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Expression and production of recombinant *cis*-epoxysuccinate hydrolase in *Escherichia coli* under the control of temperature-dependent promoter

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ABSTRACT

cis-Epoxysuccinate hydrolase (ESH) from *Nocardia tartaricans* CAS-52 could catalyze the stereospecific hydrolysis of *cis*-epoxysuccinate to L-(+)-tartrate. The ESH gene of 762 bp was cloned and its open reading frame (ORF) sequence predicted a protein of 253 amino acids. An expression plasmid carrying the ESH gene under the control of the P_LP_R promoter was introduced into *Escherichia coli*, and the ESH gene was successfully expressed in the recombinant strain. The expression conditions and scale-up production were also studied. Fed-batch fermentation of *E. coli* Trans 1-T1 transformant was carried out in a 2000 L fermentor to product recombinant ESH. The results showed that wet cell concentration reached to 62.45 g L^{-1}, and the specific activity of ESH was 380.17 U mg^{-1}, which could meet the requirements of industrial production of L-(+)-tartaric acid.

1. Introduction

Epoxide hydrolases (EHs, EC 3.3.2.3) are a group of functionally related enzymes which have gained considerable attention due to their roles in xenobiotic detoxification (Arand et al., 2005) and environmental pollutants degradation (Swaving and de Bont, 1998). EHs are ubiquitous in nature and require no co-factors for exhibiting their functions. They are known to catalyze the hydrolysis of racemic epoxides to enantiopure epoxides and their corresponding optically pure vicinal diols. Recently, bacterial EHs have been recognized as versatile biocatalysts for preparation of enantiopure pharmaceuticals and other fine chemicals owing to the advantages of high stereospecificity, availability and environmental friendliness (Steinreiber and Faber, 2001). The enzymatic production of L-(+)-tartaric acid is the first industrial application of an EH (Archelas and Furstoss, 1998).

L-(+)-Tartaric acid is a well-known natural acid that is distributed in many kinds of fruits (e.g. grapes). It is widely used in food, wine, pharmaceutical, cement, resin and polyester industries as acidifier, taste enhancer, antioxidant, chemical resolving agent (Freddi et al., 1996). The most effective way for industrial production of L-(+)-tartaric acid is biocatalysis. The enantiomer L-(+)-tartaric acid can be produced microbially from the inexpensive and easily available *cis*-epoxysuccinate (ES) by an epoxide hydrolase, *cis*-epoxysuccinate hydrolase (ESH). ESH can be produced in various microorganisms, such as *Acinetobacter tartarogenes*, *Agrobacterium aureum*, *Rhizobium validum*, *Pseudomonas* sp. (Kamatani et al., 1977), *Acetobacter curtus*, *Corynebacterium* sp. (Tsurumi and Fujioka, 1978), *Nocardia tartaricans* (Miura et al., 1977), *Rhodococcus rhodochrous* (Willaert and Vuyst, 2006).

For microbial production of L-(+)-tartaric acid, whole cells are more usual in terms of process economy (Kurillová et al., 2000). Therefore, it becomes important to enhance the apparent activity of ESH to improve the catalytic efficiency. The apparent activity can be increased by the addition of detergents (Miura et al., 1977) and various ions (Miková et al., 1998) during the bioconversion. The biotransformation efficiency can also be improved by immobilizing the microorganisms. *N. tartaricans* cells immobilized in pectate gel beads have shown higher enzymatic activity (51 U mg^{-1}) than free cells (8.9 U mg^{-1}) (Rosenberg et al., 1999). Also, *N. tartaricans* cells entrapped in sodium alginate–cellulose sulfate–poly(methylene-co-guanidine) capsules have shown a two fold increase in activity compared with cells entrapped in hardened calcium pectate beads (Bučko et al., 2005).

Escherichia coli expression systems have been the preferred option for producing many recombinant proteins in high quantities and low production costs. ESH is a monomeric enzyme produced in prokaryote and can be expressed as a soluble form in *E. coli*. Several engineering strains have been constructed, such as *E. coli* JM109/pTrc99a-ESH (Liu et al., 2007), *E. coli* BL21 (DE3)/pET11a-ESH (Wang et al., 2012), *E. coli* BL21 (DE3)/pET24-ESH (Vasu et al., 2012). The strong inducible promoters employed for ESH

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expression, T7 phage promoter and tac hybrid promoter, require the addition of chemical inducer, e.g. IPTG, which is expensive and not suitable for industrial production. $\lambda P_L P_R$ promoter, a temperature-dependent promoter widely used in *E. coli* expression system, can be finely regulated by the thermolabile cIts857 repressor. This thermo-regulated expression system can avoid the use of special media, toxic or expensive chemical inducers (Valdez-Cruz et al., 2010). Furthermore, this system can be easily scalable and suitable for all kinds of *E. coli* hosts. In this contribution, an engineering bacterium was constructed to express recombinant ESH using thermo-regulated *E. coli* expression system. The culture conditions were optimized and the scale-up production was successfully carried out.

2. Materials and methods

2.1. Organism and plasmid

N. tartaricans CAS-52 was provided by Great Wall Bio-chemical Engineering Co., Ltd. (Zhangjiakou, Hebei, China). Bacteria were grown aerobically at 30 °C for 20–24 h in 50 mL medium containing K_2HPO_4 (0.1%), KH_2PO_4 (0.05%), glucose (1%), corn steep liquor (1%), $MgSO_4$ (0.05%), $(NH_4)_2SO_4$ (0.2%), yeast extract (0.5%), $FeSO_4$ (0.001%) and distilled water with pH adjusted to 7.2. Then the medium was inoculated into 500 mL flasks, and 1% (w/v) sodium *cis*-epoxysuccinate (pH 7.5) was added to the culture medium.

The expression vector pBV220 was obtained from Shine gene Molecular Biotechnology Co., Ltd. (Shanghai, China). It was a widely used prokaryotic vector constructed in China. It was a high-copy number plasmid with ampicillin resistance gene, $P_L P_R$ promoter, cIts857 gene, multiple cloning sites (MCS), Shine-Dalgarno (SD) sequence, and two strong transcription terminators, and suitable for all kinds of hosts.

2.2. Expression plasmid construction

N. tartaricans chromosomal DNA was extracted using a Bacterial Genomic DNA Extraction Kit (Sunbiotech, Beijing, China). The ESH gene flanked by EcoRI and BamHI sites was amplified by the polymerase chain reaction (PCR) using the forward primer (ESH-F: 5′-CCGGAATTCATGCAACTGAACAATGCGAAC-3′) and reverse primer (ESH-R: 5′-CGCGGATCCTCAATCGATACCGGCAGTT-3′), which were designed based on ESH sequences from *N. tartaricans* CAS-52 (GenBank accession no. JQ267565). PCR was carried out in a 50 μL volume under the following conditions: 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, and one final step of 10 min at 72 °C. The PCR products were purified and double digested with EcoRI and Bam HI (NEB, Ipswich, MA, USA), recovered through agarose gel electrophoresis, and ligated with pBV220 plasmid DNA by T4 DNA ligase (NEB), followed by digestion with EcoRI and Bam HI. The recombinant plasmid pBV220-ESH was then transformed into *E. coli* DH5α (Cwbiotech, Beijing, China). A single transformant colony was selected, transferred into 5 mL of Luria-Bertani medium (LB medium) with 100 μg mL^{-1} ampicillin, and incubated overnight with vigorous shaking (200 rpm) at 37 °C. The recombinant plasmid was extracted using a Bacterial plasmid DNA Extraction Kit (Sunbiotech) and double digested with EcoRI and BamHI, and its DNA was sequenced.

2.3. Expression of recombinant ESH

The *E. coli* DH5α transformant was cultivated at 37 °C in LB medium with 100 μg mL^{-1} ampicillin and induced by raising temperature to 42 °C when OD600 was about 0.6–0.8. Cells were grown for additional 5 h and harvested by centrifugation at 8000 × *g* for 20 min, 4 °C.

2.4. Enzyme assay

ESH was expressed intracellularly, and its specific activity was determined as follows: the *E. coli* cells were centrifuged and resuspended in phosphate buffer (20 mM; pH 7.5). Then cells were disrupted using an ultrasonic device, followed by centrifugation at 12,000 × *g* for 30 min to remove unbroken cells and debris. The supernatant was used directly as crude enzyme to measure the enzyme activity. 0.9 mL of 100 mM ES in phosphate buffer (20 mM; pH 7.5) was incubated with 0.1 mL of enzyme solution at 37 °C for 30 min. Similarly, its apparent activity was measured using 0.5 g whole *E. coli* cells. The reaction was terminated by immersion in boiling water for 5 min. One unit of ESH was defined as the amount of enzyme that generated 1 μmol of L-(+)-tartaric acid per minute under the above conditions.

HPLC analysis of L-(+) tartaric acid was performed using a Waters 2695 HPLC system fitted with a Hypersil GOLD C18 column (5 μm × 4.6 mm × 250 mm; Dikma) and a 2996 diode array detector operated at 210 nm. The mobile phase consisted of 0.02 mol L^{-1} potassium dihydrogen phosphate buffer (pH 2.1–2.5, adjusted with phosphoric acid) and acetonitrile, at a 95:5 ratio. The elution was carried out at 25 °C with a flow rate of 1.0 mL min^{-1}. The injection volume was 10 μL. Commercially available tartaric acid was used as external standard.

The expression of ESH was analyzed by SDS-PAGE as described by Laemmli (1970), and the protein concentration was determined by the Bradford method (Bradford, 1976), with bovine serum albumin as a standard.

2.5. Effect of induction-starting time and post-induction time on enzyme activity

The induction-starting time was determined by the cell optical density at 600 nm. To determine the effect of induction-starting time on ESH activity, *E. coli* DH5α transformant was induced at different initial OD600 in range from 0.3 to 1.2 with the same post-induction time. The effect of post-induction time on ESH activity was determined by beginning to induce at optimal initial OD600 with different holding time from 2 h to 7 h. The ESH activity was measured after cultivation.

2.6. Effect of host cells on enzyme activity

Three types of *E. coli* strains have been chosen as hosts for expression of recombinant ESH, including *E. coli* DH5α, *E. coli* BL21 (DE3) and *E. coli* Trans 1-T1. The transformants containing pBV220-ESH were cultivated and induced with same conditions. The ESH expression level was measured after cultivation.

2.7. Large- scale production of recombinant ESH

Scale-up experiments were carried out in different sizes of fermentors by fed-batch culture technology for production of recombinant ESH. In the 2000 L scale production process, the fermentation medium was composed of the following ingredients: tryptone (15 g L^{-1}), yeast extract (7.5 g L^{-1}), glucose (5 g L^{-1}), NaCl (10 g L^{-1}) and ampicillin (100 μg mL^{-1}) at pH 7.0. The pH was controlled at 7.0 ± 0.2 by automatic addition of $NH_3 \cdot H_2O$ or H_3PO_4. The aeration rate and stirring speed were manually changed to maintain the dissolved oxygen (DO) level above 20% of air saturation. When DO suddenly increased, feeding medium was continuously added, consisting of tryptone (100 g L^{-1}), yeast extract (50 g L^{-1}) and glycerol (200 g L^{-1}). The temperature was raised to 42 °C at an