Construction of Whole-Cell Biocatalysts for (S)-Acetoin Synthesis Using Synthetic Biology Strategies: Postprint

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Date: 2018-11-24T00:00:00+00:00

Abstract

Objective: To overexpress diacetyl reductase (DAR) in an Escherichia coli host while concurrently constructing an in situ NADH regeneration system, employing whole-cell catalysis for the efficient asymmetric reduction of diacetyl to synthesize (S)-acetoin.

Methods: The dar gene from Paenibacillus polymyxa was cloned via PCR and ligated into plasmid pETDuet-1, which was subsequently transformed into Escherichia coli BL21(DE3) to construct the recombinant strain E. coli BL21(DE3)-DAR. The expressed DAR enzyme was purified using HiTrap TALON affinity chromatography, and its specific activity and kinetic parameters were determined. An in situ NADH regeneration system was established in the recombinant strain E. coli BL21(DE3)-DAR through co-expression of glucose dehydrogenase (GDH) from Bacillus subtilis, yielding the recombinant strain E. coli BL21(DE3)-DAR/GDH. This recombinant strain was utilized as a whole-cell biocatalyst, with catalytic conditions optimized to enhance (S)-acetoin yield and productivity.

Results: Recombinant engineered strains E. coli BL21(DE3)-DAR and E. coli BL21(DE3)-DAR/GDH were successfully constructed. The Michaelis constant (Km), maximum reaction rate (Vmax), and catalytic constant (kcat) for DAR-catalyzed diacetyl reduction using NADH as cofactor were 2.59 mM, 1.64 mol/L · min · mg, and 12.3 s^{-1}, respectively. The optical purity of (S)-acetoin produced from diacetyl reduction was 95.86%, demonstrating excellent catalytic efficiency and stereoselectivity. Following establishment of the in situ NADH regeneration system, the recombinant strain E. coli BL21(DE3)-DAR/GDH efficiently catalyzed acetoin synthesis from diacetyl. Under optimal catalytic conditions with fed-batch operation, acetoin concentration reached 51.26 g/L, with a conversion rate of 81.37% and a productivity of 5.13 g/(L · h).

Conclusion: This approach enables production of the high-value chiral compound (S)-acetoin from the achiral substrate diacetyl using recombinant whole-cell biocatalysts without requiring external addition of expensive cofactors, thereby demonstrating significant potential for industrial application.

Full Text

Preamble

Constructing Whole-cell Biocatalysts via Synthetic Biology Strategy for (S)-Acetoin Synthesis

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Abstract

Objective: To construct a whole-cell biocatalyst that overexpresses diacetyl reductase (DAR) in *Escherichia coli* hosts while establishing an in situ NADH regeneration system for the efficient asymmetric reduction of diacetyl to (S)-acetoin. **Methods:** The *dar* gene from *Paenibacillus polymyxa* was cloned via PCR and ligated into plasmid pETDuet-1, then transformed into *E. coli* BL21(DE3) to construct the recombinant strain *E. coli* BL21(DE3)-DAR. The expressed DAR enzyme was purified using HiTrap TALON affinity chromatography, and its specific activity and kinetic parameters were determined. An NADH in situ regeneration system was constructed in the recombinant strain *E. coli* BL21(DE3)-DAR by co-expressing glucose dehydrogenase (GDH) from *Bacillus subtilis*, creating the recombinant strain *E. coli* BL21(DE3)-DAR/GDH. This recombinant strain was used as a whole-cell biocatalyst, and catalytic conditions were optimized to improve (S)-acetoin yield and productivity. **Results:** Recombinant strains *E. coli* BL21(DE3)-DAR and *E. coli* BL21(DE3)-DAR/GDH were successfully obtained. DAR exhibited a Michaelis constant (Km) of 2.59 mM, maximum reaction rate (Vmax) of 1.64 $\mathrm{mol/L \cdot min \cdot mg}$, and catalytic constant (Kcat) of 12.3 s^{-1} for diacetyl reduction using NADH as cofactor. The optical purity of (S)-acetoin produced from diacetyl reduction reached 95.86%, demonstrating excellent catalytic efficiency and stereoselectivity. After establishing the NADH regeneration system, the recombinant strain *E. coli* BL21(DE3)-DAR/GDH efficiently catalyzed diacetyl conversion to acetoin. Under optimal fed-batch conditions, acetoin concentration reached 51.26 g/L with a conver-

sion rate of 81.37% and productivity of 5.13 g/(L · h). **Conclusion:** This study demonstrates the production of high-value chiral compound (S)-acetoin from the non-chiral substrate diacetyl using recombinant whole-cell biocatalysts. The system eliminates the need for expensive exogenous cofactors and shows significant potential for industrial application.

Keywords: Whole-cell biocatalyst; Cofactor regeneration; Diacetyl reductase; (S)-Acetoin

Funding: This work was supported by the Guangxi Science and Technology Program (No. 14125008-2-22) and the National Natural Science Foundation of China (No. 21466007 and No. 31400079).

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Introduction

Acetoin (AC), also known as 3-hydroxy-2-butanone or methylacetylcarbinol, naturally occurs in foods such as corn, strawberries, grapes, cocoa, and alcoholic beverages. It possesses a distinctive creamy, buttery aroma that becomes pleasantly milky when highly diluted, making it a widely used food flavor additive that complies with national standard GB2760-86 [1,2]. As an important four-carbon compound, acetoin was listed by the U.S. Department of Energy in 2004 as one of 30 priority platform chemicals for development and utilization, with extensive applications in tobacco, alcohol, cosmetics, pharmaceuticals, and chemical industries, and global demand exceeding 10,000 tons per year [3-5]. The acetoin molecule contains a chiral carbon atom, existing as two stereoisomers: (S)-acetoin and (R)-acetoin. Single-configuration acetoin not only maintains the basic functions of regular acetoin but also offers distinct advantages in asymmetric synthesis due to its unique stereochemical structure, particularly for high-value chiral pharmaceutical intermediates, chemical intermediates, and liquid crystal materials. Consequently, optically pure (S)-acetoin has far broader applications and market prospects than conventional acetoin.

Currently, acetoin production methods primarily involve chemical synthesis and microbial fermentation. The most studied chemical synthesis approaches are partial hydrogenation of diacetyl and selective oxidation of 2,3-butanediol [6]. Both methods suffer from low product recovery, severe environmental pollution, and contamination with carcinogenic or pathogenic compounds. With increasing living standards, demand for natural green products has grown substantially. Microbial fermentation produces "natural" acetoin as a flavoring agent, commanding significantly higher market prices than chemically synthesized products. Many microorganisms in nature can metabolize carbon sources such as glucose through glycolysis to produce acetoin. In 2007, Xiao et al. [7] used *Bacillus subtilis* CICC 10025 for acetoin fermentation, achieving 34.5 g/L with a productivity of 0.627 g/(L · h). In 2012, Zhang et al. [8] employed *Paenibacillus polymyxa* LY107 with glucose/xylose as substrates, producing 23.9 g/L at 0.569 g/(L · h). In 2013, Hao [9] used *B. subtilis* CCTCC M 208175 with fed-batch

fermentation to reach 58.7 g/L and 1.22 g/(L · h). Despite these achievements, natural fermentation—like chemical synthesis—typically yields a mixture of (S)- and (R)-acetoin stereoisomers [3,10]. The spontaneous oxidative decarboxylation of α-acetolactate to diacetyl is inefficient, and natural strains predominantly produce (R)-acetoin, with (S)-acetoin as a minor byproduct. Moreover, the physicochemical properties of (R)- and (S)-acetoin are similar, requiring complex and costly chiral resolution processes to obtain optically active acetoin, which keeps (S)-acetoin prices high and limits its development and application.

Enzymatic catalysis employs microbial enzymes directly for redox reactions. Reports describe using diacetyl reductase to catalyze diacetyl synthesis of (S)-acetoin or (R,R)-2,3-butanediol dehydrogenase to oxidize (R,R)-2,3-butanediol to (R)-acetoin [11,12], yielding single products with high optical purity. Gao et al. [3] used purified carboxyl reductase and glucose dehydrogenase for in vitro (S)-acetoin synthesis, achieving 12.2 g/L with a productivity of 9.76 g/(L · h). Although the productivity was high, the yield was low, and enzyme extraction and purification involve complex processes, high costs, and poor stability.

Whole-cell biocatalysis utilizes intact cells as catalysts, essentially employing intracellular enzymes while combining the advantages of fermentation and enzymatic catalysis with high stereoselectivity [13]. This study first overexpressed diacetyl reductase (DAR) from *Paenibacillus polymyxa* DSM 365 in *E. coli* [14], then introduced an NADH in situ regeneration system in the recombinant strain *E. coli* BL21(DE3)-DAR by co-expressing glucose dehydrogenase (GDH) from *B. subtilis* 168. The resulting whole-cell biocatalyst efficiently catalyzed the asymmetric reduction of diacetyl to acetoin. Using a fed-batch strategy, acetoin concentration reached 51.26 g/L with a productivity of 5.13 g/(L · h).

Materials and Methods

1.1.1 Strains, Plasmids, and Primers

Strains, plasmids, and primers are listed in Table 1 . *E. coli* DH5α and *E. coli* BL21(DE3) were used as cloning and expression hosts, respectively, with pETDuet-1 as the parental expression vector.

1.1.2 Media and Buffers

LB medium (per liter): 10 g peptone, 5 g yeast extract, 10 g NaCl, pH 7.0, used for *E. coli* seed culture and recombinant strain induction.

Potassium phosphate buffer (100 mM): Solution A, 0.2 M KH_2PO_4; Solution B, 0.2 M K_2HPO_4. Following the pH mixing system listed in Table A.1.3 of "Short Protocols in Molecular Biology" [15], solutions A and B were mixed and diluted with ddH_2O to 200 mL to obtain potassium phosphate buffer at the desired pH.

1.1.3 Enzymes and Reagents

Restriction endonucleases, calf intestinal phosphatase (CIP), and T4 DNA ligase were purchased from NEB. PrimeSTAR GXL DNA polymerase was from TaKaRa (Dalian). Bacterial genomic DNA extraction kits, plasmid mini-prep kits, and agarose gel DNA recovery kits were from Tiangen Biotech (Beijing). Ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), and nicotinamide adenine dinucleotide (NADH/NAD^+) were from Sangon Biotech (Shanghai). Diacetyl was from Shanghai Beihe Chemical. Yeast extract and peptone were Oxoid products. Primer synthesis and DNA sequencing services were provided by GenScript (Nanjing). Other chemicals were domestic analytical grade or higher.

1.2.1 Construction of Recombinant Plasmids

Based on the *dar* gene sequence from *P. polymyxa* DSM 365 (GenBank Accession No. KT717931) and the *gdh* gene sequence from *B. subtilis* 168 (GenBank Accession No. CP019663.1), primers P1/P2 and P3/P4 were designed (Table 1). Genomic DNA was extracted from *P. polymyxa* DSM 365 and used as template for PCR amplification of the *dar* fragment with primers P1 and P2. Similarly, genomic DNA from *B. subtilis* 168 was used to amplify the *gdh* fragment with primers P3 and P4. PCR reaction systems and programs followed the instructions for TaKaRa PrimeSTAR GXL DNA polymerase. The purified *dar* fragment was digested with BamH I and Hind III, then ligated into similarly digested pETDuet-1 to construct pETDuet-dar. The *gdh* fragment was digested with Nde I and Aat II and ligated into pETDuet-dar to obtain pETDuet-dar/gdh. Plasmids pETDuet-1, pETDuet-dar, and pETDuet-dar/gdh were transformed into *E. coli* BL21(DE3) to generate *E. coli* BL21(DE3)-pETDuet, *E. coli* BL21(DE3)-DAR, and *E. coli* BL21(DE3)-DAR/GDH, respectively.

1.2.2 Expression and Purification of DAR

The recombinant strain *E. coli* BL21(DE3)-DAR was activated overnight, then inoculated at 2% into fresh LB medium and cultured at 37°C to OD_{600} of 0.6–0.8. IPTG was added to 0.2 mM, followed by induction at 20°C for 12 h. Cells were harvested by centrifugation, washed twice with saline, and resuspended in binding buffer (20 mM potassium phosphate, 500 mM NaCl, pH 7.4). Cells were lysed by sonication on ice, and the lysate was centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was purified by HiTrap TALON affinity chromatography. Elution buffer imidazole concentration was adjusted to collect target protein fractions, which were desalted using PD-10 columns and stored at -80°C.

1.2.3 Enzymatic Characterization of DAR

The enzyme activity assay was performed in a 1 mL reaction mixture containing 100 mM phosphate buffer (pH 6.0), 5 mM diacetyl, 0.2 mM NADH, and

appropriate enzyme volume. DAR activity was measured indirectly by monitoring NADH consumption at 340 nm. One unit (U) was defined as the amount of enzyme catalyzing the consumption of 1 mol NADH per minute. Kinetic parameters (Km, Vmax, Kcat) were determined at 40°C in 100 mM phosphate buffer (pH 6.0) while maintaining constant pH, buffer, cofactor, and enzyme concentrations. Diacetyl concentration [S] was varied from 0.125 to 20 mM, and reaction rates (V) were measured. Data were fitted using Origin software to obtain Km and Vmax, from which Kcat was calculated.

Stereoselectivity of diacetyl reductase: A 1 mL reaction mixture (100 mM phosphate buffer, pH 6.0) containing 100 mM diacetyl, 60 mM NADH, and appropriate enzyme was incubated at 40°C for 24 h. Products were extracted with equal volume ethyl acetate, and optical purity was analyzed by chiral gas chromatography.

1.2.4 Preparation of Resting Cells

Recombinant strains *E. coli* BL21(DE3)-pETDuet, *E. coli* BL21(DE3)-DAR, and *E. coli* BL21(DE3)-DAR/GDH were inoculated into LB liquid medium (100 μg/mL ampicillin) and cultured overnight at 37°C. Activated cultures were transferred at 2% inoculum into fresh LB medium (100 μg/mL ampicillin, 200 mL/500 mL flask) and shaken at 250 rpm, 37°C to OD_{600} = 0.6–0.8. IPTG was added to 0.2 mM, followed by induction at 20°C, 125 rpm for 12 h. Cells were harvested by centrifugation, washed twice with saline, and resuspended in 100 mM phosphate buffer for storage at 4°C. These resting cells were used for 12.5% SDS-PAGE analysis and whole-cell biocatalysis.

1.2.5 Whole-Cell Biocatalytic Synthesis of Acetoin and Condition Optimization

Catalytic conditions were optimized in shake flasks (10 mL/50 mL flask) at 150 rpm and 30°C, with pH maintained at 7.0. The standard reaction mixture contained 100 mM phosphate buffer, 8 g (DCW)/L resting cells, 30 g/L glucose, and 15 g/L diacetyl (glucose/diacetyl molar ratio 1:1), reacting for 3 h. Parameters were sequentially optimized including cell dry weight, buffer pH, reaction temperature, glucose/diacetyl molar ratio, shaking speed, and initial diacetyl concentration.

1.2.6 Analytical Methods

(1) Cell growth measurement: OD_{600} was measured using a UV spectrophotometer. Dry cell weight (DCW) was calculated from the standard curve: DCW (g/L) = 0.3 × OD_{600}.

(2) Glucose concentration: Reaction samples were centrifuged at 12,000 rpm for 5 min, and the supernatant was appropriately diluted for analysis using an SBA-40D biosensor.

(3) Diacetyl and acetoin concentrations were determined by gas chromatography (Agilent 7890A) with a Phenomenex ZB-WAXplus capillary column (30 m × 0.32 mm × 0.25 m) and FID detector. Nitrogen carrier gas flow was 1.6 mL/min. Injector and detector temperatures were 250°C. Temperature program: initial 100°C for 1 min, ramp 20°C/min to 180°C (hold 3 min), then 30°C/min to 230°C (hold 1 min).

(4) Optical purity of (S)-acetoin was analyzed using an Agilent Cyclosil-B chiral column (30 m × 0.32 mm × 0.25 m) with FID detector. Nitrogen flow was 1.6 mL/min. Injector and detector temperatures were 240°C. Temperature program: initial 100°C for 1 min, ramp 10°C/min to 120°C, 6°C/min to 130°C, then 20°C/min to 230°C. Optical purity was calculated as: [S]/([S] + [R]) × 100%, where [S] and [R] represent peak areas of (S)- and (R)-acetoin, respectively.

Results

2.1 Construction of DAR and GDH Expression Vectors

The PCR-amplified *dar* fragment (720 bp, BamH I/Hind III) was ligated into pETDuet-1 to construct pETDuet-dar. Similarly, the *gdh* fragment (786 bp, Nde I/Aat II) was ligated into pETDuet-dar to create pETDuet-dar/gdh. Restriction analysis confirmed the constructs: pETDuet-dar digested with BamH I alone yielded a single 6,092 bp band, while BamH I/Hind III double digestion produced 5,372 bp and 720 bp fragments (Figure 1 [Figure 1: see original paper]a). For pETDuet-dar/gdh, Nde I single digestion generated a 6,836 bp band, and Nde I/Aat II double digestion yielded 6,050 bp and 786 bp bands (Figure 1 [Figure 1: see original paper]b). Sequencing of the correctly digested plasmids by GenScript confirmed 100% identity with GenBank sequences, verifying successful construction.

2.2 Induced Expression of DAR and GDH and Purification of DAR

Recombinant plasmids pETDuet-dar and pETDuet-dar/gdh were transformed into *E. coli* BL21(DE3). Transformants were cultured in LB medium with IPTG induction, and cells were analyzed by SDS-PAGE. As shown in Figure 2 [Figure 2: see original paper], recombinant strains *E. coli* BL21(DE3)-DAR and *E. coli* BL21(DE3)-DAR/GDH showed specific bands at approximately 27 kDa and 29 kDa, respectively, indicating efficient expression of DAR and GDH. The DAR protein from *E. coli* BL21(DE3)-DAR was purified by HiTrap TALON affinity chromatography after sonication, yielding a single ~27 kDa band.

2.3 Enzymatic Properties of DAR

Kinetic analysis revealed that DAR had a Km of 2.59 mM, Vmax of 1.64 mol/L·min·mg, and Kcat of 12.3 s^{-1} for diacetyl reduction using NADH. The optical

purity of (S)-acetoin produced was 95.86%, demonstrating excellent catalytic efficiency and stereoselectivity suitable for whole-cell catalyst construction.

2.4 Whole-Cell Biocatalytic Performance

Resting cells of *E. coli* BL21(DE3)-pETDuet, *E. coli* BL21(DE3)-DAR, and *E. coli* BL21(DE3)-DAR/GDH were used for whole-cell biocatalysis. Since each (S)-acetoin molecule synthesis consumes one NADH molecule, *E. coli* BL21(DE3)-DAR relying on endogenous cofactor regeneration produced only 2.21 g/L acetoin with a productivity of 0.74 g/(L · h). After establishing the cofactor regeneration system, catalytic efficiency improved dramatically. Under identical conditions, *E. coli* BL21(DE3)-DAR/GDH synthesized 5.03 g/L acetoin at 1.68 g/(L · h), representing a 2.28-fold improvement over DAR expression alone (Table 2).

2.5 Optimization of Whole-Cell Biocatalysis Conditions

To enhance biocatalytic efficiency, key parameters affecting acetoin synthesis were investigated. Cell concentration determines total biocatalyst quantity, while pH and temperature affect catalyst activity and stability. Optimization of cell dry weight, pH, and temperature revealed optimal conditions of 12 g(DCW)/L, pH 6.5, and 40°C in 100 mM phosphate buffer (Figure 3 [Figure 3: see original paper]a–c).

Subsequent investigation of glucose/diacetyl molar ratio and shaking speed showed that acetoin yield increased significantly with molar ratio, peaking at 3.0 before declining (Figure 3 [Figure 3: see original paper]d). Shaking speeds of 75–100 rpm provided higher efficiency, with yields decreasing at higher speeds, suggesting microaerobic conditions favor intracellular redox status for DAR catalysis (Figure 3 [Figure 3: see original paper]e). Initial diacetyl concentration optimization showed acetoin yield increased with substrate concentration, reaching 16.24 g/L at 92.8% conversion when initial diacetyl was 17.5 g/L. Further increasing diacetyl to 22.5 g/L gave maximum acetoin titer of 18.85 g/L but reduced conversion to 83.7%, likely due to substrate inhibition of cell viability (Figure 3 [Figure 3: see original paper]f).

2.6 Fed-Batch Synthesis Under Optimized Conditions

High initial diacetyl concentrations inhibited cell activity. An effective feeding strategy can significantly improve product titer [16]. Therefore, fed-batch bioconversion was performed under optimal conditions. Samples were taken every 1.5 h to monitor residual diacetyl and glucose, acetoin concentration, and pH was adjusted to 6.5 while supplementing substrates as needed. As shown in Figure 4 [Figure 4: see original paper], after 10 h of catalysis, 63 g/L diacetyl was consumed to produce 51.26 g/L acetoin at 81.37% conversion with a productivity of 5.13 g/(L · h).

Discussion

Paenibacillus polymyxa is the only natural microorganism known to efficiently produce optically pure (R,R)-2,3-butanediol [14,17-19]. Our previous whole-genome sequencing and annotation of *P. polymyxa* DSM 365 identified the key enzyme for (S)-acetoin synthesis—diacetyl reductase (DAR) [14]. This study marks the first cloning, expression, and purification of this DAR gene. Enzymatic characterization showed DAR had a Km of 2.59 mM, Vmax of 1.64 mol/L · min · mg, and Kcat of 12.3 s^{-1} for diacetyl reduction, producing (S)-acetoin with 95.86% optical purity. These properties demonstrate excellent catalytic efficiency and stereoselectivity for (S)-acetoin synthesis using either purified enzyme preparations or whole-cell biocatalysts.

Whole-cell biocatalysis leverages intracellular enzymes, overcoming many limitations of fermentation and isolated enzyme methods while offering high stereoselectivity. Compared to purified enzymes, intracellular enzymes exhibit better stability and lower costs. *E. coli* is a non-pathogenic strain with rapid growth, ease of high-density cultivation, clear genetic background, and mature genetic manipulation techniques, making it an ideal host for constructing microbial cell factories for (S)-acetoin synthesis. Gao et al. [20] introduced the *dar* gene from *P. polymyxa* ZJ-9 into *E. coli* Rosetta (DE3), with NADH supplied by endogenous glucose metabolism, achieving 39.4 g/L (S)-acetoin. However, each acetoin synthesis requires one NADH/NADPH molecule, and the low efficiency of endogenous cofactor regeneration resulted in a prolonged 20 h reaction with productivity of only 1.97 g/(L · h). Xiao et al. [21] introduced NADH oxidase from *Lactobacillus brevis* into *E. coli* BL21(DE3) to establish intracellular cofactor regeneration, reaching 36.7 g/L (S)-acetoin—2.06-fold higher than expressing (2R,3R)-2,3-butanediol dehydrogenase alone (17.8 g/L). These results demonstrate that appropriate cofactor regeneration systems effectively enhance whole-cell biocatalysis efficiency. However, their substrate (optically pure meso-2,3-butanediol) is costly, limiting scalable production.

Bacillus subtilis glucose dehydrogenase exhibits excellent stability and broad applicability (NAD^+ and $NADP^+$ dependent) and is widely used for cofactor regeneration in biocatalytic redox reactions [3,22,23]. This study constructed an exogenous cofactor regeneration system in *E. coli* by co-expressing DAR and GDH, achieving substantially improved catalytic efficiency. Under identical conditions, *E. coli* BL21(DE3)-DAR/GDH produced 2.28-fold higher acetoin titer and productivity than DAR expression alone. After optimization, fed-batch bioreduction of diacetyl using *E. coli* BL21(DE3)-DAR/GDH as whole-cell biocatalyst yielded 51.26 g/L acetoin at 5.13 g/(L · h). Using non-chiral diacetyl as substrate solves the cost problem associated with chiral substrates, offering significant industrial potential. Notably, the optical purity of (S)-acetoin from whole-cell catalysis was slightly lower than that from purified enzyme catalysis, possibly due to non-specific catalysis by endogenous *E. coli* enzymes such as diacetyl reductase and glycerol dehydrogenase.

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Note: Figure translations are in progress. See original paper for figures.