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Bioproduction of a betalain color palette in *Saccharomyces cerevisiae*

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ABSTRACT

Betalains are a family of natural pigments found exclusively in the plant order *Caryophyllales*. All members of this chemical family are biosynthesized through the common intermediate betalamic acid, which is capable of spontaneously condensing with various primary and secondary amines to produce betalains. Of particular interest is the red-violet betanin, most commonly obtained from *Beta vulgaris* (beet) as a natural food dye. We demonstrate the first complete microbial production of betanin in *Saccharomyces cerevisiae* from glucose, an early step towards a fermentation process enabling rapid, on-demand production of this natural dye. A titer of 17 mg/L was achieved, corresponding to a color intensity obtained from 10 g/L of beetroot extract. Further, we expanded the spectrum of betalain colors by condensing betalamic acid with various amines fed to an engineered strain of *S. cerevisiae*. Our work establishes a platform for microbial production of betalains of various colors as a potential alternative to land- and resource-intensive agricultural production.

1. Introduction

Dyes improve the desirability of food and provide a visual cue of freshness (Esatbeyoglu et al., 2015). A substantial fraction of the currently approved colorants on the market are chemically synthesized from petroleum (Downham and Collins, 2000; König, 2015); however, there is growing demand for natural pigments as consumers become increasingly concerned with synthetic additives in their diet as well as the sustainability of product supply chains (Downham and Collins, 2000; Esatbeyoglu et al., 2015). Plant cultivation for the extraction of natural dyes has been considered a promising solution; however, the seasonal nature of harvests and the use of arable lands for non-essential foodstuffs is not ideal (Marienhagen and Bott, 2013; Neelwarne, 2012). Plant-cell culture systems have been proposed as an alternative production platform, but these systems will be difficult to scale and can lack genetic tractability (Marienhagen and Bott, 2013; Neelwarne, 2012). Microbial metabolic engineering has the potential to address these concerns and limitations. Specifically, genetically tractable production hosts such as *Saccharomyces cerevisiae* (baker's yeast) can be engineered with heterologous biochemical pathways and rapidly optimized for high production titers (Marienhagen and Bott, 2013).

One clade of useful natural pigments are the betalains, a set of tyrosine-derived compounds exclusively restricted to the *Caryophyllales* order of plants (Brockington et al., 2011). Betalains are divided into the yellow-orange betaxanthins and red-violet betacyanins; of the betalains, betanin is the most utilized in commercial applications (Khan and Giridhar, 2015). Betanin has applications in a variety of short shelf-life foodstuffs, cosmetics, and pharmaceuticals owing to several favorable properties: high water solubility, robust stability and color intensity over a broad range of neutral and acidic conditions (pH 3–7), lack of intrinsic flavor, high extinction coefficient compared to most artificial red dyes, and stability to certain forms of pasteurization in high-sugar solutions (Esatbeyoglu et al., 2015; Hendry and Houghton, 1996; Neelwarne, 2012). Betanin is principally obtained *via* specialized cultivars of *Beta vulgaris* (beet) and sold in the form of "beetroot extract" (E number 162) at price points that can reach $100 per kg of extract (Frost & Sullivan, 2007).

As illustrated in Fig. 1, all betalains share a common betalamic acid chromophore. This central molecule is formed from tyrosine *via* two enzymatic reactions. First, the monophenolase activity of various P450s (of the CYP76AD clade in *B. vulgaris*) generates L-3,4-dihydroxyphenylalanine (L-DOPA) from L-tyrosine (Sunnadeniya et al., 2016). Then, DOPA-4,5-dioxygenase (DOD) catalyzes the ring cleavage at the catechol moiety of L-DOPA allowing subsequent spontaneous

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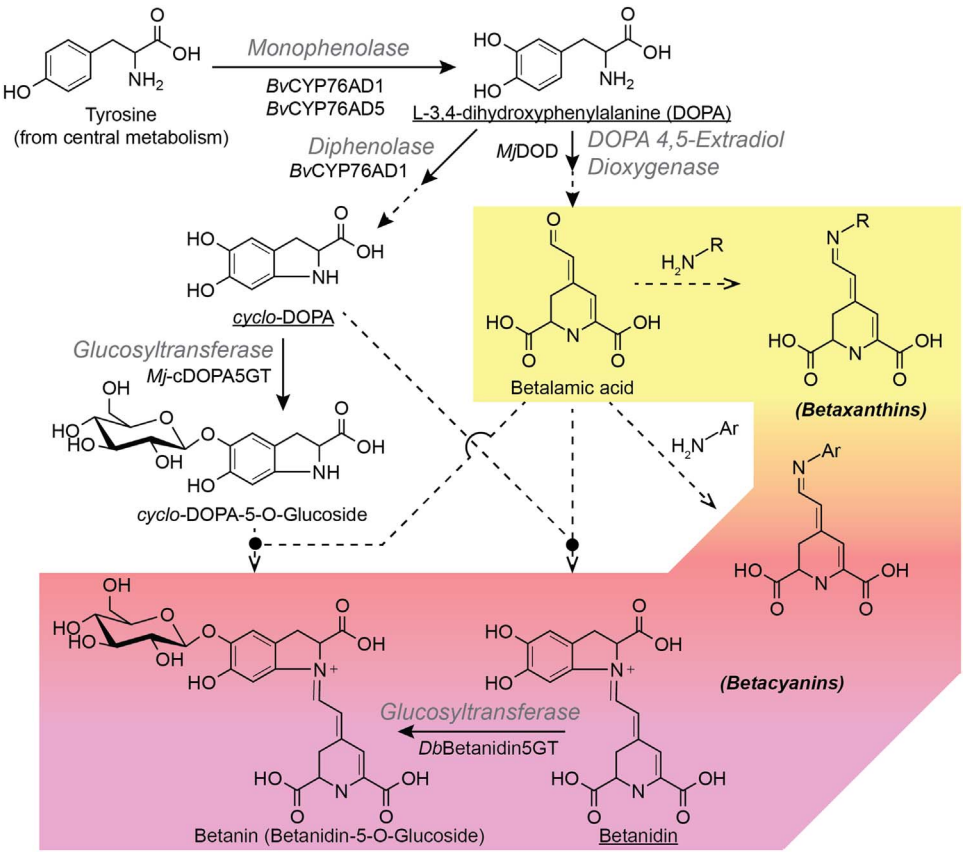

Fig. 1. Betalain biosynthesis. Diagram of the biosynthetic pathway with general enzyme activities in gray and the specific recombinant genes encoding these activities used in this work indicated in black. Solid lines are enzymatic reactions and dashed lines indicate spontaneous reactions. Unstable compounds in absence of reducing agent have their names underlined. Abbreviations: R = any organic chemical; Ar = any organic aromatic chemical.

cyclization with the alpha-amino group to form betalamic acid (Christinet, 2004). Betalamic acid spontaneously undergoes a Schiff-base condensation with free primary or secondary amines *via* its reactive aldehyde group to produce betalains possessing yellow to violet color (Schliemann et al., 1999).

Biosynthesis of the red-violet betanin requires two additional enzymatic activities that supplement the betalamic acid pathway. The first step is an enzymatic oxidation of L-DOPA to form dopaquinone, which spontaneously cyclizes into *cyclo*-DOPA. In *B. vulgaris*, CYP76AD1 is the sole enzyme capable of providing this additional diphenolase activity necessary to produce *cyclo*-DOPA (Hatlestad et al., 2012). Next, the condensation of *cyclo*-DOPA with betalamic acid results in the unstable, red-violet intermediate betanidin. A second enzymatic reaction, glucosylation, produces the dramatically more stable betanin (betanidin 5-*O*-beta-glucoside) pigment. Alternatively, the order of condensation and glucosylation can be reversed: *cyclo*-DOPA can be preemptively glucosylated prior to condensation with betalamic acid (Khan and Giridhar, 2015). The reaction order differs among plant species. For example, *Mirabilis jalapa* (four o'clock flower) produces the *cyclo*-DOPA-5-*O*-glucoside (Sasaki, 2005), whereas *Dorotheanthus bellidiformis* (Livingstone daisy) utilizes a betanidin glucosyltransferase (Vogt et al., 1999). Although specially bred cultivars of *B. vulgaris* are currently the predominant source of betanin (Khan and Giridhar, 2015), the putative glucosyltransferase (*Bv*GT) for betanin biosynthesis in beets (Sepulveda-Jimenez, 2005) has yet to be definitively confirmed by enzymatic assays.

In addition to the food dye betanin, applications of other betalains have been proposed. For example, the yellow betaxanthins have been proposed as replacements for artificial yellow dyes (Martins et al., 2017). Betalains have also shown applicability as photocell sensitizers, spectrofluorometric probes, and medical diagnostic reagents (Gonçalves et al., 2013a, 2013b; Khairy et al., 2016; Zhang et al., 2008). One such chemical is the condensation product of betalamic acid with 7-amino-4-methylcoumarin that has reported utility as a live-cell imaging probe for *Plasmodium*-infected erythrocytes (Gonçalves et al., 2013b). Such results highlight how derivatization with betalamic acid can be used to tune molecular properties and lead to value-added compounds. Although previously studied amines have yielded yellow, orange, and violet pigments upon condensation with betalamic acid (Gandía-Herrero et al., 2010, 2006; Gonçalves et al., 2013b; Khan and Giridhar, 2015), it is unclear what range of spectral and physical properties can be obtained from "designer" betalains.

To our knowledge, we herein provide the first description of betanin production from glucose in a heterologous microbial host. Additionally, we demonstrate that a heterologous system can be used to obtain novel betalain derivatives directly from yeast culture by feeding diverse amines. These results have implications for the fermentative production of natural colorants and further expand the spectrum of betalain colors obtainable *via* amine feeding.

2. Materials and methods

2.1. Chemicals and quantification

Amines used for *in vitro* and *in vivo* feeding were obtained from the following sources: L-DOPA (D9628, Sigma Aldrich), leucine (E811-100G, Amresco), para-aminobenzoic acid (100536-250G, Sigma Aldrich), anthranilic acid (A89855 Sigma Aldrich), 6-aminoindole (018336, Matrix Scientific), and o-dianisidine (01936, Chem-Impex International). Ascorbic acid was obtained from Gibco (13080-023) and iron(II) sulfate heptahydrate was obtained from Sigma Aldrich (215422).

Because pure betanin molecule is not commercially available, we used a > 98% pure beetroot extract (A10132, AdooQ Bioscience) for the majority of experiments, in conjunction with beetroot extract diluted in dextrin (B0397, TCI). Using the Beer-Lambert law and a