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A facile pretreatment method for efficient immobilization of penicillin G acylase

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ABSTRACT

A facile pretreatment method was developed to obtain high specific activity of PGA from recombinant *E. coli* cells for preparing immobilized enzyme with high activity, which coupled selective extraction of PGA with butyl acetate and adsorption of butyl acetate with active carbon. Butyl acetate (5%, v/v) led to a 92.0% release of PGA, the specific activity of which in the extracting solution was twice that obtained by sonication. The negative effect on PGA immobilization due to residual butyl acetate in the extracting solution was removed by adding active carbon (8%, w/v). The final PGA solution meets requirement for industrial immobilization while eliminating the high cost of a traditional purification process. This pretreatment method developed in this work is simple, highly performing and cost-effective. It has been employed in a 10 tons/year immobilized PGA production line.

1. Introduction

Penicillin G acylase (PGA) (penicillin G amidohydrolase, E.C. 3.5.1.11), one of the most important industrial enzymes, is used commercially to catalyze the production of 6-aminopenicillianic acid (6-APA) and 7-amino-3-desacetoxycephalosporanic acid (7-ADCA), which are further processed in the manufacture of semisynthetic antibiotics [1–3]. In addition, PGAs are useful biocatalysts in many potentially valuable reactions such as protection of amino and hydroxyl groups in peptide synthesis [4], as well as in the resolution of racemic mixtures of chiral compounds [5,6]. Therefore, many efforts have been directed to isolate new PGA from different microorganisms, including bacteria, yeast and filamentous fungi [7]. The PGA from *Alcaligenes faecalis* (*A. faecalis* PGA), which shares only 49% identity at the protein level and 53% DNA sequence homology to *E. coli* PGA, is a novel one. It shows some outstanding features such as broad pH optimum [8], significantly higher thermal stability, higher enantioselectivity for amines [5] and high affinity for 6-nitro-3-phenylacetamidobenzoic acid (NIPAB) and benzylpenicillin [8] and has therefore attracted much attention.

In our laboratory, we have obtained high activity of *A. faecalis* PGA in recombinant *E. coli* by optimizing the conditions of the enzyme production, which is above 80,000 U/L in a 300 L fermentor (unpublished results), and the specific enzyme activity is about 7 U/mg protein. But biochemically active PGA in *E. coli* resides mainly in the outer periplasmic space [9], the PGA needs to be released and purified to a higher specific activity for immobilization which is the regular industrial application form. The traditional mechanical disrupting methods such as high-pressure homogenization and sonication have been performed. However, the target PGA extracted is usually contaminated by impurities with similar physicochemical properties releasing synchronously from cells [10], and it will inevitably increase the pretreating costs to remove these impurities. So the particular chemical method, which is high level of selective protein release and less subsequent purification cost, may be a potential substitute for mechanical disruption [11,12].

Many chemicals, such as ethylenediaminetetraacetate (EDTA) [13], urea, triton X-100 [14] and cetyltrimethylammoniumbromide (CTAB) [15], have been used to extract enzymes from whole microbial cells. Although these methods are able to accomplish an efficient enzyme release, they enhance the specific enzyme activity unobviously. Butyl acetate, also known as butyl ethanoate, is an organic compound commonly used as a solvent in the production of lacquers and other products. In the cell envelope of some gram-negative bacteria, for example *E. coli*, butyl acetate can dissolve lipid substances and apparently "loosen" the bindings between lipopolysaccharides (and/or lipoproteins) with enzyme protein, resulting in the release of enzymes in the cell membrane or close to it [16]. Therefore, it may be an alternative for selective extraction of *A. faecalis* PGA from recombinant *E. coli*.

To our knowledge, when chemicals were used for extracting enzymes, other studies have not paid attention to the effects of the

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residuals of chemicals in the extraction suspension on the activity of target enzymes and subsequent immobilization [13–15]. Appropriate removal procedures for residual organic extractants have to be developed, in case they negatively affect the immobilization of the target enzyme.

In this work, butyl acetate was firstly investigated as permeabilizing reagent for selective extraction of *A. faecalis* PGA from recombinant *E. coli* cell, and then active carbon was used to absorb the butyl acetate remaining in the extraction suspension.

2. Materials and methods

2.1. Chemicals and microorganism

Potassium penicillin G was purchased from Zhuhai Yuancheng Pharmaceutical & Chemical Co., Ltd. (China). Butyl acetate was purchased from Aladdin Reagent Co., Ltd. (China). Active carbon was supplied by Shanghai Xinhuoli Active Carbon Co., Ltd. (China). Aminated carrier (LHHA-01) for immobilization was provided by Shanghai Bairui Biotech. Co., Ltd. (China). All other chemicals were of the analytical grade.

E. coli strain DH5α was used as the host for the production of *A. faecalis* PGA. The recombinant plasmid, pSMLFPGA, is a medium copy-number plasmid, which carries the *A. faecalis* pga gene, rrnB transcript terminator, medium-copy replicon (p15A) and a tetracycline resistance gene. The expression of pga gene is controlled by the *trc* promoter, which needs not be induced by isopropyl-β-thiogalactopyranoside (IPTG) because of no lacI^q gene.

2.2. Release of PGA from recombinant E. coli cells

Recombinant *E. coli* cells were grown in a medium (per litre) of 45 g dextrin, 5 g yeast extract, 20 g tryptone, 1 g $MgSO_4{\cdot}7H_2O$, 3.5 g $Na_2HPO_4{\cdot}12H_2O$, 2.5 g $K_2HPO_4{\cdot}3H_2O$, 1 g KH_2PO_4, 0.05 g NaCl, 0.6 g $(NH_4)_2SO_4$, 0.1 g NH_4Cl, 0.2 mg $FeSO_4{\cdot}7H_2O$, 40 μg $CoCl_2{\cdot}6H_2O$, 20 μg $ZnCl_2$, 20 μg $Na_2MoO_4{\cdot}2H_2O$, 10 μg $CuSO_4{\cdot}5H_2O$, 7 μg $MnSO_4{\cdot}H_2O$, 5 μg H_3BO_4 with 1 ml/l of antifoam at pH 7 and temperature 30 °C. The fermentation was carried out for 120–144 h [17]. After fermentation, the cells were harvested during stationary phase and then separated by centrifugation at 12,000 × *g* and 4 °C for 10 min. The cell pellets (20 g) were resuspended in 100 ml 0.05 M pH 7.8 phosphate buffer. From the resulting suspension (RS) PGA was released using the following methods.

Sonication: the RS was 6-fold diluted with 0.05 M pH 7.8 phosphate buffer, then disrupted using the ultrasonic cell disruptor (Ningbo Scientz, JY88-II, China) in an ice bath at 400 W for 210 cycles (working 5 s and intervals 5 s as one cycle). The disrupted cells were collected by centrifugation at 12,000 × *g* for 10 min, and the supernatant was assayed for PGA activity and protein content (for methods see Section 2.6). PGA release was deemed to be complete since no further activity increase was observed upon extension of sonication cycles.

High-pressure homogenization: the RS was homogenized (homogenizer AH-Basic II, ATS Engineering Inc.) at 600 bar for up to three passes. The temperature of the sample vessel of the homogenizer was kept constant at 4 °C by a water jacket attached to a circulator and thermostat. After homogenization, the disrupted cells were collected by centrifugation at 12,000 × *g* for 10 min, and the supernatant was assayed for PGA activity and protein content.

Osmotic shock: sucrose was added to the RS to the concentration of 30% (w/v). After shaking at 200 rpm and 25 °C for 2 h, the shrunk cells were harvested by centrifugation at 12,000 × *g* and 4 °C for 10 min. The supernatant was collected, and the cell pellet was resuspended in reverse osmosis water (25 °C). After another shaking at 200 rpm and 25 °C for 2 h, the cell suspension was centrifuged at 12,000 × *g* and 4 °C for 10 min. The cell pellet was resuspended in 0.05 M pH 7.8 phosphate buffer containing 30% (w/v) sucrose for next cycle of osmotic shock. The cycle of osmotic shock was repeated for three times. Finally, all the collected supernatants were mixed and assayed for PGA activity and protein content.

Freeze–thaw: the RS was frozen at −20 °C for 4 h, and then thawed in a rotary shaker at 200 rpm and 37 °C. The thawing solution was centrifuged at 12,000 × *g* and 4 °C for 10 min. The supernatant was collected, and the cell pellet was resuspended in 0.05 M pH 7.8 phosphate buffer for freezing again. This procedure was repeated for three times, and all the collected supernatants were mixed and assayed for PGA activity and protein content.

Permeabilization with CTAB: CTAB was added to the RS to the final concentration of 0.5% (w/v), and then stirred on a rotary shaker at 150 rpm and 4 °C for 28 h. The extraction solution was centrifuged at 12,000 × *g* and 4 °C for 10 min. The supernatant was collected for determination of PGA activity and protein content.

Permeabilization with guanidine hydrochloride and EDTA: the procedure was carried out according to the reported method [12].

Permeabilization with ethyl acetate: ethyl acetate was added to the RS to the final concentration of 5% (v/v), and then shaken at 200 rpm and 25 °C for 36 h. The permeabilizing solution was centrifuged at 12,000 × *g* and 4 °C for 10 min. The supernatant was collected for determination of PGA activity and protein content.

Permeabilization with butyl acetate: butyl acetate was added to the RS to a final concentration ranging from 0.5 to 9.0% (v/v) in absence and presence of EDTA (0.01 M). The mixture was stirred at 200 rpm and different temperatures for different time. After permeabilization, it was centrifuged at 12,000 × *g* and 4 °C for 10 min. The supernatant was collected for determination of PGA activity and protein content.

2.3. Removal of butyl acetate

Adsorption with active carbon: different amounts of active carbon were added to the enzyme solutions obtained by permeabilization with butyl acetate in a sealed flask, and then shaken at 25 °C for different time. Samples were withdrawn for determination of butyl acetate content.

Ultrafiltration: a Labscale TFF system (Millipore) was used for ultrafiltration experiment. This system was equipped with Pellicon XL Biomax 10 50 cm^2 membrane of a nominal molecular mass cut off of 10 kDa. The enzyme solutions obtained by permeabilization with butyl acetate were diafiltered against 6 volumes of 0.05 M pH 7.8 phosphate buffer. The transmembrane pressure was maintained at approximately 20 PSI during the diafiltration.

2.4. Determination of butyl acetate content

A model 7694E head space autosampler (Agilent Technologies, Palo Alto, CA, USA) connected to a model 6890 gas chromatograph (Agilent Technologies), interfaced with a model 5973 inert mass selective detector (Agilent Technologies), was used to determine the content of butyl acetate. The gas chromatograph was equipped with a DB-WAX column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies). The operating procedures were as follows: 250 μl of samples were mixed with 4.75 ml of ultrapure water, and then transferred into glass "head space" 20 ml-vials, which were closed with 20 mm rubber PTFE lined septa (130 °C proof) and crimped with perforated aluminium seals. After 40 min equilibration time at room temperature (22–23 °C), samples were placed in the auto-sampler where they were heated at 80 °C for 20 min before the head space was withdrawn. The head space autosampler was provided with a 1 ml loop kept at 130 °C. The transfer line, heated at 130 °C, was connected with the injector of the GC (split 1:1) via an interface heated at 220 °C. The oven temperature