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Short communication

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

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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].

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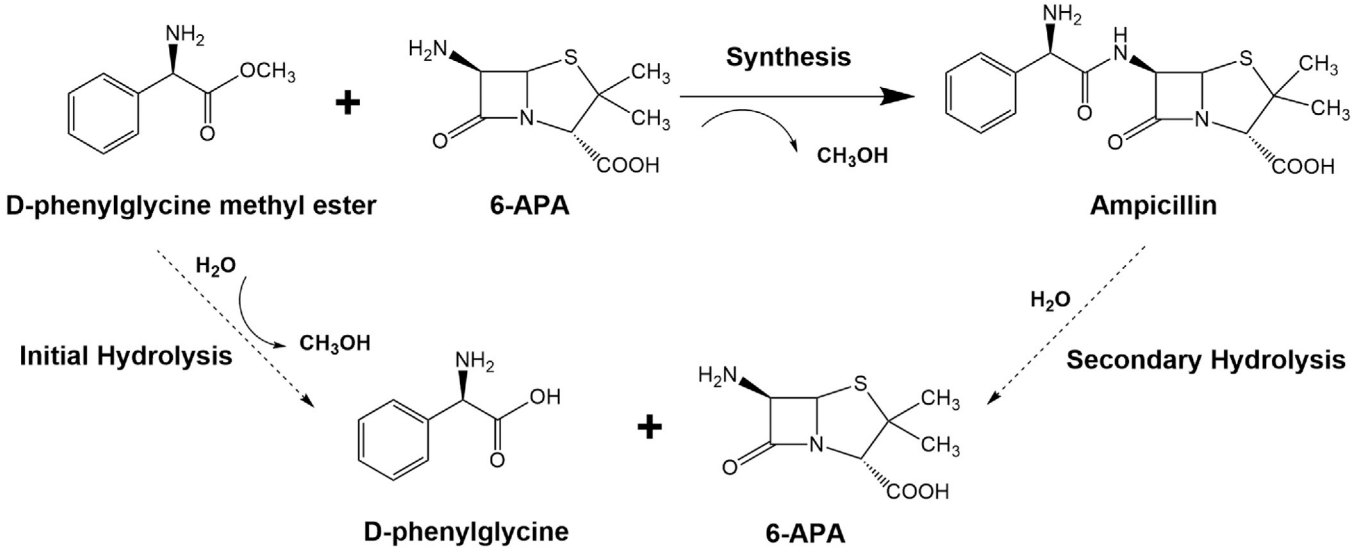

Fig. 1. Scheme of enzymatic synthesis of ampicillin.

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.

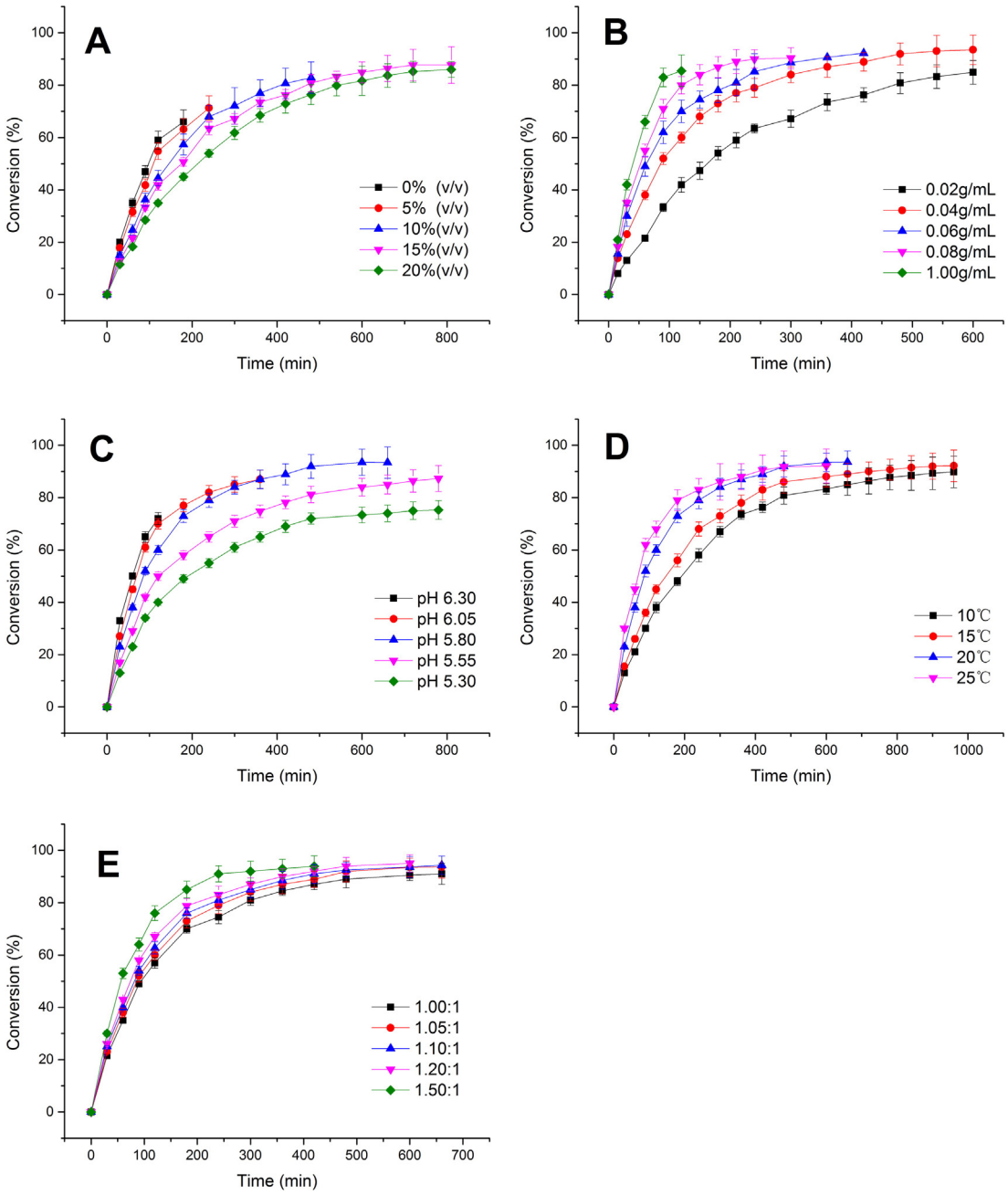

Fig. 2. Optimization of ampicillin enzymatic synthesis. (A) Time course of enzymatic synthesis of ampicillin at different concentrations of glycerol. Conditions: initial pH 5.8, 20 °C, 0.4 M 6-APA, 0.42 M D-PGME, 0.02 g/mL immobilized βF24G mutant Af PGA. (B) Time course of enzymatic synthesis of ampicillin at different concentrations of immobilized βF24G mutant Af PGA. Conditions: 15% (v/v) glycerol, initial pH 5.8, 20 °C, 0.4 M 6-APA, 0.42 M D-PGME. (C) Time course of enzymatic synthesis of ampicillin at different initial medium pH. Conditions: 15% (v/v) glycerol, 20 °C, 0.4 M 6-APA, 0.42 M D-PGME, 0.04 g/mL immobilized βF24G mutant Af PGA. (D) Time course of enzymatic synthesis of ampicillin at different temperatures. Conditions: 15% (v/v) glycerol, initial pH 5.8, 0.4 M 6-APA, 0.42 M D-PGME, 0.04 g/mL immobilized βF24G mutant Af PGA. (E) Time course of enzymatic synthesis of ampicillin at different substrate ratios. Conditions: 15% (v/v) glycerol, initial pH 5.8, 20 °C, 0.4 M 6-APA, 0.04 g/mL immobilized βF24G mutant Af PGA. All data were the average of three independent experiments.

As shown in Fig. 2B, the ampicillin synthesis was significantly influenced by the concentration of immobilized βF24G mutant Af PGA. Increased enzyme loading obviously led to faster reaction rate, as well as the accumulation of the insoluble byproduct D-PG. The reaction would be ended when the solution is concreting. In addition to higher enzyme preparation costs, increased enzyme loading could also lead to mass transfer limitations. This is unwanted for increased second hydrolysis and reduced *S*/*H* ratio [15]. When enzyme loading increased to certain amount (more than 0.04 g/mL), 6-APA conversion cannot be further improved. In consideration of enzyme cost, 0.04 g/mL of enzyme loading was thus chosen for further study.

Also, the pattern of ampicillin synthesis could be changed by initial reaction pH (Fig. 2C). Higher initial pH would increase the reaction rate, in accordance with the slightly alkaline pH optimum of PGAs. But when the initial pH increased from 5.8 to 6.3, the decrease of initial *S*/*H* ratio (from 17 to 12.9) and more byproduct D-PG accumulation could be observed at the same time [30]. When the initial pH was reduced to a certain degree, the 6-APA conversion also decreased as the decline of reaction rate. The highest 6-APA conversion was achieved at initial pH of 5.8. Meanwhile, it is noteworthy that acidic pH is beneficial for substrate stability, especially 6-APA [31].

Unlike initial pH, the change of reaction temperature exhibited smaller effect on ampicillin synthesis (Fig. 2D). The reaction rate increased slightly while temperature is rising, as well as the 6-APA conversion. The highest 6-APA conversion was achieved at 20 °C. 6-APA conversion cannot be further improved when the temperature was over 20 °C due to the balance between hydrolysis and synthesis. Also, higher temperature would speed up the chemical degradation of substrates especially 6-APA [31]. In consideration of 6-APA conversion, reaction time and substrate stability, 20 °C was chosen.

Also, the influence of changed substrate ratio on 6-APA conversion was studied (Fig. 2E). The 6-APA concentration was kept at 400 mM while 400–600 mM D-PGME was added to the reaction solution. Increased concentration of D-PGME would accelerate the reaction speed, supporting the assumption that acyl-enzyme intermediate formation is the rate-limiting step under kinetic control [13]. But the mass transfer problems could be serious at the same time. Decreased *S*/*H* ratio and more byproduct D-PG accumulation could also be observed. Though obviously higher 6-APA conversion (93.5% vs 91%) was obtained at higher D-PGME/6-APA ratio (1.05:1 vs 1.00:1) at first. But when this ratio increased from 1.05:1 to 1.10:1 and 1.20:1 (20 and 60 mM extra D-PGME were added), due to lower initial *S*/*H* ratio (decreased from 17 to 15.8 and 14.5, respectively) and the balance between hydrolysis and synthesis, only 3.2 and 6 mM more 6-APA were converted, respectively. This is not favored for the economic performance of the whole process for much higher raw material and downstream purification cost. When more extra D-PGME was added (180 mM), the reaction solution would be concreting before higher 6-APA conversion could be achieved. In conclusion, D-PGME/6-APA ratio of 1.05:1 could be regarded as a better choice.

According to the above results, the optimal reaction conditions were determined: 0.04 g/mL immobilized mutant Af PGA, 15% (v/v) glycerol, initial pH 5.8, 20 °C, D-PGME/6-APA ratio of 1.05:1. The conversion of 6-APA reached 93.5% by a 600 min procedure, higher than 90% of the chemical method. It is noted that this yield was achieved at low D-PGME/6-APA ratio (1.05:1). Less than 1.12 mol D-PGME was required to produce 1 mol ampicillin, which is much lower than the reported values [12,32,33]. All those results met the industrial requirement for reduced raw material and downstream processing costs. The catalytic performance of immobilized βF24G mutant Af PGA was in accordance with its kinetic parameters and catalysts style. High speed of ampicillin synthesis using free mutant enzyme has been observed in our previous report [13]. However, when it was using in an immobilized form at high substrate concentration, the mass transfer problems can be serious. Side reaction of hydrolysis and byproduct accumulation made the glycerol addition to be a critical choice. Besides its benefits for catalytic performance, glycerol is a cheap, nontoxic, bio-degradable and nonflammable solvent, allows an eco-efficient, bio-compatible and easily implemented process [24,25].

In conclusion, the use of immobilized mutant Penicillin G acylase and bio-based solvent glycerol at high substrate concentration and low acyl donor/nucleophile ratio was tested in this study. After process optimization, this combined strategy had been demonstrated to be easily implemented, economically efficient and environmentally benign, providing a promising model for the industrial production of SSBAs.

Conflict of interest

The authors declare that they have no conflict of interest.

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