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UNLOCKING GLYCEROL'S POTENTIAL: METABOLIC ENGINEERING FOR MUCONIC ACID BIOSYNTHESIS IN *E. coli*

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ABSTRACT

Muconic acid (MA) has been considered a bioprivileged molecule that can be converted into commercial chemicals, such as adipic acid and terephthalic acid, that are precursors of several valuable consumer bio-plastics including nylon and PET, indispensable for a modern society, and which are traditionally obtained by petrochemical routes. First, a parental *E. coli* strain was developed to enable the heterologous production of muconic acid through 3-dehydroshikimate (3-DHS) with heterologous expression of 3-DHS dehydrogenase from *Bacillus thuringiensis*, protocatechuate decarboxylase from *Enterobacter cloacae*, and catechol 1,2-dioxygenase from *Acinetobacter radioresistens*. Further a set of 8 genetic modifications were implemented into the genome of *E. coli* W to increase flux into shikimate pathway and increase intermediate biosynthesis. Final engineered strain was able to produce 1.30 g L^{-1} of muconic acid in 96 hours of small-scale cultivations, which represents a more than 3.5-fold increase in MA titer compared to the parental strain.

Keywords: Metabolic Engineering, Muconic Acid, Glycerol, Shikimate pathway, *Escherichia coli*

1 INTRODUCTION

Organic acids are versatile building block chemicals employed in a myriad of biotechnological processes, such as in the food/ beverage, cosmetics, pharmaceutical, polymer and textile industries. In particular, Muconic Acid (MA), considered a C6 bioprivileged molecule with conjugated double bonds and reactive dicarboxylic groups, has been attracting interest for its application as an intermediate for the synthesis of bulk chemicals, such as adipic acid, terephthalic acid, and trimellitic acid. These bulk chemicals have a wide variety of uses in the manufacture of commercially important bioplastics currently produced in the petrochemical industry, including nylon, terephthalic polyethylene (PET), resins, polyester polyols, and recently enabling bio-nylon and bio-PET[1]. In addition, recent studies show the potential for converting MA into new molecules, such as 3-hexenedioc and 2-hexenedioc acids, which can result in polymers with enhanced or novel properties that could not be effectively synthesized from petrochemical building blocks[2].

The bioconversion of renewable sugars and lignin-derived aromatic compounds into MA using efficient microbial platforms has been considered a promising cost-competitive and more sustainable alternative for large-scale industrial MA production. However, despite the advances already achieved, low TRY metrics (titer, yield and productivity), as well the accumulation of by-products, are still challenges that need to be overcome to enable the efficient production of MA by biological route.

In silico studies conducted by our research group indicated that the metabolic pathway derived from the natural intermediate 3-dehydroshikimate (DHS-to-MA pathway) presents the highest maximum theoretical yield of MA. This pathway is composed of three heterologous reactions: (1) conversion of DHS into protocatechuate (PCA); (2) conversion of PCA to catechol and (3) conversion of catechol to MA, catalyzed by DHS dehydrogenase (*aroZ* gene), PCA decarboxylase (*aroY* gene) and catechol 1,2-dioxygenase (*catA* gene), respectively.

In this study, *Escherichia coli* W was engineered to convert glycerol, an important biodiesel-derived waste, into muconic acid using a model-guided strategy that pre-selected the metabolic pathway with maximum theoretical MA yield (DHS-to-MA pathway) and predicted potential gene targets for overexpression and deletion to maximize MA production. The redesigned strain was able to produce 1.30 g L-1 of muconic acid after 96 hours in small-scale cultivations, which represents a more than 3.5-fold increase in MA titer compared to the parental strain.

2 MATERIAL & METHODS

Genes of the heterologous MA pathway encoding DHS dehydratase (*aroZ*) from *Bacillus thuringiensis*, PCA decarboxylase (*aroY*) from *Enterobacter cloacae*, and catechol-1,2-dioxygenase (*catA*) from *Acinetobacter radioresistens* were codon-optimized for

expression in *E. coli* and synthesized by GeneScript. Both genes were inserted between the *Nde*I and *Xho*I sites of pRSM6 vector, a high copy number plasmid from ePathBrick[3], under the control of the *lacUV5* promoter and assembled in a pseudo-operon configuration, resulting in the parental strain.

Deletion of *aroE*, *tyrR*, *trpR*, *pykA* and *pykF* genes, mutation of *aroG*^{P150L}, and overexpression of native *aroB* and heterologous overexpression of *aroD* from *Enterococcus faecalis* were performed using the Scarless Cas9-Assisted Recombineering (no-SCAR) system[4]. The method is based on the use of two plasmids: pCas9-cr4 (Addgene #62655) and pKDsg-p15 (Addgene #626566). The overexpression cassette of *aroB* and *aroD* genes were first assembled using the ePathBrick system and then subcloned between two 600-bp homology arms for integration in the *lacI* locus. The donor DNA for integration was obtained by PCR of this construct.

The effect of each genetic modification on muconic acid production was evaluated in small-scale cultivations performed in a 96-well plate setup in the Growth Profiler 960 (EnzyScreen, Netherlands). Growth was monitored for 96 hours at 37°C and 220 rpm, in triplicate. MR modified medium with supplementation of 20 g/L of glycerol was used for muconic acid production[5]. The heterologous pathway was induced by 1 mM IPTG after the culture reached an OD of 0.6. Glycerol, acetic acid, muconic acid, catechol, protocatechuate (PCA) and 3-dehydroshikimate (DHS) were quantified by HPLC.

3 RESULTS & DISCUSSION

In order to enhance MA production by *E. coli* W, the three codon-optimized heterologous genes required for DHS-to-MA pathway, *aroZ*, *aroY*, and *catA*, were cloned into a high copy number plasmid, pRSM6 from ePathBrick system, resulting in the parental strain.

In addition, a set of 8 genetic modifications were implemented into the genome of *E. coli* W to direct carbon flux into shikimate pathway, as well as to increase the biosynthesis of DHS and phosphoenolpyruvate (PEP), key precursors. **Figure 1A** shows an overview of the metabolic pathways for muconic acid production in *E. coli* and highlights all the genetic modifications evaluated in this study. First, *aroE* gene, encoding a shikimate dehydrogenase, was disrupted to prevent the conversion of DHS to shikimate. Next, *aroG* (encoding phospho-2-dehydro-3-deoxyheptonate aldolase) was mutated to increase the production of DHS, in order to avoid affecting cell growth. Two transcriptional regulators, *tyrR* (tyrosine dependent transcriptional regulator) and *trpR* (tryptophan repressor), which are mainly responsible for regulating the shikimate pathway in *E. coli*, were deleted to increase DHS level. Genes encoding pyruvate kinases, *pykA, pykF*, were also deleted to reduce competition for PEP. Finally, *aroB* (encoding 3-dehydroquinate (DHQ) synthease) and *aroD* (enconding DHQ dehydratase) were overexpressed to increase DHS production.

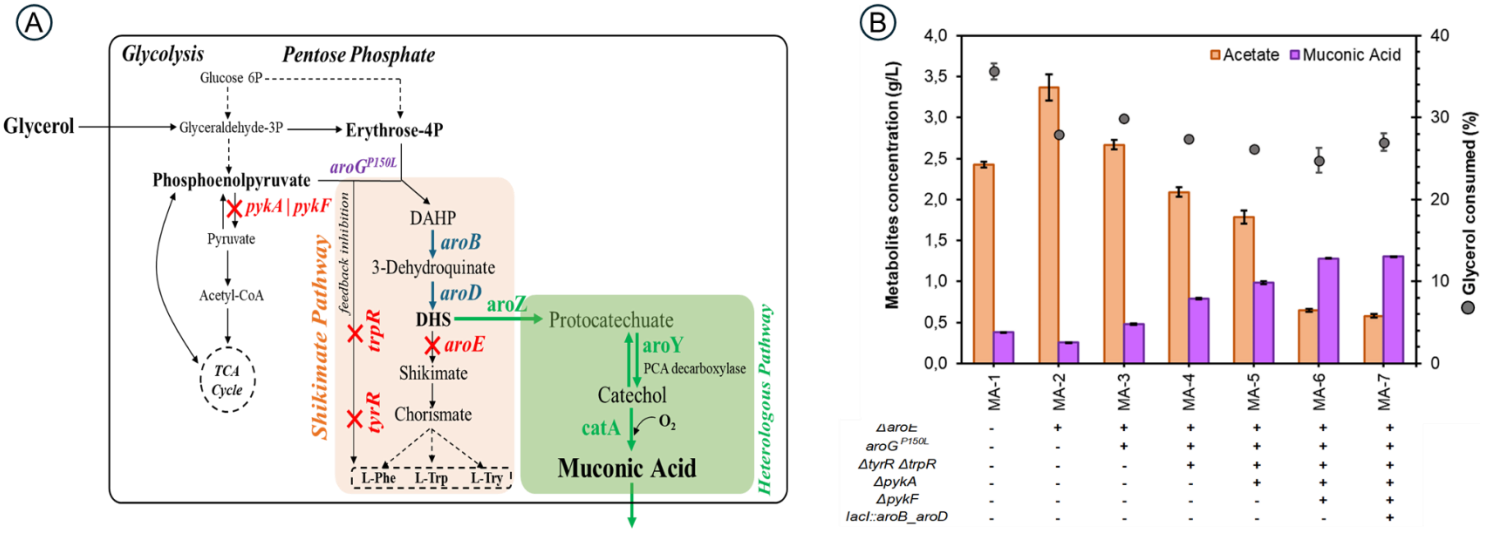

Figure 1 *– A) DHS-to-MA pathway for muconic acid production in E. coli W and the metabolic engineering strategies implemented in this study. Red crosses represent deletions, blue arrows gene overexpression, and the aroG gene mutation is highlighted in purple DAHP: 3-Deoxy-7-Phospho-Heptulonate; DHS: 3-Dehydrochiquimate; Phe: Phenylalanine; Trp: Tryptophan; Try: Tyrosine. B) Muconic acid and acetic acid production and glycerol consumption of engineered strains in 96 hours of small-scale cultivations using 20 g/L of glycerol as carbon source. Error bars represent standard deviations based on triplicate experiments.*

Muconic acid levels produced by the strains constructed in this work are shown in **Figure 1B**. The progress in genetic modifications significantly improved the production of muconic acid by developed strains. MA titer ranged from 0.25 ± 0.01 (MA-2) to 1.30 ± 0.01 g/L (MA-7) in the engineered strains, whereas the parental strain, harboring only MA heterologous pathway, produced 0.38 ± 0.01 (MA-1) in 96 hours of small-scale cultivations from 20 g/L of glycerol as carbon source. PCA and catechol were not detected in any evaluated condition. DHS accumulated in minor amounts in MA-5 to MA-7 (0.02 – 0.03 g/L of DHS). It is interesting to note that the rounds of genetic engineering not only increased the production of the product of interest, but also made it possible to reduce the production of undesired acetic acid.

Despite the low consumption of glycerol (**Figure 1B**), the MA yield of final strain was 0.06 g MA/g glycerol supplied, which is comparable to the most promising results published to date. In comparison to the parental strain, the MA titer and yield were improved by around 3.5 times.

4 CONCLUSION

In this work, we demonstrated an effective strategy for constructing muconic acid-producing strains using glycerol, an important biodiesel-derived waste, as a potential renewable feedstock.

The set of 8 genetic modifications implemented into the genome of *E. coli* W, including deletion of *aroE*, *tyrR*, *trpR*, *pykA and pykF* genes, mutation of *aroG* and overexpression of *aroB* and *aroD* genes, associated with an efficient heterologous muconic acid pathway enabled the production of 1.30 g/L of muconic acid in 96 hours of small-scale cultivations. Almost no accumulations of metabolic intermediates such as DHS, PCA, and catechol was observed. In comparison to the parental strain, the MA titer and yield were improved by around 3.5 times.

To develop a greener, more cost-effective, and sustainable MA production process, bioprocess optimization studies will be conducted to improve cell growth and glycerol consumption, and consequently maximize the production of muconic acid.

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