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Simultaneous biosynthesis of (*R*)-acetoin and ethylene glycol from D-xylose through *in vitro* metabolic engineering

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ABSTRACT

(*R*)-acetoin is a four-carbon platform compound used as the precursor for synthesizing novel optically active materials. Ethylene glycol (EG) is a large-volume two-carbon commodity chemical used as the anti-freezing agent and building-block molecule for various polymers. Currently established microbial fermentation processes for converting monosaccharides to either (*R*)-acetoin or EG are plagued by the formation of undesirable by-products. We show here that a cell-free bioreaction scheme can generate enantiomerically pure acetoin and EG as co-products from biomass-derived D-xylose. The seven-step, ATP-free system included *in situ* cofactor regeneration and recruited enzymes from *Escherichia coli* W3110, *Bacillus subtilis* shaijiu 32 and *Caulobacter crescentus* CB 2. Optimized *in vitro* biocatalytic conditions generated 3.2 mM (*R*)-acetoin with stereoisomeric purity of 99.5% from 10 mM D-xylose at 30 °C and pH 7.5 after 24 h, with an initial (*R*)-acetoin productivity of 1.0 mM/h. Concomitantly, EG was produced at 5.5 mM, with an initial productivity of 1.7 mM/h. This *in vitro* biocatalytic platform illustrates the potential for production of multiple value-added biomolecules from biomass-based sugars with no ATP requirement.

1. Introduction

Hemicellulose is one of the most abundant biomasses on earth and a renewable alternative to fossil fuels for chemicals and fuels production. D-Xylose ($C_5H_{10}O_5$), as the primary component of xylans in plant cell walls, is the most abundant pentose in nature (Martín del Campo et al., 2013). Thus, chemical and biochemical routes for converting D-xylose into value-added products is responsive to environmental concerns and dwindling petroleum reserves (Cherubini, 2010). The microbial metabolic pathway of D-xylose involves its conversion to D-xylulose, which is further oxidized to yield D-xylulose-5-phosphate and then metabolized *via* pentose phosphate (PP) pathway (Kuhad et al., 2011). Intermediate metabolites, glyceraldehyde-3-phosphate and fructose-6-phosphate, can be further converted to pyruvate through the Embden-Meyerhoff-Parnas (EMP) pathway. However, there are other pathways starting with the oxidation of D-xylose (Dahms, 1974; Weimberg, 1961). For example, D-xylose can be oxidized by NAD(P)$^+$-dependent D-xylose dehydrogenase to form D-xylonolactone. This metabolite can be spontaneously or enzymatically hydrolyzed to yield D-xylonate, and further converted to 2-keto-3-deoxy-D-xylonate that can turned into pyruvate and glycolaldehyde (Dahms, 1974).

Acetoin (3-hydroxy-2-butanone), an important four-carbon platform compound, is widely used to produce food, pharmaceuticals, and chemicals (Xiao and Lu, 2014a, 2014b). Acetoin exists as (*R*)- and (*S*)-stereoisomers, both of which are potential drug intermediates. Optically pure acetoin has been widely used as the precursor for synthesizing novel optically active materials, including liquid crystal composites and α-hydroxyketone derivatives (Xin et al., 2016; Xu et al., 2015). (*R*)-acetoin is a female sex pheromone of *Amphimallon solstitiale* (L.), which attracts swarming males, whereas racemic acetoin was non-functional in this respect (Tolasch et al., 2003). Ethylene glycol (EG, ethane-1,2-diol) is a two-carbon platform chemical, primarily used as both an anti-freezing agent and building-block molecule for various polymers, including the ubiquitous polyethylene terephthalate (PET) (Yue et al., 2012). Both acetoin and EG have significant commercial importance, and green chemistry-based routes

Abbreviations: EG, ethylene glycol; PET, polyethylene terephthalate; ThDP, Thiamine diphosphate; FAD, flavin adenine dinucleotide; NAD$^+$, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; LB, lysogeny broth; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography; GC, gas chromatography; *ee*, enantiomeric excess; PP, pentose phosphate; EMP, Embden-Meyerhoff-Parnas

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Table 1
Primers used for gene cloning in this study.

Genes	Genomic origins	Primers	Primer sequences (5′→3′)
CC_0821	*C. crescentus* CB 2	Forward	GACGACGACAAGATGTCCTCAGCCATCTATCCCAGCCTG
		Reverse	GAGGAGAAGCCCGGTTAACGCCAGCCGGCGTCGATC
CC_0820	*C. crescentus* CB 2	Forward	GACGACGACAAGATGACCGCTCAAGTCACTTGCGTATGG
		Reverse	GAGGAGAAGCCCGGTTAGACAAGGCGGACCTCATG
yagF	*E. coli* W3110	Forward	GACGACGACAAGATGACCATTGAGAAAATTTTCACCCCGC
		Reverse	GAGGAGAAGCCCGGTTAAATTCCGAGCGCTTTTTTACCGGCG
yjhH	*E. coli* W3110	Forward	GACGACGACAAGATGAAAAAATTCAGCGGCATTATTCCAC
		Reverse	GAGGAGAAGCCCGGTTAGACTGGTAAAATGCCCTG
yagE	*E. coli* W3110	Forward	GACGACGACAAGATGCCGCAGTCCGCGTTGTTCACG
		Reverse	GAGGAGAAGCCCGGTTAGCAAAGCTTGAGCTGTTGCAGC
JW2770	*E. coli* W3110	Forward	GACGACGACAAGATGATGGCTAACAGAATGATTCTGAACG
		Reverse	GAGGAGAAGCCCGGTTACCAGGCGGTATGGTAAAGCTCTAC
CCNA_02185-Small	*C. crescentus* CB 2	Forward	GACGACGACAAGATGACCGGCGCCGAGATCGTGGTCCGC
		Reverse	GAGGAGAAGCCCGGTTAGACCAGCCCCGCGCCGTC
CCNA_02185-Large	*C. crescentus* CB 2	Forward	GACGACGACAAGATGACCGCCCACCAGACCATCGAGAGC
		Reverse	GAGGAGAAGCCCGGTTAGACCAGCCCCGCGCCGTC
CC_2101	*C. crescentus* CB 2	Forward	GACGACGACAAGATGACCGCCAACGTGCAACCCGCTCC
		Reverse	GAGGAGAAGCCCGGTTACATGCCTTCGAAGCCGCGCTC
BSU36000	*B. subtilis* shaijiu32	Forward	GACGACGACAAGATGAAACGMGAAAGCAAYATTCAAGTGCT
		Reverse	GAGGAGAAGCCCGGTTATTCMGGGCTTCCTTCRGTTGTTTC

to their production are potentially valuable (Pang et al., 2016; Werpy and Petersen, 2004).

Currently, commercially available acetoin is produced as a racemic mixture by chemical synthesis based on fossil-based feedstocks (Xiao and Lu, 2014a; Yue et al., 2012). Fermentation of biomass-derived sugars (hexoses and pentoses) can be used to produce racemic mixtures of acetoin and also generate EG (Pereira et al., 2016; Xiao and Lu, 2014b). In such fermentations, monosaccharides are first converted to pyruvate, before conversion to (*R*)-acetoin or EG (Xiao and Xu, 2007). In addition to acetoin or EG, the pyruvate generated can be subsequently channelled into other products, including 2,3-butanediol, acetate, lactate and ethanol, leading to extra recovery processes of products from fermented broths (Xiao and Lu, 2014b). Alternatively, enzyme or whole-cell biocatalysis for producing enantiomerically pure acetoin and EG production could take advantage of stereo-selectivity and high catalytic efficiency (Guo et al., 2016). As such, biocatalytic routes were reported for (*R*)-acetoin synthesis using the acetoin analogue 2,3-butanediol (Guo et al., 2016; Kochius et al., 2014; Xiao et al., 2010) or diacetyl (Heidlas and Tressl, 1990; Yu et al., 2015), as the precursor. However, biocatalytic routes have not been reported for production of (*R*)-acetoin and EG using monosaccharides as starting materials.

In vitro assembly of metabolic enzymes and coenzymes has been used to produce biofuels, specialty chemicals, biopharmaceuticals and biomaterials (Taniguchi et al., 2017; Zhang, 2015). In brief, biomanufacturing *via in vitro* metabolic engineering shows great plasticity in product yield, production rate, product purity, process control, and reaction optimization (Zhu and Zhang, 2015). In addition, enzyme-based biocatalysis, involving the necessary substrates and intermediates, has higher reaction selectivity under mild reaction conditions. So far, 'one-pot', *in vitro* biotransformations with balanced cofactors and ATP have been demonstrated for bio-hydrogen (Martín del Campo et al., 2013), D-xylulose 5-phosphate (Kim and Zhang, 2016), fructose 1,6-diphosphate (Wang et al., 2017), and bio-food (Qi et al., 2014; You et al., 2013) production. Here, a coenzyme-balanced pathway for the *in vitro*, direct, simultaneous conversion of D-xylose to optically pure (*R*)-acetoin and EG was constructed and optimized by assembling seven biocatalytic steps, without ATP requirements.

2. Materials and methods

2.1. Bacterial strains and plasmids

Strain *Caulobacter crescentus* CB 2 (DSMZ 4727) was obtained from DSMZ (Braunschweig, Germany). Wild type *Bacillus subtilis* shaijiu32 and *Escherichia coli* W3110 were obtained from laboratory stocks. *E. coli* strain Top10 used as the cloning host was obtained from TianGen (Beijing, China), and strain BL21(DE3) used as the expression host was purchased from Novagen (USA). Plasmid pET28b used as the overexpression vector was also obtained from Novagen.

2.2. Chemicals

Thiamine diphosphate (ThDP) and flavin adenine dinucleotide (FAD) were purchased from Aladdin (USA). Oxidized nicotinamide adenine dinucleotide (NAD^+) and reduced nicotinamide adenine dinucleotide (NADH, disodium salt) were obtained from Sigma-Aldrich (St. Luis, MO, USA). D-xylonic acid was purchased from Zhentang Biotechnology Co., Ltd. (Shandong, China). Glycolaldehyde dimer was available from Ark Pharm, Inc. (USA). D-xylose, (*S*/*R*)-acetoin (47.3% (*S*)-acetoin and 52.7% (*R*)-acetoin), and EG were purchased from Sinopharm Group Co., Ltd. (Beijing, China), unless stated otherwise. All of the chemicals used were of analytical grade.

2.3. Genomic DNA extraction and primers designing

C. crescentus CB 2 was cultured in DSMZ 595 medium containing bacto peptone 2.0 g, yeast extract 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.2 g in 1 L tap water (pH natural) at 30 °C. *B. subtilis* shaijiu32 and *E. coli* strains were cultured in lysogeny broth (LB) medium at 37 °C. Genomic DNA of *C. crescentus* CB 2, *B. subtilis* shaijiu32 and *E. coli* W3110 was extracted after overnight cultivation by using a TIANamp Bacteria DNA