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Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae*

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ABSTRACT

The dicarboxylic acid muconic acid has garnered significant interest due to its potential use as a platform chemical for the production of several valuable consumer bio-plastics including nylon-6,6 and polyurethane (via an adipic acid intermediate) and polyethylene terephthalate (PET) (via a terephthalic acid intermediate). Many process advantages (including lower pH levels) support the production of this molecule in yeast. Here, we present the first heterologous production of muconic acid in the yeast *Saccharomyces cerevisiae*. A three-step synthetic, composite pathway comprised of the enzymes dehydroshikimate dehydratase from *Podospora anserina*, protocatechuic acid decarboxylase from *Enterobacter cloacae*, and catechol 1,2-dioxygenase from *Candida albicans* was imported into yeast. Further genetic modifications guided by metabolic modeling and feedback inhibition mitigation were introduced to increase precursor availability. Specifically, the knockout of *ARO3* and overexpression of a feedback-resistant mutant of *aro4* reduced feedback inhibition in the shikimate pathway, and the *zwf1* deletion and over-expression of *TKL1* increased flux of necessary precursors into the pathway. Further balancing of the heterologous enzyme levels led to a final titer of nearly 141 mg/L muconic acid in a shake-flask culture, a value nearly 24-fold higher than the initial strain. Moreover, this strain has the highest titer and second highest yield of any reported shikimate and aromatic amino acid-based molecule in yeast in a simple batch condition. This work collectively demonstrates that yeast has the potential to be a platform for the bioproduction of muconic acid and suggests an area that is ripe for future metabolic engineering efforts.

1. Introduction

Worldwide pressures to reduce petroleum footprints have increased interest in alternative, renewable methods to produce nearly all commodity and specialty chemicals. To this end, the field of metabolic engineering has begun to answer this demand through the development of organisms that can produce an increasingly diverse array of chemicals (Curran and Alper, 2012; de Jong et al., 2012; Jang et al., 2012; Lee et al., 2011, 2012). In particular, bio-plastics have become an especially potent area as demonstrated by the metabolic engineering of strains for production of precursors such as succinic acid, ethylene glycol (from bio-ethanol), 1,3-propanediol, 1,4-butanediol, ρ-hydroxystyrene, styrene, as well as the development of novel bio-plastics such as polylactides and polyhydroxyalkanoates (Antoniewicz et al., 2007; Ikushima et al., 2009; Li et al., 2010; McKenna and Nielsen, 2011; Qi et al., 2007; Stols and Donnelly, 1997; Yim et al., 2011; Yumoto and Ikeda, 1995; Zhou et al., 2011, 2005). Beyond this list, muconic acid serves as another interesting precursor and platform chemical for producing several bio-plastics. Muconic acid is easily converted via hydrogenation into adipic acid, a chemical used to produce nylon-6,6 and polyurethanes. Additionally, muconic acid can be converted via the Diels–Alder reaction with acetylene and subsequent oxidation into terephthalic acid, one of two primary constituents in the plastic polyethylene terephthalate (PET). Terephthalic acid is also used in the production of polyester. World production of adipic acid and terephthalic acid is over 2.8 and 71 million tonnes, respectively (Burridge, 2011; Mirasol, 2011). At present, both of these chemicals are primarily produced from non-renewable petroleum feedstock and toxic intermediates, thus warranting a sustainable, biosynthetic production platform.

Muconic acid is not endogenously produced from carbohydrates by any known organism. However, muconic acid can be found during the catabolism and detoxification of aromatic compounds by some organisms, including yeast such as *Candida sp.*, and bacteria such as *Acinetobacter sp.*, *Rhodococcus sp.*, and

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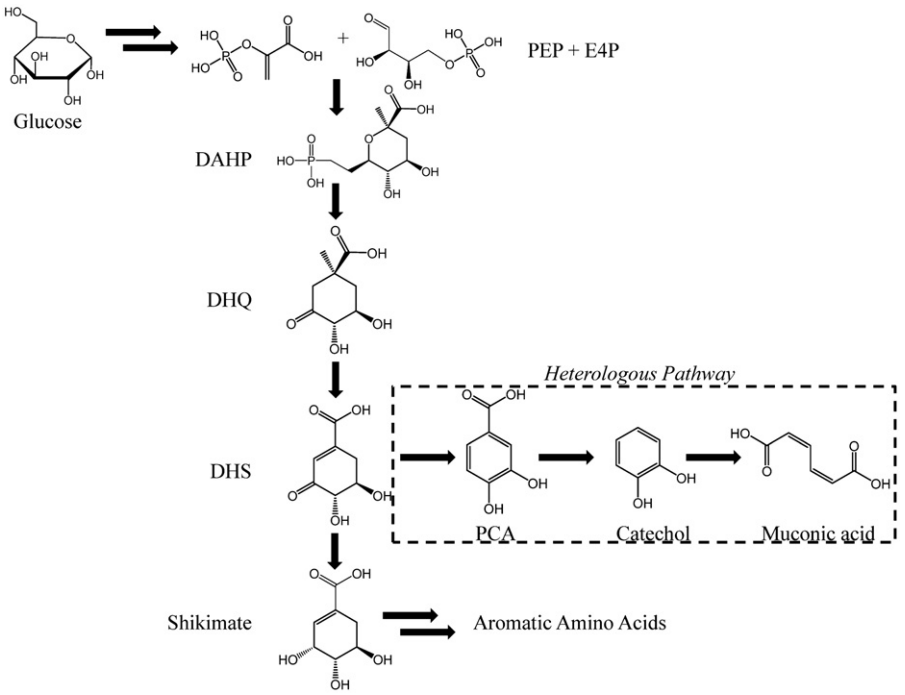

Fig. 1. *Composite heterologous pathway for muconic acid production*. The synthetic pathway for muconic acid is depicted in the context of the shikimate pathway in yeast. The following metabolite abbreviations are used: PEP is phosphoenolpyruvate, E4P is erythrose-4-phosphate, DAHP is 3-deoxy-D-arabinoheptulosonate-7-phosphate, DHQ is dehydroquinate, DHS is dehydroshikimate, and PCA is protocatechuic acid.

Sphingobacterium sp., among others (Neidle et al., 1988; Tsai et al., 2005; Warhurst et al., 1994; Wu et al., 2004). Previously, Draths and Frost engineered a recombinant *Escherichia coli* to produce muconic acid from glucose via a heterologous synthetic pathway drawing from a naturally occurring intermediate in the shikimate pathway, 3-dehydroshikimate (DHS) (Draths and Frost, 1994; Niu et al., 2002). In this synthetic, composite pathway, DHS is converted to protocatechuic acid (PCA) via a DHS dehydratase cloned from *Klebsiella pnemoniae*, PCA is then converted to catechol via a PCA decarboxylase from *K. pnemoniae*, and finally catechol is converted to *cis*,*cis*-muconic acid via a catechol 1,2-dioxygenase from *Acinetobacter baylyi* (Fig. 1). This pathway along with some minor modifications of metabolism enabled the production of muconic acid in *E. coli*.

Many industrial biotechnological processes are moving toward using yeasts as platform organisms due to their many advantages. The yeast *Saccharomyces cerevisiae* is an ideal host organism for industrial chemical production because it offers advantages including withstanding lower temperatures, easier separations, no phage contaminations, suitability in large-scale fermentation, lower pH fermentations, and generally higher tolerances. *S. cerevisiae* has been explored as a host for producing heterologous models that utilize precursors in the shikimate and aromatic amino acid pathways such as vanillin, ρ-hydroxybenzonic acid, ρ-amino benzoic acid, ρ-hydroxycinnamic acid, resveratrol and naringenin (Hansen et al., 2009; Jiang et al., 2005; Krömer et al., 2012; Naesby et al., 2009; Vannelli et al., 2007; Wang et al., 2011; Wang and Yu, 2012). These examples and advantages raise the possibility of using yeast as a platform for the production of muconic acid. Additionally, *S. cerevisiae* naturally prefers a lower pH environment than *E. coli*, a condition better suited for producing a di-acid. Here, we present the first reported production of muconic acid in the yeast *S. cerevisiae*. Through a series of strain modifications, yields were increased more than 20-fold from the initial parental strain and resulted in the highest titer of an aromatic-based molecule in a yeast shake-flask (over 140 mg/L) and among the highest yields.

2. Materials and methods

2.1. Strains and media

S. cerevisiae strain BY4741 (*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) was used as the host strain in this study (obtained from EUROSCARF). Yeast strains were routinely propagated at 30 °C in Yeast Extract Peptone Dextrose (YPD) medium, yeast synthetic complete (YSC) medium, or yeast synthetic minimal (YSM) medium. YPD medium is composed of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. YSC medium is composed of 6.7 g/L yeast nitrogen base, 20 g/L glucose, and either CSM-Ura, CSM-His, CSM-Leu, CSM-Ura-His, CSM-His-Leu, CSM-Ura-Leu, or CSM-Ura-His-Leu (MP Biomedicals, Solon, OH), depending on the required auxotrophic selection. YSM medium is composed of 6.7 g/L yeast nitrogen base, 20 g/L glucose, 20 mg/L methionine, and 10 mg/L adenine. *E. coli* strain *DH10β* was used for all cloning and plasmid propagation. *DH10β* was grown at 37 °C in Luria–Bertani (LB) broth supplemented with 50 μg/mL of ampicillin. All strains were cultivated with 225 RPM orbital shaking. Yeast and bacterial strains were stored at −80 °C in 15% glycerol.

2.2. Plasmid construction

Standard cloning and bacterial transformations were performed according to Sambrook and Russell (Sambrook, 2000). PCR reactions used Phusion High-Fidelity DNA Polymerase from New England Biolabs (Ipswich, MA) and followed supplier instructions; primers were purchased from Integrated DNA Technologies (Coralville, IO). Antarctic phosphatase and all restriction enzymes were purchased from New England Biolabs (Ipswich,