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Short communication

# Efficient enzymatic synthesis of ampicillin using mutant Penicillin G acylase with bio-based solvent glycerol

Senwen Deng [a,c,1], Xiaoqiang Ma [c,1], Ming Sun [c,*], Dongzhi Wei [c], Erzheng Su [b,d,**]

[a] School of Life Science, Hunan University of Science and Technology, Xiangtan 411201, Hunan, PR China
[b] Enzyme and Fermentation Technology Laboratory, College of Light Industry Science and Engineering, Nanjing Forestry University, Nanjing 210037, PR China
[c] State Key Laboratory of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, PR China
[d] State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, PR China

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ABSTRACT

To fulfill the industry demand of ampicillin enzymatic synthesis, immobilized mutant Penicillin G acylase and bio-based solvent glycerol were employed at high substrate concentration and low acyl donor/nucleophile ratio. After process optimization, good yield and low operation costs were achieved.



## 1. Introduction

Semi-synthetic β-lactam antibiotics (SSBAs) are the most important family of β-lactam antibiotics in the world market [1,2]. They were produced by the condensation of β-lactam moiety with the acyl side chain [3]. Chemical synthesis of SSBAs has dominated the industrial production of SSBAs for high yield (90%) [4]. But the complicated process steps, harsh reaction conditions and large volume of organic solvent needed, make them environmentally unsustainable [3,5]. Enzymatic synthesis of SSBAs is an environmental friendly alternative, which is mainly catalyzed by Penicillin G acylase (PGA: EC 3.5.1.11) [4], and can be carried out under kinetically control (Fig. 1). But its relatively low yield (40–60%), high process costs (raw material and catalyst), complicated downstream processing (isolation and recycle) needed, make it hard to fulfill the industry demand at present [3,5].

Low yield of kinetically controlled synthesis can be mainly ascribed to enzyme-catalyzed initial hydrolysis of the activated acyl donor and the secondary hydrolysis of antibiotic product (Fig. 1) [6]. For natural PGAs, low *S*/*H* (synthesis to hydrolysis) ratio is often observed [7–10]. Protein engineering is commonly used to tailor wild PGAs for SSBAs synthesis [6,11–13]. For example, βF24G mutant of Penicillin G acylase from *Alcaligenes faecalis* (Af PGA), in which the 24th Phenylalanine of the β-subunit was replaced by Glycine was isolated for high *S*/*H* ratio in this lab [13]. Also, this process can be improved by the addition of water-miscible organic solvents, excess acyl donor and high substrate concentration [6,14,15]. But these measures have to be taken carefully in consideration of nucleophile conversion, raw material and downstream purification process costs. For example, the molar ratio acyl donor/nucleophile should be as low as possible to reduce the costs of raw materials and downstream processing. In the case of ampicillin, amoxicillin and cephalexin, synthesis at high substrate concentration has been proven to be beneficial [16–18]. However, this method could be limited by substrate solubility, which could influence the conversion of the nucleophile significantly. Medium engineering could improve synthesis by increasing the *S*/*H* ratio. This has been proven for the synthesis of cephaloglycine, ampicillin and cephalexin in methanol or ethylene glycol [19–21]. But toxicity and biohazard of these solvents have to be considered seriously. Ionic liquids were regarded as the substitution of organic solvents: e.g. Pereira et al. and Zhu et al. reported enzymatic synthesis at the presence of ionic liquids [22,23]. But the use of ionic liquids is still limited by high prices and lack of data about the toxicity and bio-compatibility. As a bio-based solvent, glycerol may combine the advantages of water and ionic liquids (low toxicity, low price, large availability, renewability, high boiling point, low vapor pressure), allows its use in the synthesis of pharmaceutically active ingredients [24,25].

* Corresponding author.
** Correspondence to: E. Su, Enzyme and Fermentation Technology Laboratory, College of Light Industry Science and Engineering, Nanjing Forestry University, Nanjing 210037, PR China.
*E-mail addresses:* sunmingecust@163.com (M. Sun), ezhsu@njfu.edu.cn (E. Su).
[1] These authors contributed equally to this work.

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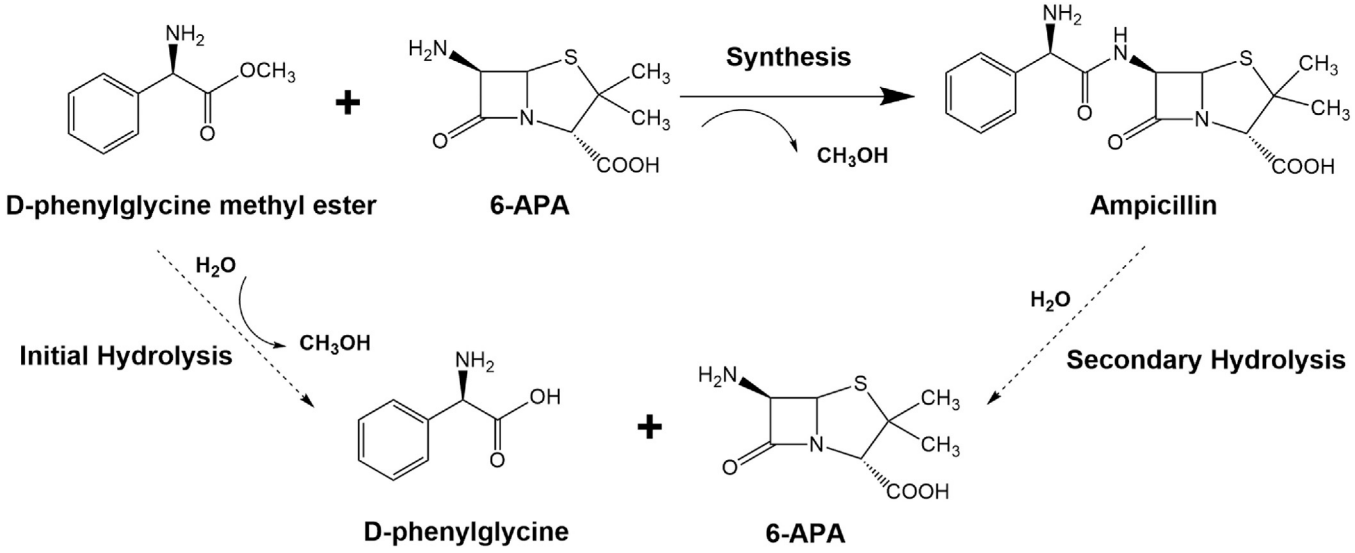


**Fig. 1.** Scheme of enzymatic synthesis of ampicillin.

In this work, we tried to establish a process for ampicillin synthesis from an industrial view, through the use of immobilized mutant Penicillin G acylase and bio-based solvent glycerol at the condition of high substrate concentration and low acyl donor/nucleophile ratio.

## 2. Experimental

### 2.1. General

All reagents were purchased from commercial sources. Plasmid pET-28a-Af PGA-24G (βF24G mutant) was reserved in this lab [13]. The transformation, cultivation, harvest and disruption of recombinant cells followed the methods reported previously [13,26]. The obtained supernatant was collected and fractionated with ammonium sulfate. Protein that precipitated between 20 and 30% (w/v) ammonium sulfate was redissolved by potassium phosphate buffer (100 mM, pH 8.0) as partly purified enzyme for immobilization. The partly purified acylases were immobilized onto an epoxy carrier LH-EP under the optimized conditions as described before [27].

### 2.2. General procedure for enzymatic synthesis of ampicillin

The 100 mL reaction mixture contained 400 mM 6-aminopenicillanic acid (6-APA), appropriate amount of D-phenylglycine methyl ester (D-PGME) and immobilized mutant Af PGA. The reaction was conducted without pH control. The conversion of 6-APA was calculated by measuring the decrease of the 6-APA by HPLC.

### 2.3. Analysis

The enzyme activity was determined by a previously reported method [28]. Specific activity is defined as $U_{PGK}$ per milligrams of protein. Protein concentration was determined using the Bradford method [29]. HPLC analysis was performed by a previously reported method [13].

## 3. Results and discussion

Approximately 73% of the total protein was covalently linked to the support (Table 1). The activity recovery was 45%. The activity of the immobilized βF24G mutant acylase was 37 U/g (Table 1). The *S/H* ratio of immobilized acylases was 4.0, slightly lower than 4.26 of the free enzymes under the same condition (0.1 M 6-APA) [13], which can be mainly ascribed to the mass transfer problem of the immobilized enzymes [15].

At first, the reaction was performed without the addition of glycerol (Fig. 2A). The observed increase of initial *S/H* ratio (from 4.0 to 11.5), could be mainly ascribed to the rise of 6-APA concentration (from 0.1 to 0.4 M), in accordance with the previous reports [16–18]. However, as the process continued, we unexpectedly found that reaction solution was concreting as byproduct D-phenylglycine (D-PG) with a poor solubility accumulated and precipitated [3]. Increased kcat/Km value of βF24G mutant Af PGA for ampicillin and high concentration of produced ampicillin [3,13] might lead to more second hydrolysis and byproduct D-PG accumulation. Gradient concentration of glycerol (5–20%, v/v) was added at the beginning of the reaction to reduce the side reaction of hydrolysis. The conversion of 6-APA increased from 66 to 87% and the concretion time of reaction system were delayed as glycerol concentration increased. This could be ascribed to the increased initial *S/H* ratio (from 11.5 to 17.3) and decreased second hydrolysis as results of reduced water activity. But the reaction speed was also significantly reduced by the glycerol addition, which might be the results of changed enzyme structure [24,25]. When high concentration of glycerol was employed (20%, v/v), dramatically reduced reaction speed was unfavorable for 6-APA conversion. In consideration of 6-APA conversion, 15% (v/v) glycerol is suitable.

**Table 1**
Protein expression and immobilization.

| Offered enzyme (mg/g) | Specific activity ($U_{PGK}$/mg) | Immobilization yield(%)[a] | Activity recovery(%)[b] | Activity ($U_{PGK}$/g)[c] | *S/H* $ratio_{ini}$[d] |
|---|---|---|---|---|---|
| 34.9 | 2.37 | 73 | 45 | 37 | 4.0 |

All data were the average of three independent experiments.
[a] The immobilization yield (%, w/w) was calculated as described before [27].
[b] The activity recovery was calculated as described before [27].
[c] Activity toward PGK hydrolysis expressed per gram of support ($U_{PGK}$/g).
[d] *S/H* $ratio_{ini}$: Initial rate of antibiotic formation to initial rate of D-PG formation.