicillin V by penicillin V acylase from *S. lavendulae*.

that the solvent concentration (% v/v) was referred to a final reaction volume of 300 μL . All of the experiments were carried out in triplicate. The measure of the solvent's hydrophobicity was the log P value, where P is the partition coefficient for the solvent between octanol and water. The log P values provided were taken from, or calculated on the basis of, Rekker (1977). Dielectric constants (ϵ) at 20 $^{\circ}\text{C}$ were taken from the monographs by Reichardt (1988) and Riddick et al. (1986).

Estimation of Deactivation Rate Constants at Different Glycerol and Glycol Concentrations. The stability of PVA was determined in 100 mM phosphate buffer pH 8.0 containing different concentrations of organic cosolvents and 0.33 units of purified enzyme. The samples were incubated at 40 and 50 $^{\circ}\text{C}$ in closed vessels, and aliquots of 150 μL were withdrawn at different storage times to check the residual activity. The residual activity was determined by using the enzyme activity assay described above (PDAB method).

Results and Discussion

Effect of Solvent Concentration on Catalytic Activity. The effect of several concentrations of glycerol and the different glycols on PVA activity at 40 $^{\circ}\text{C}$ has been studied. If we suppose a catalytic mechanism for PVA similar to that for penicillin G acylase from *E. coli* (Duggleby et al., 1995), the acyl-enzyme could be attacked by other competing nucleophiles such as glycerol and glycols to release the product in a higher rate, thus enhancing the catalytic activity. As shown in Figure 1, the highest relative hydrolysis rate was achieved in the

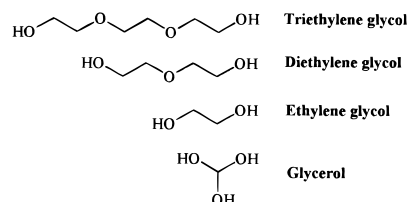


Figure 2. Chemical structures of glycerol and glycols.

presence of ethylene glycol. The other glycols and glycerol also enhanced PVA activity but to lower extents. This behavior may be attributed to the nucleophilicity of the polyols, which depends on their chemical structures (Figure 2). As a matter of fact, there may be higher or lower PVA activation depending on their nucleophilicity in the following trend: ethylene glycol \approx glycerol \approx diethylene glycol $>$ triethylene glycol. On the other hand, an effect of solvents on a better solubility of substrate and/or products cannot be discarded (Arroyo et al., 1999).

The maximal rate of penicillin V hydrolysis catalyzed by PVA depends on the concentration of the cosolvent in a threshold manner (Figure 1). After a critical concentration has been reached, further addition produces a gradual decrease in enzyme activity. This behavior has been extensively reported for other enzymes in water/organic cosolvent mixtures (Mozhaev et al., 1989; Khmel'nitsky et al., 1991). In a previous work, we found a linear correlation between the dielectric constant and the maximal concentration of the solvent that PVA can accept without any loss of enzymatic activity (Arroyo et al., 1999). The solvents were divided into two groups, alcohols and aprotic polar solvents, which showed different linear trends. Those solvents with high ϵ -values in both groups could be used at high concentrations without any deleterious effect, better than solvents with low ϵ -values that drastically diminished PVA activity at low concentrations. However, alcohols were less deleterious than the other water-miscible organic solvents. In the present work, we studied a wider range of cosolvent concentrations. We found out that glycerol and ethylene glycol show broad bell-shaped curves (Figure 1), indicating that both solvents exert a very low denaturing effect on PVA and the enzyme can tolerate high solvent concentration. In fact, these solvents have high ϵ -values (Table 1), and this effect was expected. In addition, they are very mild denaturants according to Khmel'nitsky's scale (Khmel'nitsky et al., 1991). On the other hand, diethylene glycol and triethylene glycol seem to be more harmful for PVA, as shown in Figure 1. Their lower ϵ -values (Table 1) would explain this fact supporting the linear correlation observed for alcohols in our previous work (Arroyo et al., 1999).

Effect of Glycol Concentration on PVA Thermal Stability. The influence of several concentrations of glycerol and glycols on PVA deactivation at 40 $^{\circ}\text{C}$ have been studied. Deactivation rate constants (k_d) of PVA at different cosolvent concentrations were estimated from the slope of the linear equation of first-order inactivation kinetics $\ln(E/E_0) = -k_d t$ where E/E_0 is the residual activity of the enzyme in the presence of cosolvent respect to its counterpart in buffer, and t is the storage time (h). We could also calculate the stabilization factor, considered as the ratio between PVA half-life in the presence of the cosolvent and soluble PVA half-life. The decrease of the values of first-order deactivation constants (k_d) (Table 2) with the increase in glycol concentration reflects a stabilizing effect. On the other hand, the thermodynamic parameter standard free energy (ΔG°) of the

Table 1. Physicochemical Parameters of the Cosolvents

solvent	log <i>P</i>	ε	DC ^a
ethylene glycol	-1.8	37.7	18.7
diethylene glycol	-2.3	31.7	<i>b</i>
triethylene glycol	-2.8	23.7	<i>b</i>
glycerol	-3.03	42.5	20.2

^a DC = denaturation capacity (taken from Khmelnitsky et al., 1991). ^b Not determined.

Table 2. First-Order Deactivation Constants for PVA in the Presence of Glycols at 40 °C

solvent concn (%)	ethylene glycol		diethylene glycol		triethylene glycol	
	<i>k_d</i> (h ⁻¹)	Δ <i>G</i> ^o (kJ/mol)	<i>k_d</i> (h ⁻¹)	Δ <i>G</i> ^o (kJ/mol)	<i>k_d</i> (h ⁻¹)	Δ <i>G</i> ^o (kJ/mol)
0	0.003	113.2	0.003	113.2	0.003	113.2
3	0.00379	112.59	0.00237	113.81	0.00136	115.26
5	0.00291	113.28	0.00186	114.44	0.00127	115.44
8	0.00280	113.38	0.00178	114.56	0.00105	115.93
10	0.00303	113.17	0.00131	115.35	0.00095	116.19
20	0.00251	113.66	0.00065	117.18	0.00073	116.88

deactivation process can be calculated from the following equation:

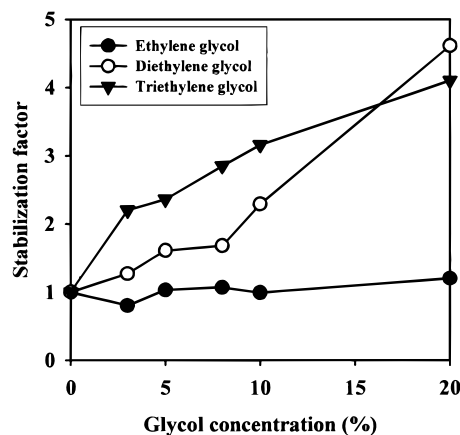
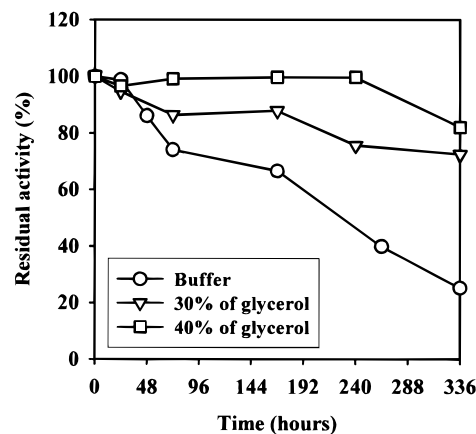
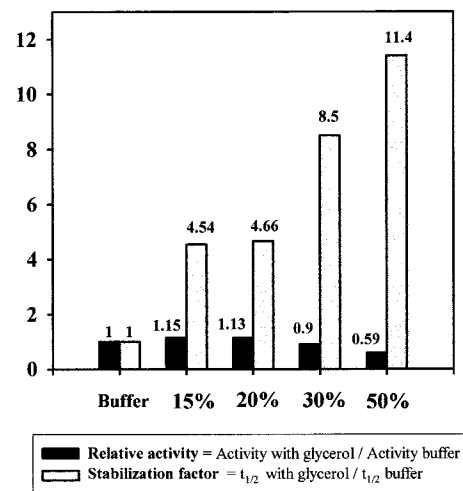
$$-\Delta G_d^o = RT \ln[(k_B T)/(k_d \hbar)]$$

where *k_d* is the first-order deactivation constant (h⁻¹), *k_B* is Boltzmann's constant (1.38×10^{-23} J K⁻¹), \hbar is Planck's constant (1.84×10^{-37} J h), *R* is the gas constant (8.314 J mol⁻¹ K⁻¹), and *T* is the temperature (K). As it is shown in Table 2, the protective effect exerted by glycols is also supported by an increase in the standard free energy of denaturation. These increments should stabilize PVA efficiently since the net free energy for stabilization of proteins is small in general (Gekko and Timasheff, 1981a). Furthermore, we can conclude that there is an improvement of the stabilization related to the polarity of the glycol: the lower the log*P* value, the higher stabilization factor value (Figure 3). Ethylene glycol does not affect enzyme stability, and we can consider it as an inert solvent for PVA, whereas the other glycols are protective agents. The protective effect increases proportionally when the concentration of diethylene glycol and triethylene glycol is increased in the medium. The stabilization exerted by triethylene glycol at 20% was lower than expected since its threshold concentration had been reached.

Effect of Glycerol Concentration on PVA Thermal Stability. The influence of glycerol on PVA thermal stability at 40 and 50 °C was studied. The stabilization of PVA at 40 °C in the presence of glycerol was so high (Figure 4) that we decided to establish more extreme conditions of temperature. Glycerol has the lowest log *P* value, so the stabilization was even higher than that observed in the presence of glycols. Once again, there is an improvement of stability proportional to glycerol concentration (Figure 5). Nevertheless, we could check that the catalytic activity was increased to a lower extent than as observed at 40 °C.

Conclusions

There are many enzymes, i.e., yeast alcohol dehydrogenase (Larreta-Garde et al., 1988), invertase (Monsan and Combes, 1988), α-amylase (Graber and Combes, 1989; De Cordt et al., 1994), α-chymotrysin (Lozano and Combes, 1992), papain (Lozano et al., 1993), thermolysin (Mejri et al., 1998), β-xylanase (Breccia et al., 1998), penicillin G acylase (Erarslan, 1994; Kazan and Erarslan, 1997, 1999; Azevedo et al. 1999) whose activities and

**Figure 3.** Influence of glycol concentration on thermal stability of penicillin V acylase from *S. lavendulae* at 40 °C.**Figure 4.** Thermal stabilization of penicillin V acylase from *S. lavendulae* at 40 °C in the presence of glycerol.**Figure 5.** Stability and activity of penicillin V acylase from *S. lavendulae* at 50 °C in the presence of glycerol.

thermal stabilities are enhanced in the presence of polyols proportionally to their concentration. In fact, polyols and glycols are the best cosolvents to maintain both the solvophobic interactions essential for the native structure of the enzyme and the water shell around the protein molecule (Back et al., 1979; Gekko and Timasheff, 1981b; Lee and Lee, 1987). The stabilizing effect exerted by these cosolvents is attributed to a positive change in the standard free energy of denaturation as reported for other enzymes such as chymotrypsinogen and ribonuclease (Gekko and Timasheff, 1981a). Penicillin V acylase

from *S. lavendulae* is another enzyme whose longevity can also be increased by the presence of glycerol and glycols. This fact could be very interesting for industrial processes, even more so when one knows the concentration of cosolvent in which PVA displays enhanced activity.

Acknowledgment

Financial support from the CICYT (QUI97-00490) is gratefully acknowledged.

References and Notes

- Arroyo, M.; Torres, R.; de la Mata, I.; Castillón, M. P.; Acebal C. Interaction of penicillin V acylase with organic solvents. Catalytic activity modulation on the hydrolysis of penicillin V. *Enzyme Microb. Technol.* **1999**, *25*, 378–383.
- Azevedo, A. M.; Fonseca, L. P.; Prazeres, D. M. F. Stability and stabilisation of penicillin acylase. *J. Chem. Technol. Biotechnol.* **1999**, *74*, 1110–1116.
- Back, J. B.; Oakenfull, D.; Smith, M. B. Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry* **1979**, *18*, 5191–5196.
- Batra, R.; Gupta, M. N. Enhancement of enzyme activity in aqueous–organic solvent mixtures. *Biotech. Lett.* **1994**, *16*, 1059–1064.
- Brescia, J. D.; Morán, A. C.; Castro, G. R.; Siñeriz, F. Thermal stabilization by polyols of β -xylanase from *Bacillus amyloliquefaciens*. *J. Chem. Technol. Biotechnol.* **1998**, *71*, 241–245.
- De Cordt, S.; Hendrickx, M.; Maesmans, G.; Tobback, P. The influence of polyalcohols and carbohydrates on the thermostability of α -amylase. *Biotechnol. Bioeng.* **1994**, *43*, 107–114.
- Duggleby, H. J.; Tolley, S. P.; Hill, C. P.; Dodson, E. J.; Dodson, G.; Moody, P. C. E. Penicillin acylase has a single amino acid catalytic centre. *Nature* **1995**, *373*, 264–268.
- Eraslan, A. Effect of polyol compounds on thermostability of penicillin G acylase obtained from a mutant of *Escherichia coli* ATCC 11105. *Process Biochem.* **1994**, *30*, 133–139.
- Gekko, K.; Timasheff, S. N. Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry* **1981a**, *20*, 4677–4686.
- Gekko, K.; Timasheff, S. N. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol–water mixtures. *Biochemistry* **1981b**, *20*, 4667–4676.
- Graber, M.; Combes, D. Effect of polyols on fungal α -amylase thermostability. *Enzyme Microb. Technol.* **1989**, *11*, 673–677.
- Gupta, M. N. Enzyme function in organic solvents. *Eur. J. Biochem.* **1992**, *203*, 25–32.
- Kazan, D.; Eraslan, A. Influence of polyethylene glycols on the thermostability of penicillin G acylase obtained from a mutant of *Escherichia coli* ATCC 11105. *Appl. Biochem. Biotechnol.* **1997**, *62*, 1–13.
- Kazan, D.; Eraslan, A. Effect of dextran polymers on the stability of soluble *Escherichia coli* penicillin G acylase. *J. Chem. Technol. Biotechnol.* **1999**, *74*, 1157–1164.
- Khmelnitsky, Y. L.; Mozhaev, V. V.; Belova, A. B.; Sergeeva, M. V.; Martinek, K. Denaturation capacity: a new quantitative criterion for the selection of organic solvents as reaction media in biocatalysis. *Eur. J. Biochem.* **1991**, *198*, 31–41.
- Larreta-Garde, V.; Xu, Z. F.; Thomas, D. Behaviour of enzymes in the presence of additives. Influence of alcohols, polyols and sugars on activity and stability of yeast alcohol dehydrogenase. *Ann. N.Y. Acad. Sci.* **1988**, *542*, 294–298.
- Lee, L. L.-Y.; Lee, J. C. Thermal stability of proteins in the presence of poly(ethylene glycols). *Biochemistry* **1987**, *26*, 7813–7819.
- Lozano, P.; Combes, D. α -Chymotrypsin in plastein synthesis: influence of hydroxylated additives on enzyme activity. *Appl. Biochem. Biotechnol.* **1992**, *33*, 51–65.
- Lozano, P.; Cano, J.; Iborra, J. L.; Manjón, A. Influence of polyhydric cosolvents on papain thermostability. *Enzyme Microb. Technol.* **1993**, *15*, 868–873.
- Mejri, M.; Pauthe, E.; Larreta-Garde, V.; Mathlouthi, M. Effect of polyhydroxylic additives on the catalytic activity of thermolysin. *Enzyme Microb. Technol.* **1998**, *23*, 392–396.
- Monsan, P.; Combes, D. Enzyme stabilization by immobilization. *Methods Enzymol.* **1988**, *137*, 584–598.
- Mozhaev, V. V.; Khmelnitsky, Y. L.; Sergeeva, M. V.; Belova, N. L.; Klyachko, N. L.; Levashov, A. V.; Martinek, K. Catalytic activity and denaturation of enzymes in water/organic cosolvent mixtures: α -chymotrypsin and laccase in mixed water/alcohol; water/glycol and water/formamide solvents. *Eur. J. Biochem.* **1989**, *184*, 597–602.
- Reichardt, C. *Solvents and Solvent Effects in Organic Chemistry*, 2nd ed.; VCH: Weinheim, 1988.
- Rekker, R. F. *The Hydrophobic Fragmental Constant*; Elsevier: New York, 1977.
- Riddick, J. A.; Bunger, W. B.; Sakano, T. K. *Organic solvents. Physical Properties and Methods of Purification*, 4th ed.; Wiley: New York, 1986.
- Shewale, J. G.; Sudakaran, V. K. Penicillin V acylase: its potential in the production of 6-amino penicillanic acid. *Enzyme Microb. Technol.* **1997**, *20*, 402–410.
- Shewale, J. G.; Kumar, K. K.; Ambedkar, G. R. Evaluation of determination of 6-amino penicillanic acid by *p*-dimethylamino benzaldehyde. *Biotechnol. Tech.* **1987**, *1*, 69–72.
- Tan, K. M.; Lovrien, R. Enzymology in aqueous–organic cosolvent binary mixtures. *J. Biol. Chem.* **1972**, *247*, 3278–3285.
- Torres, R.; Ramón, F.; de la Mata, I.; Acebal, C.; Castillón, M. P. Enhanced production of penicillin V acylase from *Streptomyces lavendulae*. *Appl. Microbiol. Biotechnol.* **1999**, *53*, 81–84.
- Torres, R.; de la Mata, I.; Castillón, M. P.; Arroyo, M.; Torres, J.; Acebal, C. Purification and characterization of penicillin V acylase from *Streptomyces lavendulae*. In *Stability and Stabilization of Biocatalysts*; Ballesteros, A., Plou, F. J., Iborra, J. L., Halling, P. J., Eds.; Elsevier Science: New York, 1998, 719–724.

Accepted for publication March 15, 2000.

BP000023H