GREEN ROUTE FOR AMOXICILLIN PRODUCTION THROUGH THE INTEGRATION WITH THE RECYCLE OF THE BY-PRODUCT (*P*-HYDROXYPHENYLGLYCINE)

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ABSTRACT – In the kinetically-controlled enzymatic synthesis of amoxicillin catalyzed by penicillin G acylase (PGA, E.C.3.5.1.11) *p*-hydroxyphenylglycine (PHPG) is a by-product continuously formed in the reaction. Also, methyl ester is the standard substrate used in the production of amoxicillin generating methanol as a by-product. Thus, we assessed the recovery and reuse of PHPG as reactant for the synthesis of *p*-hydroxyphenylglycine ethyl ester substrate (PHPGEE) and the integration to the process through the recycle of PHPGEE for the reactor of enzymatic synthesis of amoxicillin generating ethanol as a by-product. Recovery of the by-product of the enzymatic synthesis of amoxicillin was effective reaching a purity of 99% for the PHPG. Purified PHPG was successfully employed in the production of PHPGEE with a conversion of 93%. The enzymatic synthesis of amoxicillin employing the previously synthesized PHPGEE was feasible following the characteristic profile that is expected for these reactions.

1. INTRODUCTION

β-Lactam antibiotics are among the most used pharmaceuticals (Giordano *et al.*, 2006). Among them, amoxicillin occupies a prominent position due to its broad spectrum of activity, high rate of absorption and its stability under acid conditions (Rolinson & Geddes, 2007). Although the high yields, the current industrial process to produce these antibiotics follows a drastic chemical route, generating non-recyclable wastes and harmful to the environment. Therefore, alternative routes for the production of semi-synthetic β-lactam antibiotics are required. In this context, the enzymatic synthesis of these antibiotics, firstly proposed by Cole (1969), is a "green chemistry" strategy to this important process. However, the enzymatic synthesis of β-lactam antibiotics is not yet economically competitive with the fine-tuned conventional chemical processes.

Penicillin G acylase (PGA, E.C.3.5.1.11) can be used for the enzymatic synthesis of β-lactam antibiotics. In this reaction, the enzyme catalyzes the condensation of an acyl group to a β-lactam nucleus (Giordano *et al.*, 2006). Moreover, the industrialized production of these nuclei, for instance, 6-aminopenicillanic acid (6-APA), which is an intermediate for semi-synthetic penicillins, including amoxicillin, is performed by enzymatic hydrolysis of penicillin G, which is the major application of PGA in industry (Parmar *et al.*, 2000). It is important to note that the most studied PGA is the strain ATCC 11105 from *Escherichia coli* (Calleri *et al.*, 2004), especially

immobilized-stabilized to insoluble carriers, because the implementation of soluble enzymes in industrial practice is much hindered and often cannot be recovered and reused, which will become unfeasible the operational cost of the industrial processes.

Semi-synthetic antibiotics can be enzymatically produced using thermodynamic controlled synthesis (TCS) or kinetically controlled synthesis (KCS) (Diender *et al.*, 1998; Fernández-Lafuente *et al.*, 1996). In TCS, yield is determined by the thermodynamic equilibrium constant of the process, while for KCS the competition between different catalytic activities of the same enzyme determines the overall selectivity (synthesis/hydrolysis ratio) of the synthetic process. In the case of amoxicillin in aqueous medium, TCS alternative is not feasible (Margolin *et al.*, 1980). In KCS of β-lactam antibiotics, an activated derivative of the acyl donor reacts with 6-APA and produces the antibiotic. In this reaction, PGA acts as a transferase. However, PGA is also a hydrolase. So, 6-APA and water molecules compete in the nucleophilic attack to the acyl-enzyme intermediate.

In these series-parallel reactions, the by-product (PHPG) is continuously formed throughout the course of the enzymatic synthesis of amoxicillin, as result of the hydrolase action of PGA. Both for economic and environmental reasons, the by-product (PHPG) that is formed must be recovered and reused for the synthesis of the ethyl ester substrate (PHPGEE), which would be recycled to the reactor for the subsequent enzymatic production of amoxicillin. It should be stressed that the *p*-hydroxyphenylglycine methyl ester (PHPGME) is the standard side-chain derivative in the literature. Nevertheless, PHPGME leads to formation of methanol, which is in disagreement with the concept of an environmental-friendly process, since methanol is more toxic than ethanol. For instance, methanol has a threshold limit value of toxicity inhalation (200 ppm) 5 times smaller compared to ethanol (1000 ppm).

In this context, the adoption of enzymatic catalysis and use of reactants that can contribute to lower environmental impacts are part of the green chemistry scope and extremely important to modern society. With this motivation, this study assessed the development of an integrated process of recovery, reuse and recycle of the by-product (PHPG) from KCS of amoxicillin catalyzed by PGA. The recovery of PHPG from the crystals mixture of amoxicillin and PHPG was performed through a selective variation in the pH of the reaction medium. Following, PHPG was reused in the production of PHPGEE substrate, that was, finally, recycled to the enzymatic reactor for further synthesis of amoxicillin.

2. MATERIALS AND METHODS

2.1. Materials

The chemicals, amoxicillin, 6-aminopenicillanic acid (6-APA), *p*-hydroxyphenylglycine (PHPG), *p*-hydroxyphenylglycine methyl ester (PHPGME), and *p*-dimethylaminobenzaldehyde (PDAB) were obtained from Sigma-Aldrich Corporation (Wisconsin, U.S.). Penicillin G acylase (PGA) from *E. coli* covalently immobilized on Sepabeads® (Mitsubishi Chemical Corporation) was kindly donated by the Department of Enzymatic Biocatalysis, Institute of Catalysis, CSIC, Spain (Mateo *et al.*, 2007).

2.2. Methods

Recovery of *p*-hydroxyphenylglycine: An amount of the crystals mixture of amoxicillin and PHPG was used so as to exceed the solubility limit of the compounds. Then, the pH of this solution was raised to 8.5 with concentrated sodium hydroxide (NaOH) solution at room temperature. Filtration of this solution was performed to obtain practically pure crystals of the PHPG. The filtrate containing a mixture of amoxicillin and PHPG was acidified at 4 °C with concentrated HCl solution lowering the pH to 4.89 (pI of amoxicillin). Amoxicillin precipitate was separated by filtration. The final purity of the compounds was obtained by HPLC analyzes.

Synthesis of *p*-hydroxyphenylglycine ethyl ester: For the synthesis of PHPGEE in lab scale a reflux system was used, with HCl acting as a catalyst. Also, the initial concentrations employed in this reaction were 16.4 M of ethanol, 0.3 M of HCl and 0.03 M of PHPG. The reaction medium was kept under reflux for 24 hours. During the course of the reaction, aliquots were withdrawn and analyzed by HPLC in order to monitor the process. At the end of the reaction, the solvent was completely evaporated leaving only dry crystals in the flask. The final crystals were analyzed by HPLC and conversion of the esterification process was determined.

Spectrometric characterization of *p*-hydroxyphenylglycine ethyl ester: The characterization of PHPGEE was carried out by spectrometry techniques. In the proton nuclear magnetic resonance (1H NMR), PHPGEE was solubilized in deuterated methanol (CD_3OD) for analyzes in a Bruker ARX-200 spectrometer. For infrared (IR) analyzes, PHPGEE was ground with potassium bromide (KBr) and the mixture was pressed to obtain transparent disks. The IR spectra were obtained in a Bomem M102 spectrometer. Then, PHPGEE was characterized using Waters Xevo TQ mass spectrometer. For this purpose, an infusion of PHPGEE solubilized in methanol was carried out with a syringe pump. For this MS analyzes, it was employed an electrospray source in the positive ionization mode and a triple quadrupole mass analyzer.

Enzymatic activity: Enzymatic activity of PGA immobilized on Sepabeads® was measured by the *p*-dimethylaminobenzaldehyde (PDAB) method at a wavelength of 415 nm (Balasingham *et al.*, 1972). For this purpose, an Ultrospec 2000 UV-Vis spectrophotometer from Pharmacia Biotech was used. The operational conditions were as follows: solution of Pen G 5% (w/v) in 100 mM phosphate buffer, pH 8.0, and 37 °C. One unit of enzyme (UI) was defined as the amount of enzyme that hydrolyzes 1 µmol de Pen G per minute at 37 °C and pH 8.0.

Synthesis of amoxicillin: Enzymatic reactions for the synthesis of amoxicillin were conducted under kinetic control. In these experiments, the reaction between PHPGEE and 6-APA was catalyzed by PGA immobilized on Sepabeads®. Initial substrate concentrations were 50 mM for both PHPGEE and 6-APA. In addition, the ratio of enzyme to substrate (E/S) was 52 IU/mmol of substrate and the total reaction volume was 30 mL; thus, the amount of enzyme derivative used in all the experiments was 0.3 g. Every synthesis was conducted at 25 °C, 100 mM phosphate buffer pH 6.5. Throughout the course of the reactions, aliquots were withdrawn for HPLC analyzes.

Analytical method: Concentrations were determined by HPLC in a Waters 996 Photodiode Array Detector (PDA) liquid chromatograph equipped with a Phenomenex Gemini C18 column (150 x 4,6 mm, 5 µm). The mobile phase was prepared as follows: 1.4 g of sodium dodecyl sulfate (SDS), 0.6805 g of phosphate monobasic anhydrous (KH_2PO_4), 650 mL of ultrahigh-purity water (Millipore Milli-Q System), 350 mL of acetonitrile, and correction to pH 3.0 using phosphoric acid (H_3PO_4). Analyzes were carried out at 25 °C and detection at 225 nm.

3. RESULTS AND DISCUSSION

The overall concept of the integrated process, with re-activation and recycling of PHPG, is presented in Figure 1. After the enzymatic synthesis of amoxicillin, run in a fed-batch reactor with simultaneous crystallization of the products, the result is a mixture of crystals of amoxicillin and PHPG. Purified PHPG goes to the reactor for synthesis of the ethyl ester substrate (PHPGEE), together with surplus ethanol. Then, the synthesized ester is recycled to the first reactor. The efficiency of the separation of PHPG from the crystals mixture of amoxicillin and PHPG was assessed in this study. The final purity was 99% for PHPG and 96% for amoxicillin.

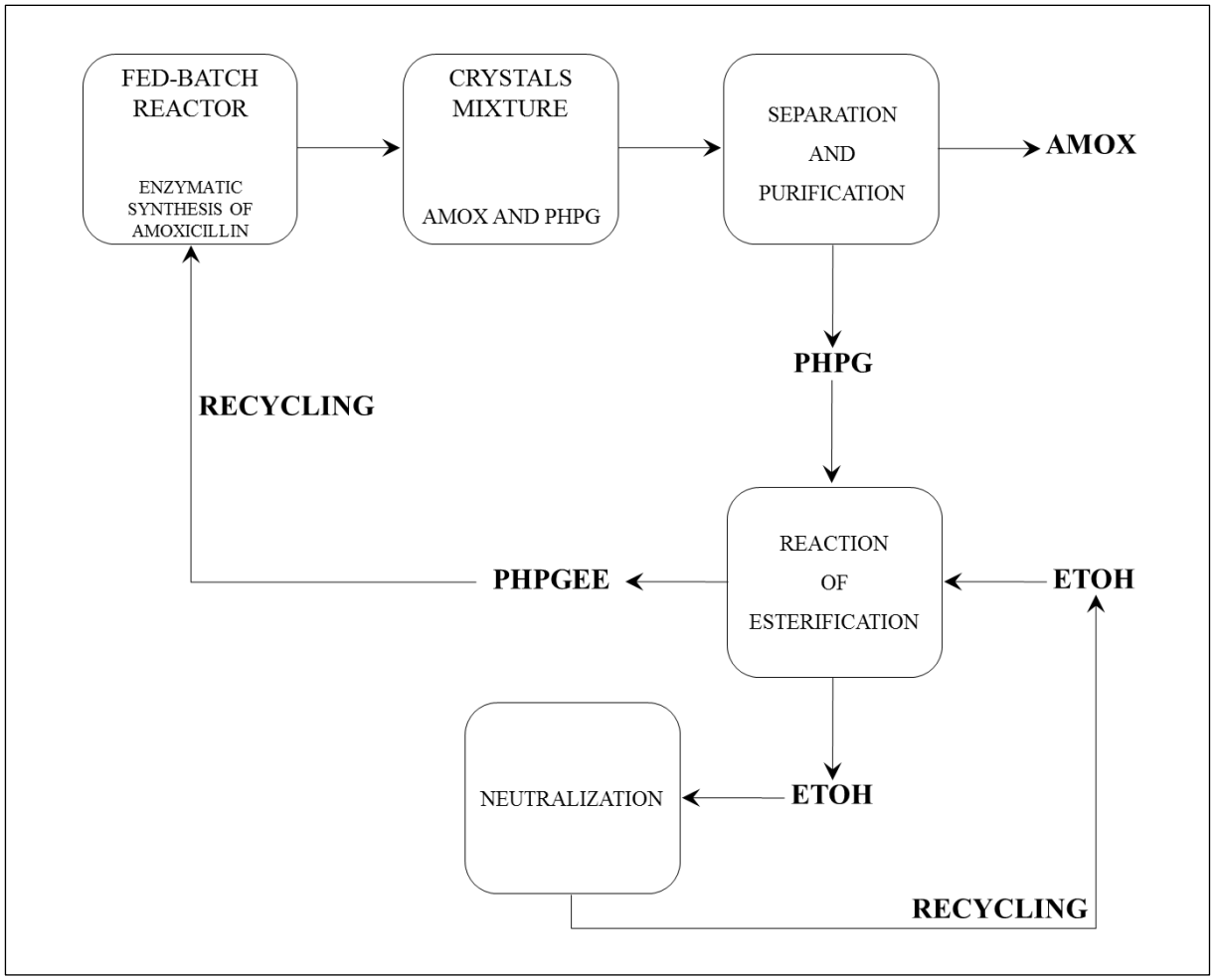
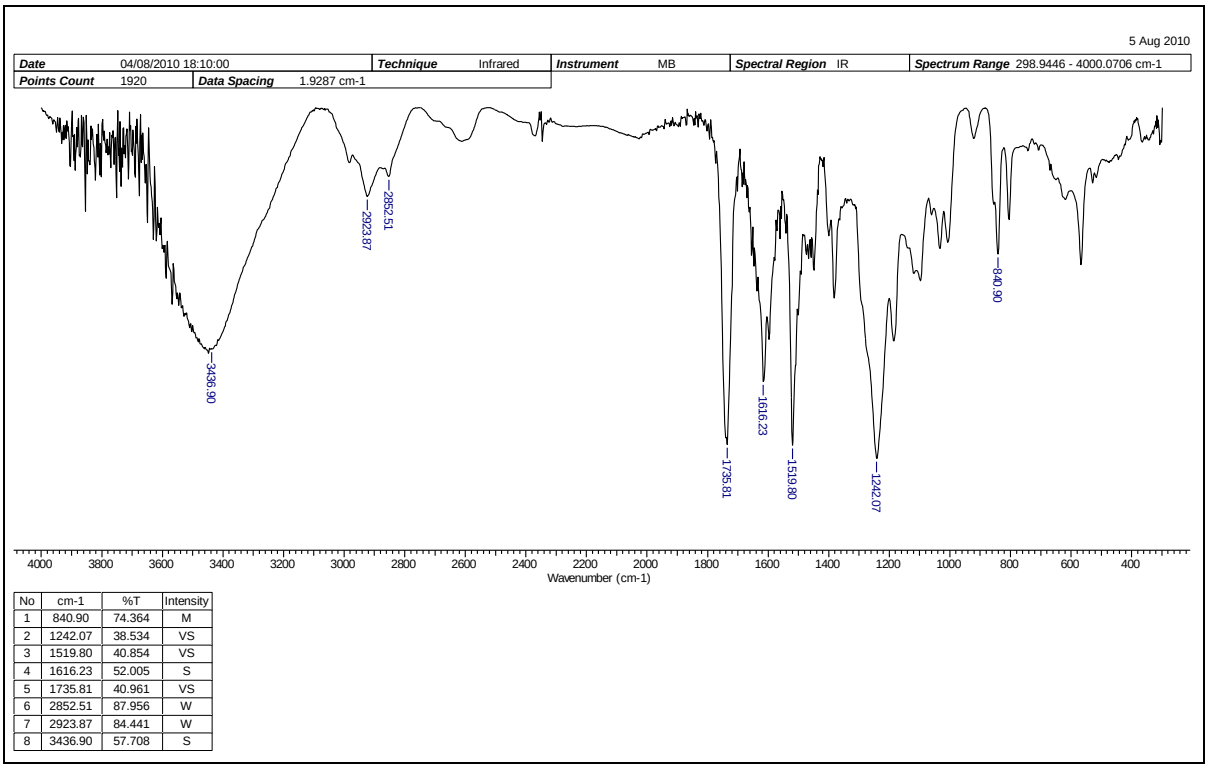


Figure 1 – Overall concept of the integrated process, with recovery of PHPG, production of PHPGEE, and recycle of PHPGEE to the enzymatic synthesis reactor.

Previously purified PHPG was reused for the synthesis of the ethyl ester substrate (PHPGEE). Under the reaction conditions studied here, the production process of PHPGEE reached a conversion of 93%. It is noteworthy that the conversion was calculated based on the concentration of PHPG used at the starting point of the esterification. It was found that after 24

hours of reaction the system was close to the chemical equilibrium, so this time of reaction was chosen for all experiments of PHPGEE synthesis.

Proton nuclear magnetic resonance (1H NMR), infrared (IR) and mass spectrometry (MS) were applied to characterize the synthesized PHPGEE according to the procedure described previously. Figure 2 shows the IR spectrum of the synthesized PHPGEE. A broad band in the region of 3600-3200 cm^{-1} is present, with the bell-shape characteristic of phenolic O-H bonds. Some of the bands relating to the stretching of C-H bond can be observed in the region of 3300-2800 cm^{-1}. Besides this, a band of strong absorption is observed in the region close to 1736 cm^{-1}, related to the stretching of C=O bond of the esters. A broader and stronger band than this last one, in the region of 1242 cm^{-1}, is characteristic of the stretching of C-CO-O bonds of the esters, as can be observed in 2.



No	cm-1	%T	Intensity
1	840.90	74.364	M
2	1242.07	38.534	VS
3	1519.80	40.854	VS
4	1616.23	52.005	S
5	1735.81	40.961	VS
6	2852.51	87.956	W
7	2923.87	84.441	W
8	3436.90	57.708	S

Figure 2 – IR spectrum (νmax, KBr) of PHPGEE.

The PHPGEE 1H NMR spectrum is in Figure 3. It is observed two doublets in the chemical shift at 6.86 and 7.28 ppm, characteristic of the hydrogens present in the di-substituted aromatic rings, as well as a singlet referring to the hydrogen of the methine group at 5.03 ppm. Besides, there is a quartet at the chemical shift 4.26 ppm, which is characteristic of hydrogens present in the methylene group, and a triplet at 1.22 ppm, referring to the hydrogens of the methyl group. All these observations indicate that the PHPGEE was successfully synthesized. It is noteworthy that the integration of the signals in the 1H NMR spectrum was consistent with the numbers of hydrogens present in the chemical structure of PHPGEE (see Figure 3).

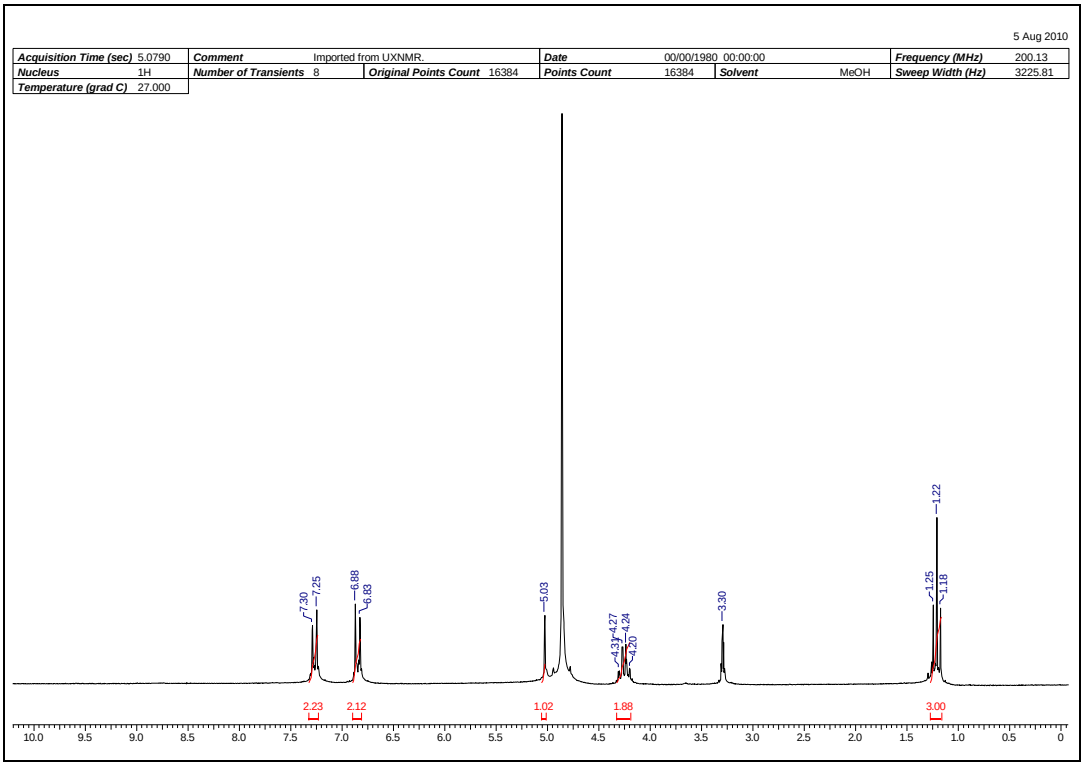


Figure 3 – 1H NMR spectrum (200 MHz, CD_3OD) of PHPGEE.

In order to prove the reactivity of the synthesized PHPGEE from the by-product (PHPG) of the enzymatic production of amoxicillin, a standard reaction of synthesis of this antibiotic was run using this substrate. The evolution of the KCS of amoxicillin catalyzed by PGA immobilized on Sepabeads® followed the expected pattern for the processes carried out in these operational conditions, as can be seen in Figure 4. It is important to mention that synthesized PHPGEE was obtained with high final purity, and it can be employed for the enzymatic synthesis of amoxicillin without further purification, despite it is produced in the form of a hydrochloride (PHPGEE.HCl). This fact does not affect the enzymatic reaction of amoxicillin production, since the pH of the reaction medium is previously adjusted by adding concentrated NaOH solution, generating the harmless salt NaCl.

4. CONCLUSION

The integration of the process of recovery of PHPG, a by-product of the kinetically controlled enzymatic synthesis of amoxicillin catalyzed by PGA, with its reuse for the subsequent production of ethyl ester substrate (PHPGEE), which is recycled to the reactor for the further enzymatic synthesis of amoxicillin, was assessed in this work. The integration of the process with the step of recycling of the synthesized PHPGEE for the enzymatic synthesis of amoxicillin proved to be feasible, with the evolution of the reaction following the same profile that is found when methyl ester (standard substrate) is used as activated substrate, presenting the important advantage to generate ethanol instead of methanol as by-product.

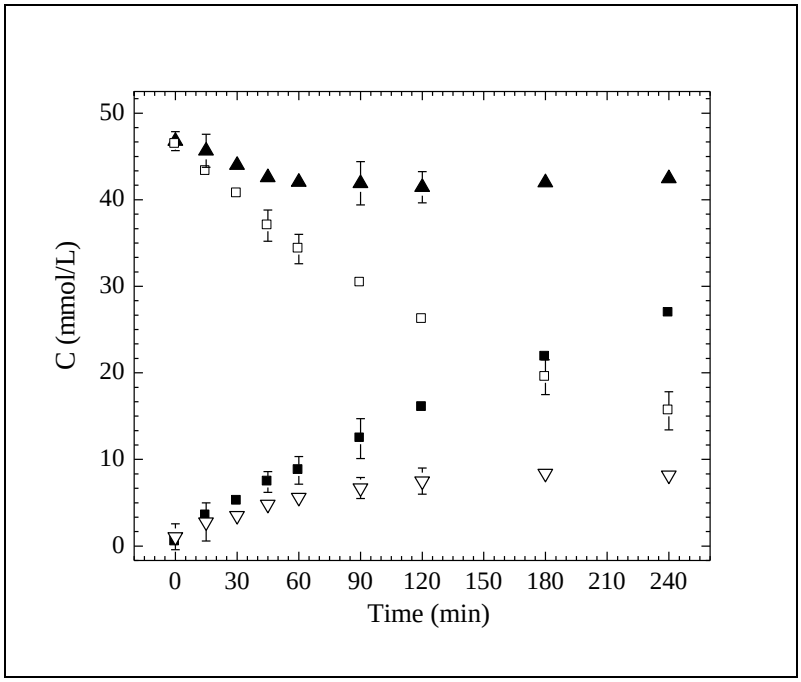


Figure 4 – Amoxicillin synthesis at 25 °C and pH 6.5. Initial bulk concentrations: 50 mM PHPGEE, 50 mM 6-APA, and 0.3 g of 260 IU/g of derivative in 100 mM phosphate buffer pH 6.5. In the graphic: (□) PHPGME, (▲) 6-APA, (■) PHPG and (∇) amoxicillin. Error bars: standard deviation (SD), estimated from triplicates.

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