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Synthesis of cephalexin with immobilized penicillin acylase at very high substrate concentrations in fully aqueous medium

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Abstract

The presence of organic cosolvents was previously considered necessary to obtain high conversion yields in the synthesis of β-lactam antibiotics with immobilized penicillin acylase, and it is so when working at moderate substrate concentrations. Conversion yields close to stoichiometric and high productivities were recently reported for the synthesis of cephalexin at high substrate concentrations in ethylene glycol medium. Under such conditions, the effect of cosolvent concentration on yield is not significant so we raised the hypothesis that stoichiometric yields and high productivities are attainable at very high substrate concentrations in fully aqueous medium leading to substantial process improvement in terms of costs and environment. To test the hypothesis, the kinetically controlled synthesis of cephalexin with immobilized penicillin acylase was conducted in aqueous medium at substrates concentrations up to and beyond their solubilities at varying temperature, pH, enzyme to substrate and acyl donor to nucleophile ratios. At the best conditions, 99% conversion yield was attained with volumetric productivity of 300 mM/h and specific productivity of 7.8 mmol/h g_{cat}. These values are slightly higher than those previously obtained under optimized conditions in organic medium so that the hypothesis has been confirmed, which opens up the possibility of efficiently produce the antibiotic through an environmentally friendly process.

Keywords: Penicillin acylase; Cephalexin; β-lactam antibiotics; Kinetically controlled synthesis; Immobilized enzymes

1. Introduction

6-Aminopenicillanic acid and 7-amino 3-desacetoxicephalosporanic acid are industrially produced mainly by hydrolysis of penicillin G and cephalosporin G with immobilized penicillin acylase [1,2], which has replaced the former cumbersome chemical processes [3,4]. Penicillins and cephalosporins, mostly semi-synthetic derivatives, are the most relevant members of that family accounting for 60% of the total antibiotic market [5–10]. Traditionally, these molecules have been synthesized chemically from the corresponding β-lactam nuclei and suitable side chain precursors [9,10]. Enzymatic synthesis is becoming an attractive option since penicillin acylase can be used also as a catalyst for the reverse reactions of synthesis [11–14], either by thermodynamic [15–17] or kinetic control [18,19], the latter being a better strategy when conversion yield is the main issue [20–22]. In both strategies conversion yields can be improved by using organic solvents [23] precipitation-driven [24] and biphasic systems [25,26]. Reduction of water activity by the use of organic cosolvents [27–29] or very high substrates concentrations [30–33] is beneficial to the reaction of synthesis and has been thoroughly evaluated to make enzyme synthesis competitive. Synthesis in the presence of organic cosolvents can lead to conversion yields not attainable in aqueous medium [27,34] and, in fact, we have reported stoichiometric yields in the synthesis of cephalexin at high substrates concentrations in ethylene glycol medium [35]. However, the presence of organic solvent contradicts the concept of clean technology associated to biological processes. We have observed though, that at very high substrates concentrations, cosolvent concentration is no longer a key variable with respect to conversion yield. Therefore, in this study we present results on the kinetically controlled synthesis of cephalexin with immobilized penicillin acylase at very high substrate concentrations in fully aqueous medium to test

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the hypothesis that under such conditions stoichiometric yields and high productivities are attainable, representing substantial process improvement in terms of costs and environment.

2. Experimental

2.1. Materials

Polyacrylamide gel surface bound penicillin acylase (PGA-450) from *Escherichia coli* with 380 ± 20 IU/g was from Roche Molecular Biochemicals (Mannheim, Germany). Immobilized biocatalyst spherical particles were around 0.1 mm in diameter. The biocatalyst was stored wet at 5 °C with no loss of activity during the whole working period.

(*R*)-(−)-2-phenylglycine methyl ester hydrochloride (97% pure) and cephalexin hydrate were from Sigma Chemical Company Inc. (St. Louis, MO, USA); (*R*)-(−)-2-phenylglycine (PG) was from Aldrich (Milwaukee, WI, USA); 7-amino 3-desacetoxicephalosporanic acid (7ADCA) was kindly provided by Antibióticos S.A. (León, Spain); penicillin G potassium salt (PenGK) was donated by Natsus S.A. (Lima, Perú). All other reagents were analytical grade either from Sigma–Aldrich or Merck (Darmstadt, Germany).

2.2. Analysis

Enzyme activity was measured using a pHstat (Mettler Toledo, DL50), which recorded the evolution of NaOH consumption to keep pH constant as the protons coming from phenyl acetic acid were produced. Initial slope of the reaction was automatically provided by the equipment and, from that slope, a straightforward stoichiometric calculation allowed to convert the rate of equivalents of base consumed to the rate of phenylacetic acid production and then to the rate of penicillin G hydrolysis.

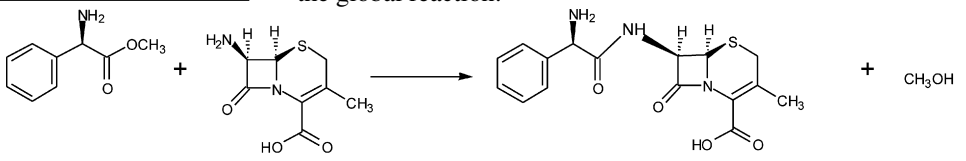
One international unit of activity (IU) of penicillin acylase was defined as the amount of enzyme that hydrolyzes 1 μmol of PenGK per minute from a10 mM PenGK solution in 0.1 M phosphate buffer pH 7.8 at 30 °C.

Substrates and products of synthesis were identified and analyzed by HPLC using a Shimadzu delivery system LC-10AS with a Shimadzu UV SPD-10AV UV–vis detector and a CBM-101 Shimadzu HPLC/PC integrator. The column used was a μ-Bondapack C_{18} (300 mm × 3.9 mm) from Waters (Milford, MA, USA). Samples were eluted under gradient with a sonicated mixture of methanol and 10 mM phosphate buffer pH 7.0 at a flow rate of 1 mL/min, and analyzed in the UV detector at 220 nm. Elution times were 2.9, 4.5, 5.7 and 7.4 min for 7ADCA, PG, cephalexin and phenylglycine methyl ester (PGME), respectively. Concentration of substrates and products were calculated from calibration curves using stock solutions. HPLC samples were always assayed in triplicate, differences among them never exceeding 3%.

2.3. Synthesis of cephalexin

Syntheses were performed batch-wise, with temperature and pH control, in 50 mL Pyrex glass reactors with a working volume of 30 mL, equipped with a paddle impeller, working at a stirring speed of 200 rpm to keep biocatalyst particles in suspension. Samples were taken at intervals and were properly diluted prior to be assayed by HPLC. At high substrate concentrations the system is highly heterogeneous and a fraction of the substrates and the products are in solid state. Samples with solids in suspension were diluted prior to assay so that the solids were dissolved except for the biocatalyst particles that were filtered out (the volume occupied by the biocatalyst was insignificant).

Syntheses of cephalexin with PGA-450 proceed according to the global reaction:



PGME 7ADCA Cephalexin Methanol

Syntheses were performed under kinetic control, whose reaction mechanism has been described elsewhere [36–38].

The study was conducted in a pH range from 6.5 to 7.4 and a temperature range from 10 to 20 °C, at 125 IU/mmol 7ADCA, 200 mM 7ADCA and 600 mM PGME. A 200 mM 7ADCA was close to the solubility limit at the lowest temperature and lowest pH of the range, so that the substrates were soluble at all the conditions tested (solubility of 7ADCA increases both with temperature and pH). Temperature and pH ranges were determined from previous results obtained in water–ethylene glycol medium. Temperatures higher than 20 °C produce a substantial decrease in yield, while temperatures below 10 °C produce a substantial decrease in enzyme activity, which reflects in a low volumetric productivity; a similar trend occurred over pH 7.5 and below pH 6.5 [35]. Once the best pH and temperature conditions were established, the effect of increasing the substrate concentration beyond the solubility limit, the effect of reducing the enzyme to substrate ratio and the effect of reducing the excess acyl donor were studied. Syntheses were evaluated in terms of molar conversion yield (*Y*), volumetric productivity (*P*) and specific productivity (P_{sp}). *Y* was determined as the maximum molar conversion of limiting substrate (7ADCA) into product (cephalexin); *P* was defined as the moles of cephalexin produced per unit time and unit reaction volume at maximum *Y*; P_{sp} was defined as the moles of cephalexin produced per unit time and unit of biocatalyst mass at maximum *Y*. Experiments were done in duplicate and samples assayed in triplicate with variations below 5% among them; data points in figures represent the average of such measurements. Reactions were monitored up to the point in which product concentration levelled off or began to decline and *Y* was evaluated at its maximum value.