Bioresource Technology 196 (2015) 176–183

Contents lists available at ScienceDirect

Bioresource Technology

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

Extracellular expression of natural cytosolic arginine deiminase from *Pseudomonas putida* and its application in the production of L-citrulline

Lingqia Su, Yue Ma, Jing Wu *

State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China
School of Biotechnology and Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China

HIGHLIGHTS

- The natural cytosolic *P. putida* arginine deiminase was extracellularly expressed.
- An extracellular arginine deiminase activity of 101.2 U mL^{-1} was obtained.
- The arginine deiminase activity represents the highest yield reported to date.
- L-Citrulline was prepared using the extracellularly expressed arginine deiminase.
- The conversion rate reached 100% with high substrate concentration at 650 g L^{-1}.

ARTICLE INFO

Article history:
Received 15 June 2015
Received in revised form 20 July 2015
Accepted 21 July 2015
Available online 29 July 2015

Keywords:
Arginine deiminase
Pseudomonas putida
Extracellular expression
Fermentation optimization
L-Citrulline

ABSTRACT

The *Pseudomonas putida* arginine deiminase (ADI), a natural cytosolic enzyme, and *Thermobifida fusca* cutinase were co-expressed in *Escherichia coli*, and the optimized cutinase gene was used for increasing its expression level. 90.9% of the total ADI protein was released into culture medium probably through a nonspecific leaking mechanism caused by the co-expressed cutinase. The enzymatic properties of the extracellular ADI were found to be similar to those of ADI prepared by conventional cytosolic expression. Extracellular production of ADI was further scaled up in a 3-L fermentor. When the protein expression was induced by IPTG (25.0 μM) and lactose (0.1 g L^{-1} h^{-1}) at 30 °C, the extracellular ADI activity reached 101.2 U mL^{-1}, which represented the highest ADI production ever reported. In addition, the enzymatic synthesis of L-citrulline was performed using the extracellularly expressed ADI, and the conversion rate reached 100% with high substrate concentration at 650 g L^{-1}.

1. Introduction

Arginine deiminase (ADI) catalyzes the hydrolysis of L-arginine to form L-citrulline and ammonia (Monstadt and Holldorf, 1991). L-Citrulline, a non-proteinogenic amino acid, is involved in multiple metabolic pathways, and plays important roles in physiological processes like immune system stimulation, blood sugar balancing. L-Citrulline is widely used in pharmaceutical and cosmetics industries with the demand increasing annually (Fike et al., 2014; Raghavan and Dikshit, 2001; Ruiz and Tejerina, 1998; van Wijck et al., 2014). L-Citrulline could be prepared by extraction from natural plants, chemical synthesis, fermentation, and enzymatic synthesis. Among these approaches, the enzymatic synthesis using arginine hydrolysis catalyzed by arginine deiminase is a favorable way, due to availability of the substrate L-arginine, high yield of the product, simple preparation process and purification steps (Kakimoto et al., 1971; Plimmer, 1916; Zheng et al., 2008). So far, a number of studies on enzymatic synthesis of L-citrulline using arginine deiminases from a variety of microorganisms, including *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Pseudomonas ovalis*, have been described, and the conversion rate reached more than 90% under the optimized conditions, but the reports about the L-citrulline production with high substrate concentration is limited in these literature (Kakimoto et al., 1971; Wu and Meininger, 1993; Yamamoto et al., 1974; Zheng et al., 2008).

As ADI is a natural cytosolic enzyme, the conventional intracellular recombinant expression method was generally used in the ADI production, and *Escherichia coli* was the most commonly used host for ADI expression (Ahn et al., 2014; Fayura et al., 2013;

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China. Tel.: +86 510 85327802; fax: +86 510 85326653.
E-mail address: jingwu@jiangnan.edu.cn (J. Wu).

http://dx.doi.org/10.1016/j.biortech.2015.07.081
0960-8524/

Misawa et al., 1994). For example, ADI from *Lactococcus lactis* ssp. *lactis* ATCC 7962 was expressed in *E. coli* BL21 by Jong-Eun Kim, with the ADI activity of 2.16 U mg^{-1} (Kim et al., 2007). Yongmei Liu et al. introduced the gene coding for ADI from *Pseudomonas plecoglossicida* CGMCC 2039 into *E. coli* BL21(DE3) strain, and ADI was expressed at 2.04 U mL^{-1} (Liu et al., 2011). Ying Wang's group provided a new strategy that in vivo solubility of overexpressed arginine deiminases (ADIs) was significantly improved by co-expression with the GroES-GroEL chaperone and combined addition of L-arginine and D-glucose in the LB medium, and the enzymatic activity of the crude extracts reached 9.43 ± 0.02 U mL^{-1} (Wang and Li, 2014).

However, compared to the intracellular expression, extracellular expression of proteins has advantages of improved protein folding, reduced inclusion body formation, as well as fewer contaminating proteins from the host, simplified extraction steps, and significantly reduced cost. Thus it is a more favorable approach for large scale protein preparation (Mergulhao et al., 2005). *E. coli* has five types of secretion pathway, and each of them can be used for extracellular expression of target protein. However, for cytosolic enzymes, even a signal peptide is attached to target protein, it still cannot be secreted extracellularly under normal conditions. Only intracellular expression can be used for production of these proteins, and extraction of protein is generally carried out by physical or chemical disruption of cell membrane, followed by difficult purification procedures (Cai et al., 2008; Jong et al., 2010; Mergulhao et al., 2005).

Previously, *Thermobifida fusca* cutinase was found to have catalytic activity to hydrolyze phospholipids. While expressed intracellularly in *E. coli*, it can fold correctly into active enzyme, which catalyzes hydrolysis of membrane phospholipids, resulted in increased membrane permeability. Due to the modification to the membrane, cutinase is released into the culture medium in large quantities. It should be noted that this process did not cause obvious physiological damage to the cells, and cell lysis was not observed (Su et al., 2013a). More importantly, other cytosolic proteins could also be "secreted" via the increased membrane permeability, which provided a novel approach for extracellular expression of recombinant cytosolic proteins in *E. coli* (Su et al., 2013b).

In the present study, the ADI from *P. putida*, a very effective enzyme for the preparation of L-citrulline (Kakimoto et al., 1971; Shibatani et al., 1975; Yamamoto et al., 1974), was extracellularly produced by co-expression with *T. fusca* cutinase in *E. coli*, and the induction strategy was optimized in a 3-L fermentor. In addition, the enzymatic synthesis of L-citrulline by the extracellular expressed ADI was investigated.

2. Methods

2.1. Bacterial strains, vectors and materials

P. putida ACCC 10185 was purchased from DSMZ (Braunschweig, Germany). *E. coli* strains JM109, BL21(DE3), and the expression vector pETDuet-1, were obtained from Novagen (Madison, USA). The plasmid pETDuet-*cut* and pET24a-cut^{opt}, which harbor the native and optimized genes encoding *T. fusca* cutinase respectively, were constructed and stocked in our previous work (Su et al., 2015, 2013b). The pMD18-T simple vector, DNA polymerase, restriction enzymes, alkaline phosphatase, and T_4 DNA ligase were obtained from Takara (Dalian, China). Genomic DNA mini preparation kit, agarose gel DNA purification kit, and plasmid mini preparation kit were obtained from TIANGEN Biotech Co., Ltd (Beijing, China). Primer synthesis and DNA sequencing were performed by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). *O*-nitrophenyl-β-D-galactopyranoside (ONPG) and N-phenyl-α-naphthylamine (NPN) were obtained from Sigma. Other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Construction of recombinant E. coli for co-expression of cutinase and arginine deiminase

The optimized gene of *T. fusca* cutinase (NCBI accession number YP_288944) was amplified from pET24a-cut^{opt} by PCR utilizing the primers 5′-CCATGGCCA ACCCTTATGAGCG-3′ and 5′-AAGCTTAAAA CGGGCAGGTGC-3′. The amplification product was isolated and cloned into pMD18-T simple vector. After confirmed by DNA sequencing, the gene was excised from recombinant pMD18-T derivative utilizing the restriction enzymes *Nco* I and *Hin*d III and then ligated into the first multiple cloning site of the expression vector pETDuet-1, which was restricted with the same restriction enzymes and alkaline phosphatase, yielding plasmid pETDuet-cut^{opt}.

The genes encoding arginine deiminase (NCBI accession number AAA16964) was amplified from genomic *P. putida* by PCR utilizing the primers 5′-CATATGTCCGCTGAAAAACAGAAGTAC-3′ and 5′-CTCGAGTTAGTAGTCG ATCGGGTCACGCAC-3′, and ligated into the second multiple cloning site of the plasmid pETDuet-*cut* or pETDuet-cut^{opt} using a similar approach as described above, resulting in pETDuet-*cut-arcA* and pETDuet-cut^{opt}-*arcA*, respectively. The plasmids were used to transform chemically competent *E. coli* BL21(DE3) for co-expression of cutinase and arginine deiminase.

2.3. Media and feeding solutions

Luria–Bertani (LB) medium, containing (g L^{-1}) yeast extract 5.0, tryptone 10.0, NaCl 10.0, was used for seed cultivation. Terrific Broth (TB) medium that consisted of (g L^{-1}) yeast extract 24.0, tryptone 12.0, K_2HPO_4 16.4, KH_2PO_4 2.3, and glycerol 5.0 was for protein production in shake-flask. A modified semisynthetic medium in a 3-L fermentor was as follows: the initial batch medium contained (g L^{-1}) yeast extract 20.0, tryptone 30.0, $MgSO_4{\cdot}7H_2O$ 2.0, K_2HPO_4 14.6, $(NH_4)_2$-H-citrate 1.0, glycerol 8.0, and trace metal solution 1.0 mL L^{-1}, pH 7.0. The feeding solution was (g L^{-1}) yeast extract 50.0, tryptone 50.0, $MgSO_4{\cdot}7H_2O$ 3.4, and glycerol 500.0.

2.4. Cultivation conditions

2.4.1. Shake-flask

The seed culture was started by inoculating 50 mL of LB medium containing 100 μg mL^{-1} ampicillin in a 250 mL shake flask with 100 μL sample of a frozen glycerol stock (kept at −80 °C), and grown for 8 h at 37 °C in a rotary shaker (200 rpm). A sample (5%, v/v) of this seed culture was inoculated into 50 mL of TB medium containing 100 μg mL^{-1} ampicillin in a 250 mL shake flask. The resulting culture was then shaken at 37 °C in a rotary shaker (200 rpm). When the OD_{600} of the culture reached 1.5, IPTG was added to a final concentration of 0.4 mM to induce protein expression. After induction, incubation was continued at 25 °C until the extracellular arginine deiminase activity reached the highest level.

2.4.2. Bioreactor

The seed culture was prepared as described above, and then inoculated into the semisynthetic medium, supplemented with 100 μg mL^{-1} ampicillin, for fed-batch cultivation in a 3.6-L fermentor (Labfors 5, Infors-HT Co., Ltd) with the temperature of 37 °C and pH 7.0. The end of initial glycerol consumption was detected by a sudden increase in both dissolved oxygen and pH. The continuous