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A Kinetic Study of Synthesis of Amoxicillin Using Penicillin G Acylase Immobilized on Agarose

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Abstract

We present a kinetic model for the synthesis of amoxicillin from *p*-hydroxyphenylglycine methyl ester and 6-aminopenicillanic acid, catalyzed by penicillin G acylase immobilized on agarose, at 25°C. Michaelis-Menten kinetic parameters (with and without inhibition) were obtained from initial velocity data (pH 7.5 and 6.5). Amoxicillin synthesis reactions were used to validate the kinetic model after checking mass transport effects. A reasonable representation of this system was achieved under some operational conditions, but the model failed under others. Nevertheless, it will be useful whenever a simplified model is required, e.g., in model-based control algorithms for the enzymatic reactor.

Index Entries: Amoxicillin; kinetic model; penicillin G acylase; agarose; synthesis.

Introduction

Amoxicillin (D-[-]-α-amino-p hydroxybenzyl penicillin trihydrate) is a semisynthetic antibiotic with a broad spectrum of bactericidal activity against Gram-positive and gram-negative microorganisms and is one of the major b-lactam antibiotics, with sales in 1994 of $2200 million (US$) as a bulk-formulated drug *(1)*. Its bactericidal action against susceptible organisms occurs during the stage of active multiplication, acting through the inhibition of the cell wall mucopeptide biosynthesis.

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For the synthesis of semisynthetic antibiotics such as amoxicillin and ampicillin, the sidechains of penicillin G and V are replaced by other chains such as phenylglycine and *p*-hydroxyphentlglycine (*p*-OH-PG).

Enzymatic synthesis today is becoming more interesting as an industrial process because it reduces the number of reaction steps and decreases the amount and toxicity of waste products per kilogram of antibiotic. In many industrial processes, the chemical removal of sidechains is replaced by an enzymatic reaction step (hydrolysis) using immobilized penicillin acylase. The same enzyme also catalyzes the reverse reaction, and this behavior allows for the coupling of different side chains. Using enzymes such as penicillin G acylase (PGA) as catalysts could be of great interest. The high selectivity, specificity, and activity of these enzymes in mild reaction conditions (aqueous media, neutral pH, and moderate temperatures) precludes the use of organic solvents as well as the reactive group's protection/deprotection sequence, low temperatures (–30°C), and chemical acylation *(2)*. However, for enzymatic coupling to economically feasible, improvements on the enzymatic process are required. PGA can act as a hydrolase as well as a transferase. Although its most important application so far is to hydrolyze penicillin G to produce the antibiotic nucleus 6-aminopenicillanic acid (6-APA), when acting as a transferase it can catalyze the reverse reaction of antibiotic synthesis if appropriate temperature and pH conditions are used. But the hydrolysis of the acylation agent is an undesirable side reaction, involving excessive amounts of the acylation agent to achieve high conversions of the antibiotic nuclei. The hydrolysis of the new antibiotic is a second side reaction to be minimized in order to obtain acceptable yields from synthesis.

PGA can be used to semisynthesize β-lactam antibiotics following two different approaches: thermodynamically controlled (equilibrium controlled) and kinetically controlled synthesis.

The first is the direct condensation of the β-lactam nucleus with the acid as acyl donor. This approach has the advantage of not requiring prior substrate modifications. However, the high concentration of organic solvents needed to shift the equilibrium makes this form of synthesis less attractive.

In kinetically controlled synthesis, the use of ester or amides as activated substrates is necessary. The yield obtained depends on the balance of three different reactions catalyzed by the same enzyme *(3)*. This process involves the synthesis of a β-lactam antibiotic-AN, the hydrolysis of the activated substrate AB (h1), and the hydrolysis of the newly formed antibiotic AN (h2). The maximum yield depends on the degree of saturation of the active enzymatic center by the nucleophile, the ratio of antibiotic synthesis/hydrolysis (s/h2), and the ratio of antibiotic synthesis/ester hydrolysis (s/h1). The yield of enzymatic synthesis, however, is low because of the mentioned side-chains reactions, which makes further research about the system necessary in order to find better operational conditions to minimize ester and antibiotic hydrolysis.

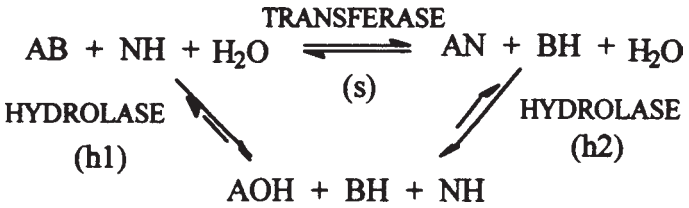

Fig. 1. Reactions involved in the enzymatic synthesis of amoxicillin, Kasche *(4)*.

In this work, we propose a model for the kinetically controlled synthesis of amoxicillin from *p*-hydroxyphenylglycine methyl ester (PHPGME) and 6-APA catalyzed by PGA from *Escherichia coli* immobilized on agarose. These equations will be used to search for optimal reaction conditions without exhausting and expensive experimental work. Because of the system's complexity, the process is decoupled and investigated step by step in order to estimate all the model parameters.

Following Kasche's *(4)* example, the scheme displayed in Fig. 1, we assume that synthesis of the antibiotic requires prior adsorption of its nucleus to the active center of the enzyme. The $V_{AN}/(V_{AN} + V_{AOH})$ ratio is the fraction of consumed activated substrate that yields the condensation product AN.

Here $(V_{AN})/(V_{AN} + V_{AOH}) = T_{max} \cdot X$, in which T_{max} is the maximum conversion ratio of the complex acyl-enzyme-nucleus into product (AN), and X is the fraction of enzyme saturated with the nucleus. It is also considered that a fraction of the acyl-enzyme complex becomes available again for the synthesis, as the nucleus is also adsorbed to the enzyme. Hence, the driving force group for hydrolysis of the antibiotic (neglecting the reverse reaction) is $C_{AN}.(1 - T_{max.}X)$.

Some advantages are added to enzymatic systems when immobilized enzymes are used, such as easy recovery and improved enzyme stability, continuous operation and better control of reaction, and higher purity and yield of products *(3)*. However, immobilized biocatalysts may cause mass-transfer problems to interfere in the reaction. Immobilization may result in diffusional limitations. Mass transport resistance, both inside the pores of the gel matrix and through the film surrounding the particle, is a factor that must be considered. When estimating kinetic parameters, these problems must be checked and considered (if important) to ensure the validity of the inherent parameters. The best way to determine whether diffusional phenomena are relevant in an immobilized biocatalyst system is highly dependent on the type of system. For instance, one may check for intraparticle resistance changing enzymatic loads into the biocatalyst particles and make identical experiments using particles with the same size distribution or change the particles' sizes and keep the enzyme load constant. A possible method to verify the presence of external mass-transfer resistance is to use different stirring rates, keeping all the other conditions constant.

Materials and Methods

Chemicals

PHPGME was synthesized at the Universidad de Salamanca, Spain. PGA was from *E. coli* and was donated by Antibioticos S/A, Spain. Agarose gel was donated by Hispanagar S/A, Spain, and amoxicillin was obtained from a drug called Glamoxyl intravenous from Spain. All other chemicals were of laboratory grade from commercial suppliers.

Preparation of PGA Derivatives (3)

Activation of agarose gel was performed by etherification with glycidol and oxidation with periodate. Further control of the PGA (amine)-agarose (aldehyde) multiple-point attachment was performed by reaction at pH 10.0, in the presence of phenylacetic acid. Final reduction of the amino double bonds was performed with sodium borohydride.

Agarose Gel Characterization

Agarose gel density was determined by picnometry. Image analysis software (Image-Pro Plus, Media Cybernetics), and an optical microscope (Olympus BX50) were used to measure the size of agarose particles.

Enzyme Activity

Enzyme activity was evaluated by colorimetric analysis of *p*-nitro-analide benzoic acid, released during 33 mg/L 6-nitro-3 fenilacetamide benzoic acid hydrolysis in a 50 m*M* phosphate buffer, pH 7.5. The difference between enzymatic activities of the supernate before and after immobilization was used to assess the enzymatic load of the gel. One unit of enzyme activity was defined as the quantity of enzyme that hydrolyses 1 μmol of penicillin G/min at pH 8.0 and 38°C.

Analysis

Concentrations of PHPGME, amoxicillin, 6-APA, and phenylacetic acid were determined using high-performance liquid chromatography with a C18 column (Analysis Vinicus; MFE-SIL 5 μm, 10 cm long and 4.6 mm diameter) and mobile phase with 27% acetonitrile, 2‰ sodium dodecyl sulfate (Lauryl sodium sulfate), and 16 m*M* phosphate buffer and pH 2.6 and 25°C.

Kinetic Studies

Kinetic parameters were determined by measuring the initial rates for different initial concentrations of substrate. Values of the parameter were estimated by nonlinear least squares fitting, using the algorithm of Marquardt *(5)*.

Experiments

A batch reactor with magnetic stirring was used in all the experiments. Ester hydrolysis and antibiotic synthesis required two reactors, with and without enzyme, because the ester was hydrolyzed even in the absence of a catalyst and this hydrolysis is deducted when hydrolysis or synthesis conversions are calculated. The pH of the solutions during the enzymatic hydrolysis and synthesis reactions was kept constant by an automatic titrator. Enzymatic hydrolysis and synthesis were carried out in a 25 m*M* phosphate buffer solution, pH 6.5 or 7.5, at 25°C.

Kinetic Model

In the kinetic model for the antibiotic hydrolysis, ester (AB) is a competitive inhibitor and 6-APA nucleus (NH) is a noncompetitive one (*see* Eq. 1). The antibiotic is a competitive inhibitor in the reaction of ester hydrolysis (*see* Eq. 2). It is also assumed that the presence of 6-APA (NH) does not interfere in the rate of ester (AB) hydrolysis (reaction h1).

For antibiotic hydrolysis:

$$v_A = \frac{k_{cat2} C_{AN} C_{EZ}}{K_{m2}[1 + (C_{AB}/k_{EA}) + (C_{NH}/k_{IN})] + C_A[1 + (C_{NH}/k_{IN})} \quad (1)$$

Ester hydrolysis:

$$v_{AB} = \frac{k_{cat1} C_{AB} C_{EZ}}{K_{m1}[1 + (C_{AN}/k_{AE})] + C_{AB}} \quad (2)$$

Amoxicillin synthesis:

$$v_{AN} = \frac{k_{cat1} C_{AB} C_{EZ}}{K_{m1}[1 + (C_{AN}/k_{AE}) + C_{AB}} \quad (3)$$

in which

$$X = [C_{NH}/(K_{EN} + C_{NH})]$$

The mathematical model for the batch reactor is as follows:

$$dC_{AB}/dt = -v_{AB} \quad (4)$$

$$dC_{AN}/dt = v_{AB} - v_A \quad (5)$$

$$dC_{NH}/dt = v_A - v_{AN} \quad (6)$$

Equations 4–6 are a set of ordinary differential equations, solved numerically.

Results and Discussion

Agarose Characterization

Table 1 presents the agarose gel characteristics.

Table 1
Agarose Gel Characteristics[a]

Parameter	10 BCL	6 BCL
Density (g/mL)	1.0434 ± 00.0017	1.02150 ± 00.0016
Average radius (μm)	150.6790 ± 78.6950	86.43800 ± 26.3560
Porosity (%)	92.0440 ± 00.0440	94.45700 ± 00.1590

[a]Ten BCL large particles (10% of crosslink bonds, small pores) and 6 BCL small particles (6% of crosslink bonds, large pores).

Mass Transport Effects: External and Internal Diffusion

Batch experiments were performed using the same biocatalyst but different stirring speeds in order to investigate the existence of external mass transport limitations. Figure 2 shows the results obtained: dimensionless concentrations of ester and antibiotic nucleus vs time. No difference was observed in the concentration profiles for different speeds (Fig. 2). If the process were limited by external diffusion, the course of reaction would be different for each speed. Therefore, we conclude that external diffusion was not significant.

Batch experiments of amoxicillin synthesis were performed for different particle diameters and pore sizes. The enzymatic load immobilized on the gel was kept low to avoid the interference of intragel diffusion. Nevertheless, experiments were run to detect whether internal mass transport effects were really absent (see Fig. 3 for the results). There is no noticeable difference among the conditions that were investigated. This is an indication that internal mass transport does not control the system, at least under the conditions used here.

Estimation of Parameters

Because of the system's complexity, the model parameters were estimated separately. First, ester hydrolysis (in the absence of antibiotic nuclei) were studied in order to estimate k_{cat1} and Km_1 values, using initial rate data (see Fig. 4). Next, amoxicillin hydrolysis was studied to determine k_{cat2} and Km_2 values, using the same approach (*see* Fig. 5). Third, inhibition studies were conducted to verify the influence of substrates and products on the kinetics of hydrolysis and synthesis: ester hydrolysis in the presence of amoxicillin (competitive inhibitor; *see* Fig. 6) and amoxicillin hydrolysis, in both the presence of ester (competitive inhibitor; *see* Fig. 7) and 6-APA (noncompetitive inhibitor; *see* Fig. 8). All experiments took initial velocities. From these experiments we estimated k_{EA}, k_{AE}, and k_{IN}. Finally, to estimate Tmax and K_{EN}, initial rates of amoxicillin synthesis were studied for different initial concentrations of antibiotic nuclei. Owing to substrate solubility problems, it was not possible to use high concentrations of 6-APA. Therefore, we kept Tmax = 0.97 and estimated K_{EN} from those data (*see* Fig. 9). Table 2 gives the estimated model parameters.

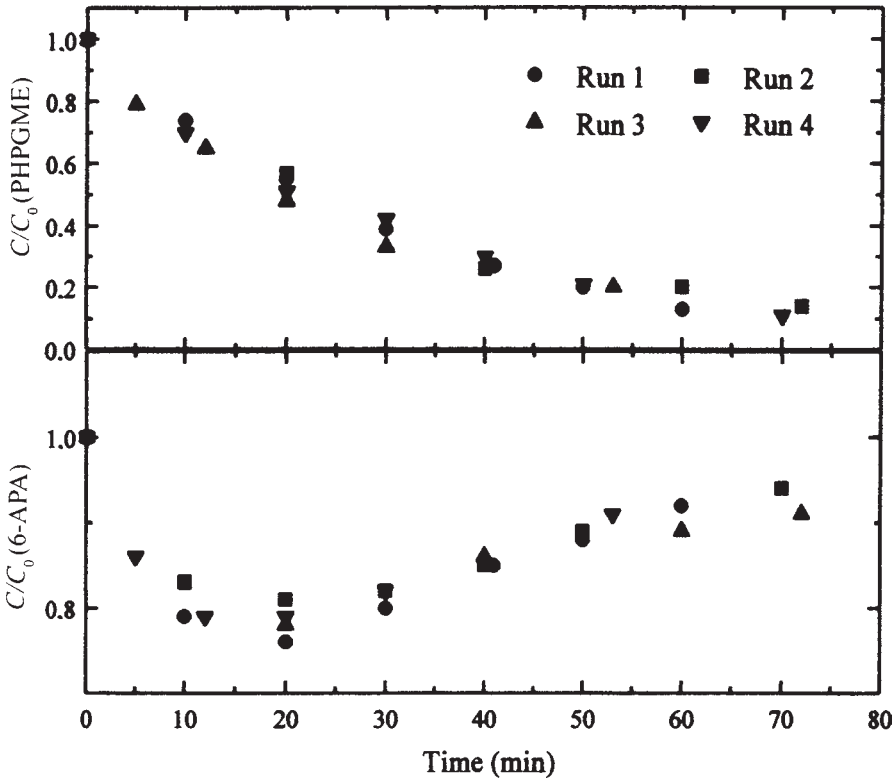

Fig. 2. Dimensionless concentration of PHPGME and 6-APA as a function of time for different stirring speeds. Runs 1–3 represent a triplicate with the following conditions: stirring, 360 rpm; biocatalyst, agarose 6 BCL large pores. Run 4 was performed with the same biocatalyst but with stirring at 120 rpm.

Model Validation

To validate the model and the estimated parameters, experiments of amoxicillin synthesis at different initial conditions were conducted.

1. pH 6.5 and 25°C with low substrate concentration: yield of synthesis was 16.60% with respect to both substrates (equimolar amounts were used).
2. pH 6.5 and 25°C with a large amount of ester: yield of synthesis was 11% with respect to ester and 44.4% with respect to 6-APA.
3. pH 7.5 and 25°C: yield of synthesis was 18% with respect to both substrates (equimolar amounts were used).

The reported yields are still low. The ratio of conversion of 6-APA into amoxicillin was 23% at pH 7.0 and 20°C, in the presence of 100 m*M* of phosphate buffer, and 60% in the absence of buffer *(6)*, and that is one of the reasons that this process is not yet used in industrial scale. The experimental yield obtained here was also low, but this result was already expected once we were not working under optimized synthetic conditions. In a pre-

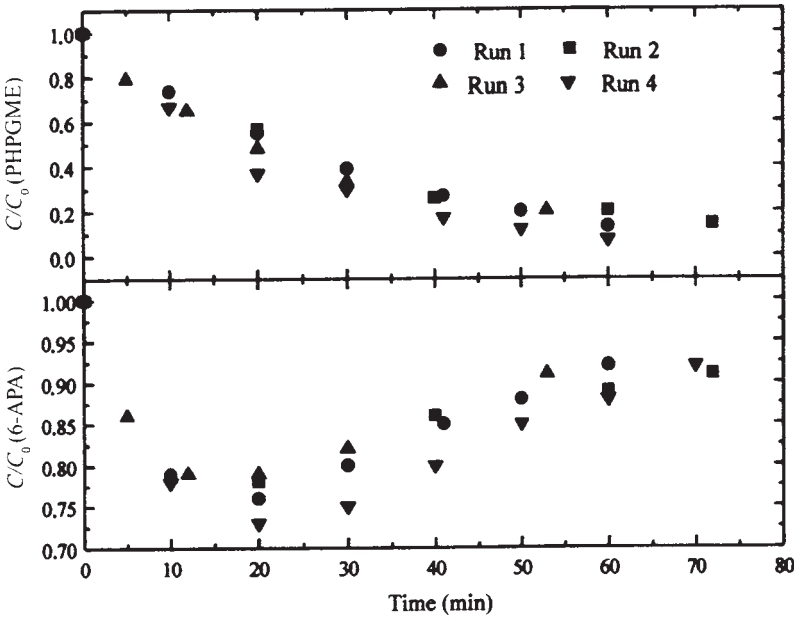

Fig. 3. Dimensionless concentration of PHPGME and 6-APA as a function of time for different particle and pore sizes. Runs 1–3: replicates, biocatalyst with small diameter and large pores (6 BCL). Run 4: biocatalyst with large diameter and small pores (10 BCL).

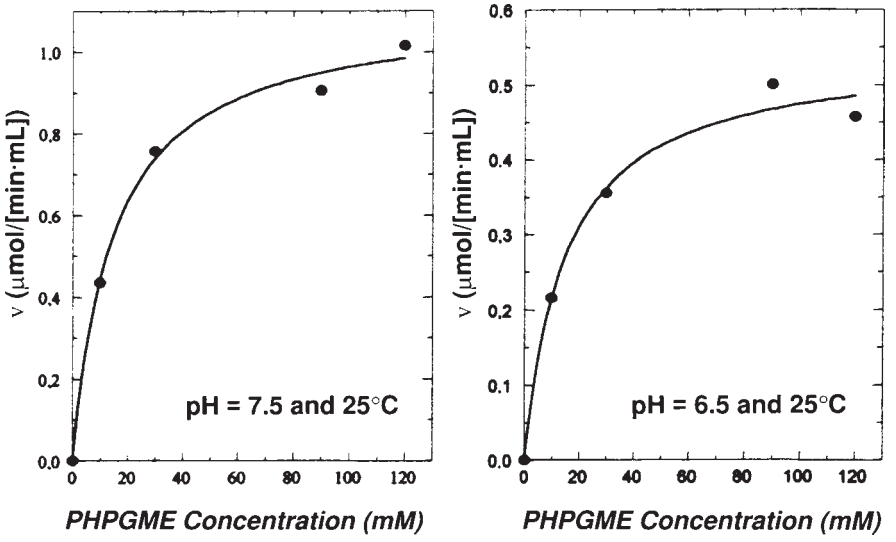

Fig. 4. Ester hydrolysis for different pH levels and temperatures. ●, Experimental data and solid line, the kinetic model.

vious work, we concluded that the optimal conditions to amoxicillin production were pH 6.5 and 4°C (*7*). These conditions were not used here, though, because the slow rates of reaction would lead to very difficult

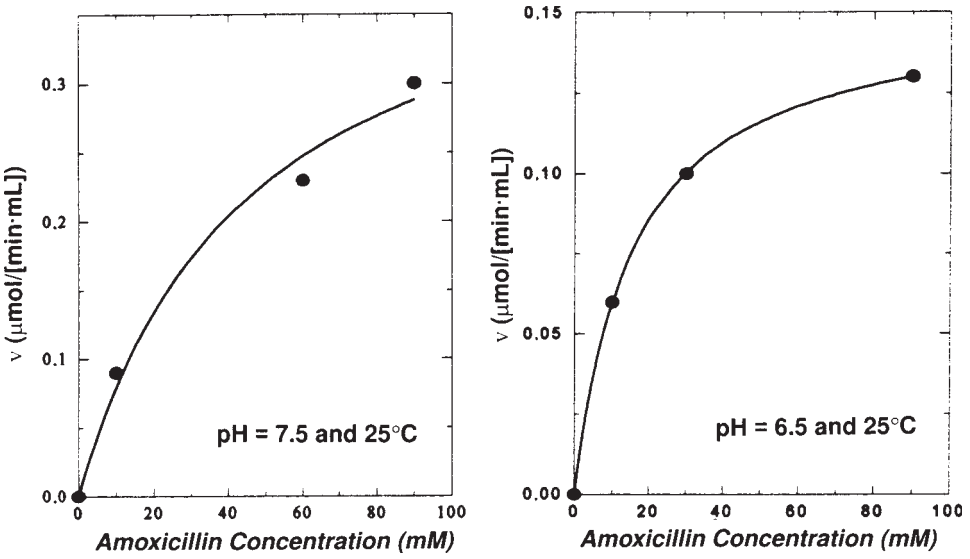

Fig. 5. Amoxicillin hydrolysis at different pH levels and temperatures. ●, Experimental data; solid line, the kinetic model.

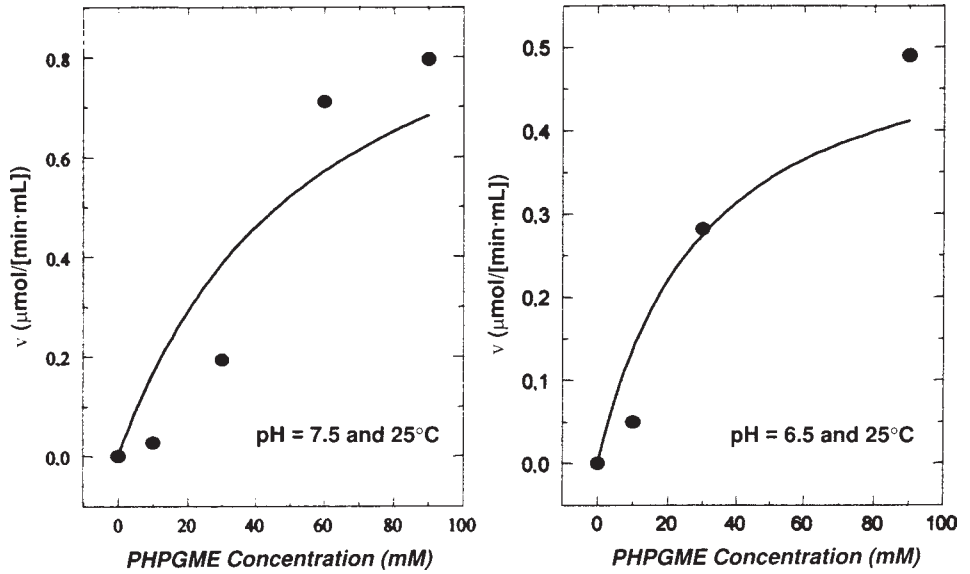

Fig. 6. Ester hydrolysis in the presence of amoxicillin. ●, Experimental data; solid line, the kinetic model.

experimental work. This work was not focused on improving experimental yield, but some studies are being done in our laboratory in order to achieve this goal by changing enzyme conformation during immobilization and conditions of enzyme reaction media.

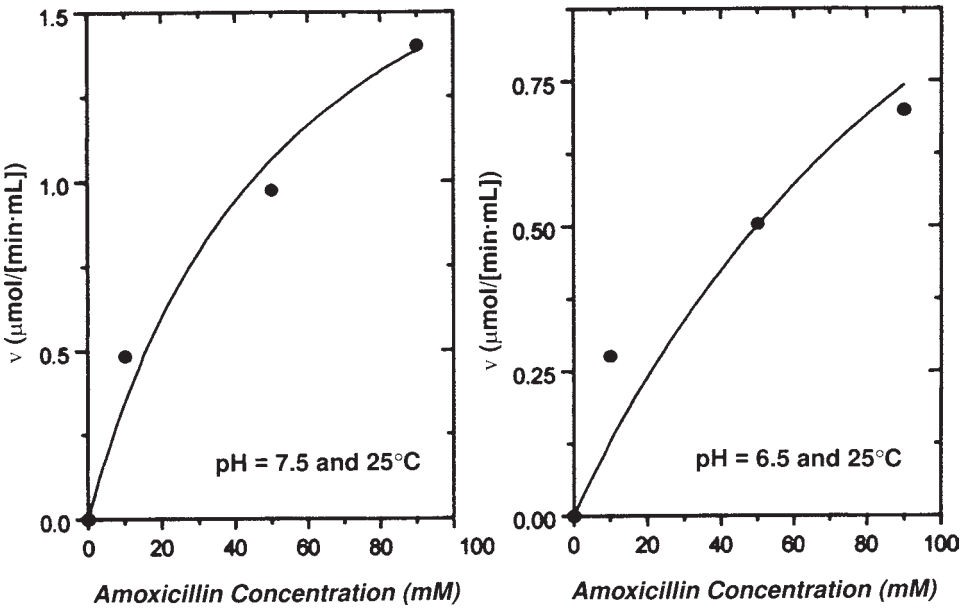

Fig. 7. Amoxicillin hydrolysis in the presence of ester. ●, Experimental data; solid line, the kinetic model.

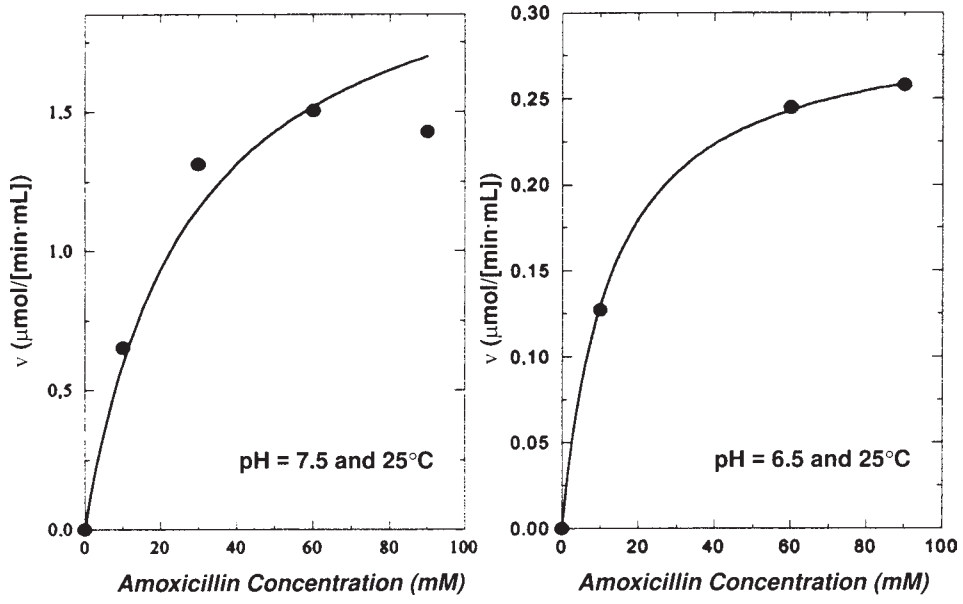

Fig. 8. Amoxicillin hydrolysis in the presence of 6-APA. ●, Experimental data; solid line, the kinetic model.

Figures 10–12 show the obtained results. One can see that the model provides a reasonable representation for some operational conditions (pH 7.5 and 25°C; and pH 6.5, 25°C, and low substrate concentration) but fails

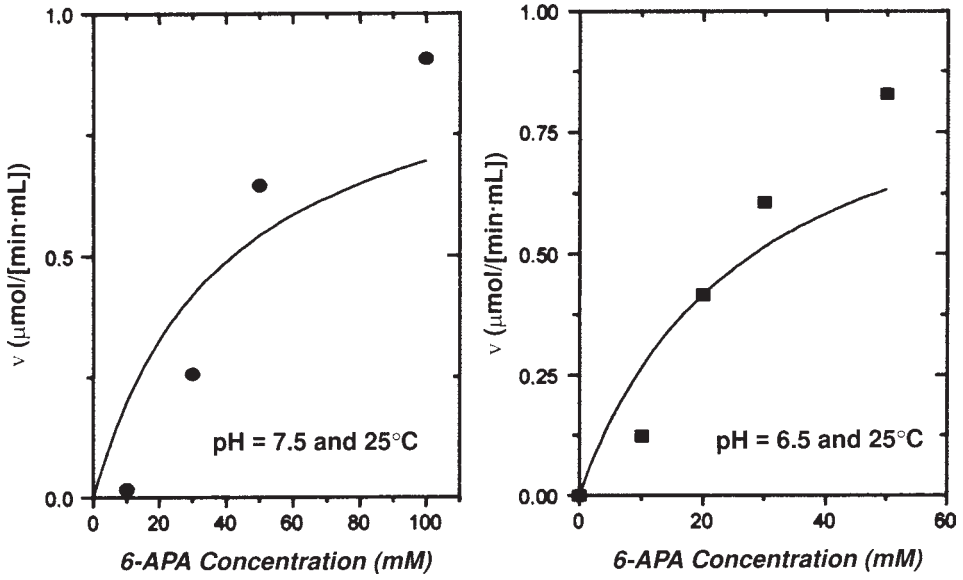

Fig. 9. Synthesis:hydrolysis ratio for different initial concentrations of nucleus. ● and ■, experimental dta; solid line, the kinetic model.

Table 2
Estimated Parameters, with 95% Confidence Interval

Parameter	pH 7.5 and 25°C	pH 6.5 and 25°C
k_{cat1} (μmol/mim UI)	1.105 ± 0.03958	0.546 ± 0.0308
k_{cat2} (μmol/mim UI)	2.218 ± 0.28264	1.857 ± 0.27108
Km_1 (m*M*)	14.95 ± 2.15535	15.32 ± 3.4452
Km_2 (m*M*)	21.46 ± 8.53358	12.83 ± 7.83065
K_{EA} (m*M*)	10.80 ± 4.57257	32.13 ± 22.96022
K_{AE} (m*M*)	6.54 ± 1.27442	1.05 ± 0.1763
K_{IN} (m*M*)	34.46 ± 26.11506	1.89 ± 0.01015
K_N (m*M*)	39.5 ± 18.23828	22.57 ± 7.15403
T_{max}	0.97	0.97

for others (pH 6.5, 25°C, and a large amount of substrate ester). These results indicate that the empirical model needs improvement. In other words, the hypothesis should be revised and/or some other inhibitory effects should be considered.

Some information is available on the synthesis of different antibiotics, such as cephalexin and ampicillin, which are quite similar to amoxicillin. Because the enzyme for these antibiotics is more specific for the acyl moiety and accepts a wide range of β-lactam rings *(8)*, we expect that the mechanism of synthesis is very similar. Although kinetic parameters and synthetic yields should be different, we believe the catalytic pathway should be very similar. In the acyl moiety of the amoxicillin molecule, that is an OH

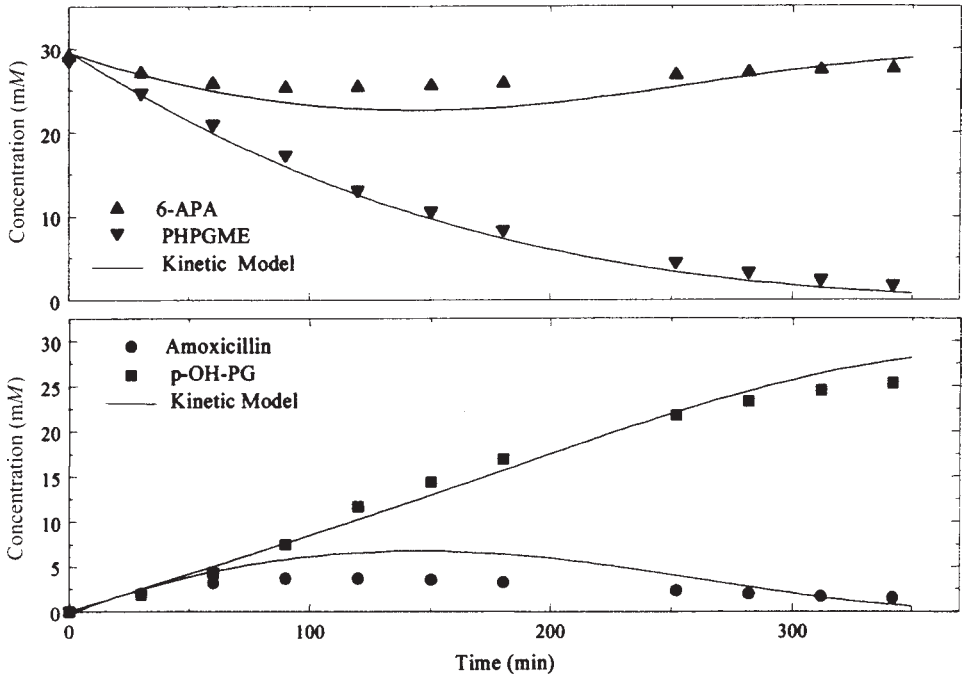

Fig. 10. Amoxicillin synthesis at 25°C, pH 6.5, and 3 mL of 30 UI/mL of biocatalyst: low substrate concentration.

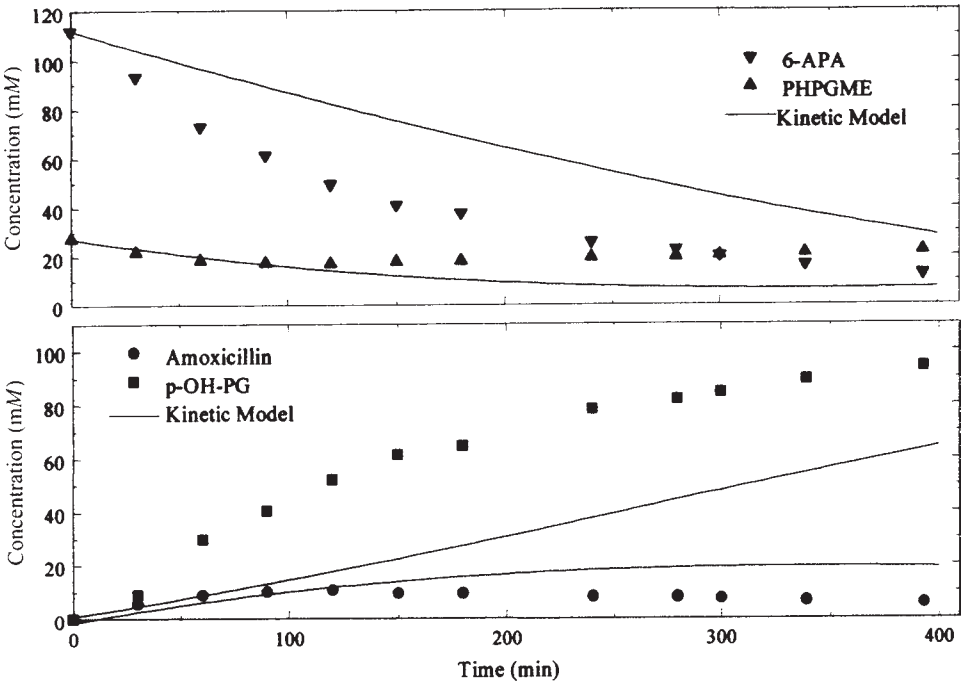

Fig. 11. Amoxicillin synthesis at 25°C, pH 6.5, and 3 mL of 30 UI/mL of biocatalyst: large initial amount of PHPGME.

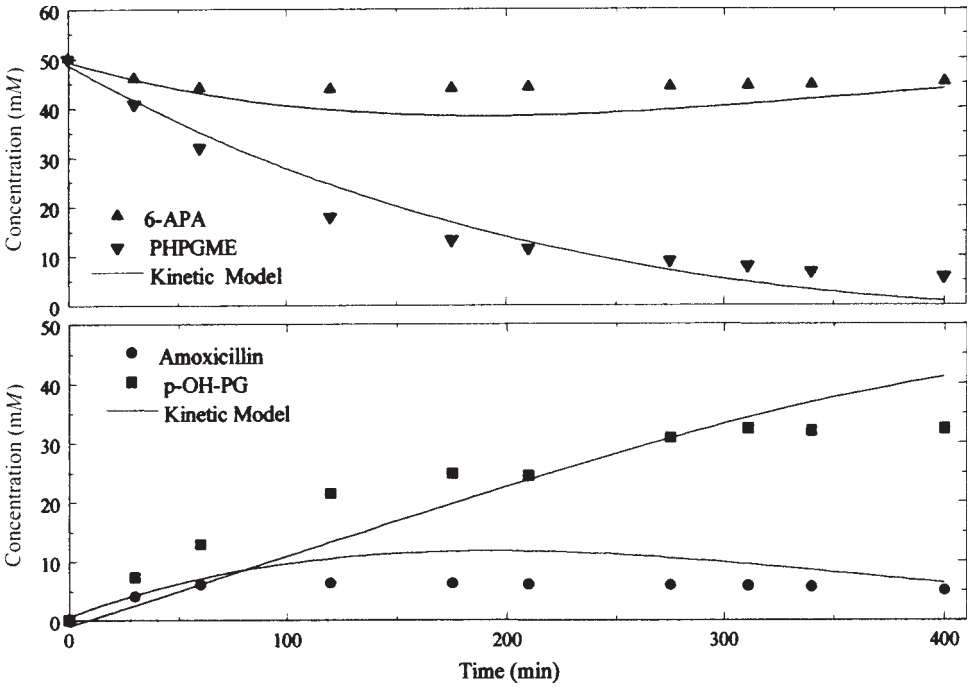

Fig. 12. Amoxicillin synthesis at 25°C, pH 7.5, and 5 mL of 10 UI/mL of biocatalyst.

group that is not present in ampicillin. This group is the only difference between the two antibiotics and is not directly involved in the breaking of amide or esterase bond. Blinkovsky and Markaryan *(9)* found that phenylglycine behaves as a competitive inhibitor in cephalexin hydrolysis, but they also report that methanol does not influence the synthesis of cephalexin up to a concentration of 1.5 m*M*. Ospina et al. *(10)*, using the same enzyme, studied the synthesis of ampicillin. They reported inhibitory effects, such as methanol noncompetitive inhibition on the ester hydrolysis and competitive phenylglycine inhibition on ampicillin hydrolysis, which are not considered herein. They also proposed a kinetic model, obtaining a good correlation between experimental results and the model predictions, except for high substrate concentrations. This behavior was also observed for the model described herein.

In view of the results obtained in the present study and taking into account the discordance among different investigators with respect to the enzyme's action mechanism during the synthesis of β-lactamic antibiotics, we conclude that the question is still open to further investigation. To improve our understanding of the problem, different inhibitory effects are currently being analyzed: 6-APA, methanol, phenylglycine and others.

Conclusion

The results obtained after batch experiments using the same biocatalyst but different stirring speeds showed that external mass transport is not

a limiting step for the process. Batch runs of amoxicillin synthesis using different particle sizes and pore diameters and different enzymatic loads showed that intraparticle diffusion is not significant either, at least under the conditions studied. For some operational conditions, the validation tests showed that the empirical model proposed herein is in reasonable agreement with the experimental data of amoxicillin synthesis; however, it fails for others, a clear indication that other variables must be considered. Nevertheless, the proposed kinetic model may be useful in situations in which a simplified model is required as, e.g., in model-based hybrid process control algorithms.

Acknowledgments

We thank the State of São Paulo Research Support Foundation (FAPESP) for the sponsorship that made this work possible.

Nomenclature

AB = Activated acyl donor (PHPGME, in the amoxicillin synthesis)
AN = Formed antibiotic
AOH = Formed acid (*p*-OH-PG in the PHPGME hydrolysis)
6-APA = 6-Aminopenicil;anic acid
BCL = Crosslink bonds
BH = Methanol
C_{AB} = PHPGME concentration
C_{AN} = Amoxicillin concentration
C_{EZ} = Enzyme concentration
C_{NH} = Nucleus concentration
$h1$ = Hydrolysis of the activated acyl donor
$h2$ = Hydrolysis of the newly formed antibiotic
k_{AE} = Inhibition constant, amoxicillin: competitive inhibitor in the PHPGME reaction
k_{cat1} = First-order constant to PHPGME consumption
k_{cat2} = First-order constant to amoxicillin hydrolysis
k_{EA} = Inhibition constant, PHPGME: competitive inhibitor in the amoxicillin reaction
k_{EN} = Nucleus adsorption constant
k_{IN} = Inhibition constant, 6-APA: noncompetitive inhibitor in the amoxicillin reaction
Km_1 = Michaelis-Menten constant to PHPGME consumption
Km_2 = Michaelis-Menten constant to amoxicillin hydrolysis
NH = β-Lactam nucleus (6-APA in amoxicillin synthesis)
v_A = Rate of antibiotic hydrolysis
v_{AB} = Rate of ester consumption
v_{AN} = Rate of antibiotic formation
v_{AOH} = Rate of acid formation

T_{max} = Maximum conversion of acyl-enzyme-nucleus complex in product (AN)
X = Portion of enzyme saturated with nucleus

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