Biochemical Engineering Journal 114 (2016) 183–190

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

High-level expression of Cephalosporin C deacetylase from *Bacillus subtilis* SIL_3 in *Escherichia coli* by a multilevel collaborative strategy

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ARTICLE INFO

Article history:
Received 13 March 2016
Received in revised form 11 July 2016
Accepted 15 July 2016
Available online 16 July 2016

Keywords:
Cephalosporin C deacetylases
Bacillus subtilis
Deacetyl-7-aminocephalosporanic acid (D-7-ACA)
Heterologous expression
Medium optimization

ABSTRACT

Cephalosporin C deacetylases (CAH) is employed to hydrolyze 7-aminocephalosporanic acid (7-ACA) to deacetyl-7-aminocephalosporanic acid (D-7-ACA), which can be used to produce the 3-vinyl substituted cephalosporin antibiotics. In this study, a multilevel collaborative strategy was conducted to achieve high-level production of CAH by recombinant *Escherichia coli* (*E. coli*). Inducible and constitutive expression systems harboring four *Bacillus subtilis* (*B. subtilis*) CAH genes were constructed and expressed in *E. coli*. Effects of expression system, spacing sequence and terminator on the CAH expression were investigated. Obvious increase in CAH activity demonstrated that the recombinant *E. coli* strain of DH5α/pB2-CAH-K-SIL_3 was the optimal CAH producing strain, and the CAH activity produced increased approximately 3 times after systematic optimization. Using glycerol feeding with pH control, an effective fermentation process for recombinant CAH production was established in 7.0 L fermenter. The cell density (OD_{600}), CAH activity and productivity reached 60, 1340 U/mL and 55 U/mL/h, which are the highest values reported in *E. coli*. The fermentation process established in this work is expected to be applicable for industrial CAH production.

1. Introduction

7-aminocephalosporanic acid (7-ACA) produced from cephalosporin C (CPC) is the most important β-lactam antibiotics intermediate used for preparation of more than 50 semi-synthetic cephalosporin antibiotics [1]. Certain types of semi-synthetic cephalosporin antibiotics such as cefcapene pivoxil [2], cefixime [3] and cefdinir [4] require deacetyl-7-Aminocephalosporanic acid (D-7-ACA) as the intermediate. D-7-ACA can be yielded by two deacetylation methods. The chemical way is energy-consuming along with harsh reaction conditions, and also gives poor yield due to side reactions [2]. As an environment-friendly "green" technology, the enzyme catalyzed method was developed on the industrial scale [5].

Esterases (EC 3.1.1.x), distributing extensively in animals, plants and microorganisms [6], have high regio- and stereo-specificity for the synthesis of optically pure compounds [7]. Esterases and deacetylases active on carbohydrate ligands are typically classified into 14 families [8]. Cephalosporin C deacetylases (EC 3.1.1.41, CAH) belong to carbohydrate esterase family seven (CE-7) and usually exhibit deacetylation activity towards cephalosporin antibiotics [8]. CAHs have been isolated from various sources such as citrus peel, actinomycetes, fungi, and bacteria. *B. subtilis* CAHs were identified as the optimal enzymes for industrial production of D-7-ACA due to their high *kcat* in hydrolysis of 7-ACA with negligible product inhibition and robust operational stability [6,9]. A packed bed reactor filling with the immobilized CAH from *B. subtilis* ATCC 6633 could be operated consecutively for 30 days without any significant activity loss [10]. CAH from *B. subtilis* SHS0133 was immobilized on an anion-exchange resin by using glutaraldehyde, and its operational tests showed only a slight loss of the CAH activity even after repeated use for 70 days [2].

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http://dx.doi.org/10.1016/j.bej.2016.07.009
1369-703X/

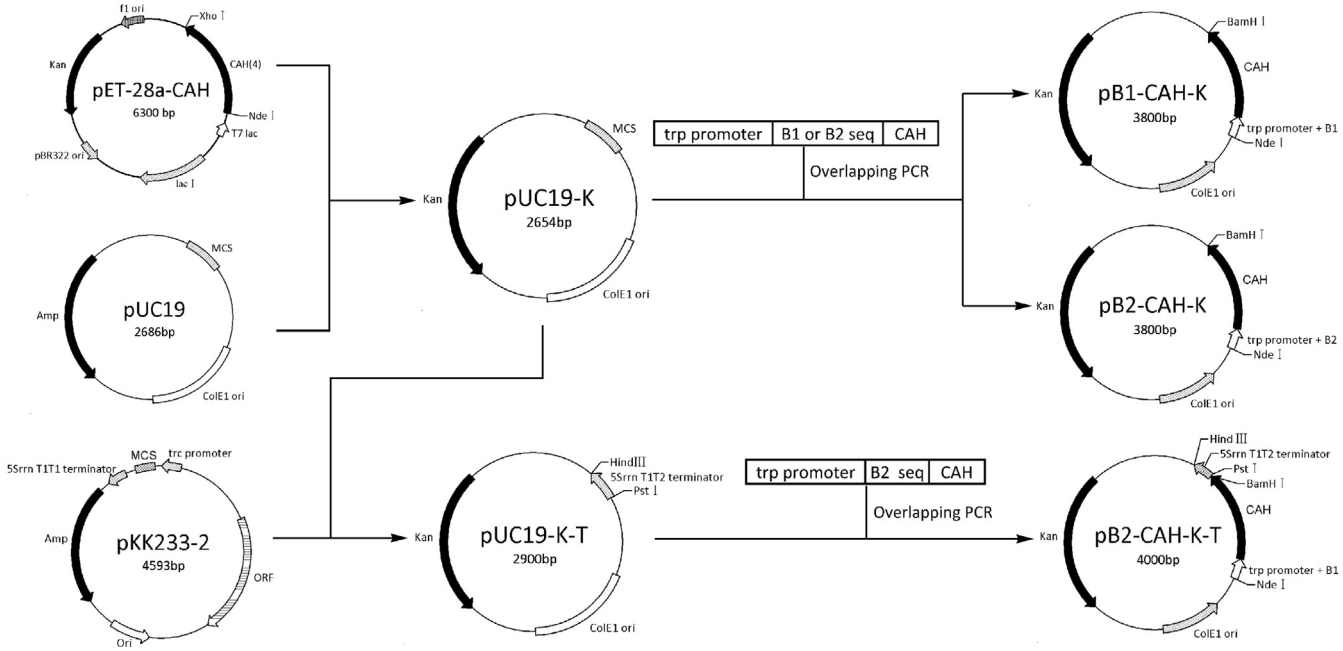

Fig. 1. Plasmids schematic of pB1-CAH-K, pB2-CAH-K and pB2-CAH-K-T. Amp, ampicillin-resistant marker; Kan, kanamycin-resistant marker; CAH, Cephalosporin C deacetylases; MCS, multiple cloning sites; ColE1 ori, replication origin of the plasmid ColE1; B1 and B2 seq, spacing sequence between the Shine-Dalgarno (SD) sequence and the initiation codon (ATG); *trp* promoter, *E.coli* tryptophan promoter.

Development of recombinant CAH to an industrial biocatalyst involves the fermentative production of enzyme, extraction, purification and immobilization of enzyme [11,12]. Establishing an effective bioprocess for CAH production has a positive impact on the economics of the whole development process. *E. coli* has been widely utilized to produce important biocatalysts for enzymatic synthesis of β-Lactams intermediates [13]. The CAH from *B. subtilis* SHS0133 was expressed via a constitutive expression plasmid in *E. coli* JM103, and the enzyme activity reached 1040 U/mL after 20 h of cultivation, and the specific activity of purified enzyme toward 7-ACA was 210 U/mg [2,14]. The CAH from *B. subtilis* CICC 20034 was expressed in *E. coli*, and the specific activity of purified enzyme toward 7-ACA was the highest among CE-7 esterases (approximately 888 U/mg) [9]. Results of amino acids sequence alignment of these two CAHs shared 84.3% sequence similarity (Table S3), suggesting that the disparity of specific activity of these two CAHs on 7-ACA is mainly attributed to the difference of protein structure. In this study, seven *B. subtilis* strains (Table S1) were collected to acquire a highly active CAH for the deacetylation of 7-ACA to D-7-ACA. Inducible and constitutive expression systems were constructed, and their effects on CAH expressions were investigated accordingly. Optimization of fermentation medium was performed to improve CAH production, and available fed-batch strategy was also established in fermenter.

2. Materials and methods

2.1. Strains, plasmids, culture mediums

E. coli DH5α and BL21 (DE3) were used for gene cloning and expression. The plasmids pET-24a, pET-28a, pKK233-2 and pUC19 preserved in our laboratory, were used to construct expression plasmids. Strains and plasmids used in this study are listed in Table S1. Primers used in this study were synthesized by Shanghai Generay Biotech Co., Ltd and are listed in Table S2. Luria-Bertani (LB) medium: 5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl. M9 medium [15]: 10 g/L glucose, 10 g/L Oxoid yeast extract, 20 g/L Oxoid tryptone and 10 × M9 basal salts (60 g/L Na_2HPO_4, 30 g/L KH_2PO_4, 5 g/L NaCl, 10 g/L NH_4Cl, 20 mM $MgSO_4$, and 1 mM $CaCl_2$). Carbon sources, nitrogen sources and inorganic salts used for medium optimization were industrial grade and purchased from domestic market.

2.2. Cloning of CAH genes and sequence analysis

Genomic DNAs of 7 *B. subtilis* strains were used as templates for CAH amplification. To clone the CAH gene, specific primers (PF, GCGATGCAACTATTCGATCTGCCG and PR, GCGTCAGCCTTTAAGATGCTGCT) were designed based on the conserved sequences of known CAH from *B. subtilis* (Genebank accession number: D10935.1, CP002468.1 and AGF25253.1). PCR conditions were as follows: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, followed by one cycle of 72 °C for 10 min. The amplified PCR products were purified and cloned into pMD19-T vector, and transformed into *E. coli* DH5α. The constructed plasmids (pMD19-T-CAH) were sequenced. The protein sequence similarities were assessed using the Basic Local Alignment Search Tool (BLAST) programs in NCBI.

2.3. Construction of CAH expression plasmids

Primers P1F and P1R (Table S2) were designed to construct CAH expression plasmids. The plasmids pMD19-T with full-length of different CAH genes were used as PCR template. PCR amplification was conducted as mentioned in Section 2.2. The obtained CAH genes and the plasmids pET-24a and pET-28a were digested with the *Nde* I and *Xho* I restriction enzymes (Fermentas, Shanghai, China) simultaneously, and ligated to construct inducible CAH expression vectors of pET-24a-CAH and pET-28a-CAH.

pUC19, a high-copy-number variant of pBR322, was selected for construction of constitutive CAH-expressing plasmids. P2F and P2R (Table S2) were used for eliminating the ampicillin resistance gene of pUC19, then these obtained fragments were ligated with the kanamycin resistance gene, which was cloned from the plasmid pET-28a using primers of P3F and P3R (Table S2). The obtained