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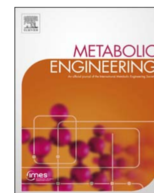

Microbial production of branched-chain dicarboxylate 2-methylsuccinic acid via enoate reductase-mediated bioreduction

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ABSTRACT

2-Methylsuccinic acid (2-MSA) is a C5 branched-chain dicarboxylate that serves as an attractive synthon for the synthesis of polymers with extensive applications in coatings, cosmetic solvents and bioplastics. However, the lack of natural pathways for 2-MSA biosynthesis has limited its application as a promising bio-replacement. Herein, we conceived a non-natural three-step biosynthetic route for 2-MSA, via employing the citramalate pathway in combination with enoate reductase-mediated bioreduction of the pathway intermediate citraconate. First, over-expression of codon-optimized citramalate synthase variant CimA* from *Methanococcus jannaschii*, endogenous isopropylmalate isomerase EcLeuCD and enoate reductase YqjM from *Bacillus subtilis* allowed the production of 2-MSA in *Escherichia coli* for the first time, with a titer of 0.35 g/L in shake flask experiments. Subsequent screening of YqjM-like enoate reductases of different bacterial origins enabled identification and characterization of a new NAD(P)H-dependent enoate reductase KpnER from *Klebsiella pneumoniae*, which exhibited higher activity towards citraconate than YqjM. Incorporation of KpnER into the 2-MSA biosynthetic pathway led to 2-MSA production improvement to a titer of 0.96 g/L in aerobic condition. Subsequent optimizations including cofactor regeneration, microaerobic cultivation and host strain engineering, boosted 2-MSA titer to 3.61 g/L with a molar yield of 0.36 in shake flask experiments. This work established a promising platform for 2-MSA bioproduction, which enabled the highest titer of 2-MSA production in microbial hosts so far.

1. Introduction

Dicarboxylic acids are important building blocks for the production of diols, polyurethanes, polyesters and polyamides with an ever-increasing market (Chung et al., 2015; Jambunathan and Zhang, 2014; Lee et al., 2011). The roaring interests in developing novel bioplastics have prompted microbial metabolic engineering for large-scale production of natural or non-natural dicarboxylic acids from renewable biomass, which is both sustainable and environmentally friendly (Lee et al., 2011). Current research mainly focused on the production of linear aliphatic dicarboxylic acids (C3-C10) such as succinate, glutarate and adipate (Chung et al., 2015; Deng et al., 2016; Park et al., 2013; Wang et al., 2017; Yu et al., 2014). Among those, only succinate production has achieved economically competitive productivities and yields in microbial hosts (Okino et al., 2008; Song and Lee, 2006). Recently, branched-chain or isomeric dicarboxylic acids like itaconate, mesaconate, malate and citramalate have drawn much more attention as alternative building block chemicals (Bai et al., 2016; Blazeck et al., 2015; Dong et al., 2016; Wang and Zhang, 2015; Wu and Eiteman, 2016). Itaconate has been recognized as one of the top 12 platform chemicals, serving as a precursor for value-added C5 chemicals like 2-methyl-1,4-butanediol (MBDO) and methyl-γ-butyrolactones (MGBLs) (Liu et al., 2016; Spanjers et al., 2016; Werpy et al., 2004).

2-Methylsuccinic acid (2-MSA) is a methylated succinate and a reduced product of itaconate that has broad applications as a chemical synthon in the pharmaceutical and polymer industries (Loos et al., 2013; Sortino et al., 2013; Xie et al., 2014; Zhou et al., 2016). It has been used as co-monomer with diols for the synthesis of biodegradable polyesters like poly (butylenes 2-methylsuccinate) (PBM), showing great applications for coatings and cosmetic solvents (Loos et al., 2013; Sonntag et al., 2014; Takasu et al., 2005; Xie et al., 2014). 2-MSA also serves as an alternative to itaconate for synthesis of C5 chemicals including MBDO and MGBLs. Generally, 2-MSA are produced from chemical reduction of itaconate by electro-catalytic valorization or chemo-

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catalytic hydrogenation via ruthenium-based catalysts, which however, are challenging owing to the costs of metal complexes and strict conditions like high temperature (Holzhäuser et al., 2017; Huang et al., 2015). Recently, the direct formation of 2-MSA from citric acid was successfully achieved via one-step dehydration, decarboxylation and hydrogenation, which also requires metal catalysts and harsh reaction conditions (Verduyckt and De Vos, 2017). Bio-based production of 2-MSA has been only achieved from ethylmalonyl-coenzyme A pathway (EMCP) that comprises seven enzymatic steps including expressing a heterologous thioesterase to convert methylsuccinyl-CoA into 2-MSA in methylotroph *Methylobacterium extorquens* AM1 (Sonntag et al., 2014, 2015). The efficiency of this CoA-dependent pathway was low and only 0.26 g/L 2-MSA was released from methylsuccinyl-CoA, which has to be significantly increased to allow a commercially attractive process (Sonntag et al., 2015).

Bioreduction of unsaturated C5 dicarboxylic acids including itaconate, mesaconate and citraconate by enoate reductases (ERs) might serve as potential pathways for 2-MSA (Guccione et al., 2010; Stueckler et al., 2007). The old yellow enzymes (OYEs) are a classic family of ERs that are flavin mononucleotide (FMN)-containing, NAD(P)H-dependent oxidoreductases. These OYE-like enzymes are capable of catalyzing the C=C bond reduction of α,β-unsaturated ketones, imides, nitroalkenes, aldehydes, carboxylic acids and their derivatives, affording hydrogenated products with a variety of industrial and pharmaceutical applications (Toogood et al., 2010). YqjM from *Bacillus subtilis* is one of the most extensively investigated OYE-like ER that has been utilized for bioreduction-based production of β-methyl-δ-valerolactone (βMδVL) and n-butanol (Reiße et al., 2016; Xiong et al., 2014). YqjM has been reported to display in vitro reduction activity towards citraconate, which is probably ascribed to its structural similarity to the ideal substrate N-ethylmaleimide (Fitzpatrick et al., 2003; Stueckler et al., 2007; Wardrope et al., 2006). Citraconate is an intermediate metabolite in the well-characterized citramalate pathway that serves as an alternative isoleucine pathway in most methanogenic archaea such as *M. jannaschii* and certain bacteria such as *Leptospira interrogans* (Atsumi and Liao, 2008; Drevland et al., 2007; Shen and Liao, 2013). These imply the feasibility of establishing the ER-mediated reduction of citraconate for bio-production of 2-MSA.

Herein, to explore the possibility of using enoate reductase for 2-MSA production, we assembled a novel CoA-independent and short-cut 2-MSA biosynthetic pathway via harnessing *Escherichia coli* as a biological chassis. First, we established and validated the total biosynthetic pathway for 2-MSA mainly by introducing the optimized (R)-citramalate pathway and enoate reductase YqjM. Then we identified and characterized a superior YqjM-like enoate reductase from *Klebsiella pneumoniae* that enabled the improved production of 2-MSA. Final incorporation of a NADH regeneration system, cultivation optimization in microaerobic condition and host engineering permitted 2-MSA titer enhancement to 3.61 g/L in shake flask experiments. This work constituted and demonstrated a novel biosynthetic pathway for 2-MSA and achieved the highest bioproduction of 2-MSA so far, which might potentially serve as an industrial platform for bio-based synthesis of 2-MSA from renewable carbon sources.

2. Methods and materials

2.1. Strains, plasmids and chemicals

All bacterial strains and plasmids used in this study are summarized in Table 1. *E. coli* strain XL-1 Blue (Stratagene) was used as the host for standard DNA cloning, BL21 Star (DE3) (Invitrogen) for protein expression and BW25113 (F′) for 2-MSA production, respectively. *E. coli* strain BW25113 (F′) is a BW25113 (*rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78*) derivative strain with F′ plasmid transduced from *E. coli* XL-1 Blue to supply $lacI_q$. *E. coli* BW25113 (F′) *ΔadhEΔldhAΔfrdBCΔfnrΔpta* was created by deleting *adhE*, *ldhA*, *frdBC*, *fnr* and *pta* (Atsumi et al., 2008). Compatible plasmids pZE12-luc (high-copy number) and pCS27 (medium-copy number) were used for the construction of 2-MSA biosynthetic pathway. Plasmid pETDuet-1 was used for gene expression and purification. Standard chemicals including (*R*)-citramalate, citraconate and 2-MSA were purchased from Sigma-Aldrich.

2.2. Plasmid construction

All DNA manipulations were carried out following standard molecular cloning protocols (Sambrook et al., 1989). Phusion high-fidelity DNA polymerase (New England Biolabs) was used for gene amplification and Quick ligase kit (New England Biolabs) was used for gene ligation. To construct pZE12-luc plasmids containing YqjM-like enoate reductase genes, *nemA* (NP_416167) from *E. coli*, *xenA* (AAN66878) from *Pseudomonas putida* and *reuER* (CAJ96943) from *Ralstonia eutropha* were respectively constructed into pZE12-luc between *Acc65*I/*Xba*I sites. Plasmids pZE12-*yqjM*, pZE12-*lmoER*, pZE12-*cglER*, pZE12-*kpnER*, pZE12-*oye2* and pZE12-*oye3* were constructed in our previous work (Sun et al., 2016). For the purification purpose, *yqjM* and *kpnER* were cloned into pETDuet-1 between *Bam*HI/*Sal*I sites, obtaining pETDuet-*yqjM* and pETDuet-*kpnER*. pZE-*cimA**-*EcleuCD* was obtained by amplifying *cimA** from pZE-*cimA** and *EcleuCD* from *E. coli*, and inserting them into pZE12-luc by *Acc65*I/*Sal*I and *Sal*I/*Xba*I sites. To construct pZE-*cimA**-*EcleuCD-yqjM* and pZE-*cimA**-*EcleuCD-kpnER*, the cassettes P_L*lacO1-yqjM* and P_L*lacO1-kpnER* were respectively amplified from pZE12-*yqjM* and pZE12-*kpnER* and inserted into pZE-*cimA**-*EcleuCD* by *Spe*I/*Sac*I sites as independent operons. pZE-*cimA**-*MjleuCD-kpnER*, pZE-*cimA**-*VfleuCD-kpnER* and pZE-*cimA**-*Scleu1-kpnER* were constructed by amplifying *MjleuCD* from *Methanococcus jannaschii*, *VfleuCD* from *Vibrio fischeri* and *Scleu1* from *Saccharomyces cerevisiae* and replacing *EcleuCD* in pZE-*cimA**-*EcleuCD-kpnER* by *Sal*I/*Xba*I sites. pCS-*EcleuCD* was constructed by amplifying *EcleuCD* from *E. coli* and inserting into pCS27 between *Acc65*I and *Bam*HI. pCS27 containing a formate dehydrogenase gene from *Candida boidinii* (pCS-*fdh*) was obtained from our previous work (Jain et al., 2014). Plasmid pCS-*EcleuCD-fdh* was obtained by amplifying the cassette P_L*lacO1-fdh* from pCS-*fdh* and inserting into pCS-*EcleuCD* between *Spe*I and *Sac*I sites as an independent operon. All plasmids involved were listed in Table 1.

2.3. Culture media and conditions

Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) was used for cell propagation. 20 mL M9 minimal medium (20 g/L glucose, 6 g/L Na_2HPO_4, 0.5 g/L NaCl, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 g/L yeast extract) was used for shake flask experiments in 125-mL shake flasks. Specifically, sodium formate was added to the M9 minimal medium if needed with a final concentration of 50 mM. When necessary, ampicillin and kanamycin were added to the medium at 100 and 50 µg/mL, respectively.

2.4. Protein expression, purification and in vitro enzyme assays

To express and purify enoate reductases, *E. coli* BL21 Star (DE3) was transformed with pETDuet-*yqjM* or pETDuet-*kpnER*. Single colonies were inoculated in 3 mL LB tubes at 37 °C. 500 µL of the overnight cultures were transferred to 50 mL fresh LB and grown for 2.5–3 h at 37 °C. When the cell optical density at 600 nm (OD_{600}) of cultures reached 0.6–0.8, the cells were induced by isopropyl-β-D-1-thiogalactopyranoside (IPTG) with a final concentration of 0.5 mM and incubated at 30 °C on a rotary shaker for 9 h. The cultures were centrifuged at 10,000 rpm for 10 min to collect the cell pellets and the cells were lysed using Mini Bead Beater (Biospec). Protein purification was performed using His-Spin Protein Miniprep Kit (Zymo Research, Irvine, CA) following manufactures' instructions. The purified protein was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using

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