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Heterologous production of pentane in the oleaginous yeast *Yarrowia lipolytica*

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ABSTRACT

The complete biosynthetic replacement of petroleum transportation fuels requires a metabolic pathway capable of producing short chain *n*-alkanes. Here, we report and characterize a proof-of-concept pathway that enables microbial production of the C_5 *n*-alkane, pentane. This pathway utilizes a soybean lipoxygenase enzyme to cleave linoleic acid to pentane and a tridecadienoic acid byproduct. Initial expression of the soybean lipoxygenase enzyme within a *Yarrowia lipolytica* host yielded 1.56 mg/L pentane. Efforts to improve pentane yield by increasing substrate availability and strongly overexpressing the lipoxygenase enzyme successfully increased pentane production three-fold to 4.98 mg/L. This work represents the first-ever microbial production of pentane and demonstrates that short chain *n*-alkane synthesis is conceivable in model cellular hosts. In this regard, we demonstrate the potential pliability of *Y. lipolytica* toward the biosynthetic production of value-added molecules from its generous fatty acid reserves.

1. Introduction

The complete replacement of petroleum-derived liquid transportation fuels can be achieved in one of two means: by using an alternative, drop-in fuel molecule (such as an alcohol) or by recreating the major constituents of gasoline in a renewable manner. Gasoline and jet fuel are complex, variable mixtures of hydrocarbons and other additives that contain large quantities of short and long-chain *n*-alkanes (Faroon et al., 1995; Harper and Liccione, 1995; IARC, 1989; Lu, 1989; Risher et al., 1998; Watkins and Krukonis, 1991). To this end, biosynthetic hydrocarbon production in engineered cellular hosts has received great attention recently. In *Escherichia coli* and *Saccharomyces cerevisiae*, heterologous pathway expression has enabled the production of isoprenoids (Kizer et al., 2008; Martin et al., 2003; Paradise et al., 2008; Pitera et al., 2007), but their abundance of carbon–carbon double bonds results in only the alkene fraction of fuels. Biosynthetic production of long chain *n*-alkanes has been achieved through the reduction and decarbonylation of fatty acids to their aliphatic backbones (Cheesbrough and Kolattukudy, 1984; Dennis and Kolattukudy, 1992; Savage et al., 1996; Schirmer et al., 2010). In particular, this pathway enabled synthesis of tridecane, pentadecene, pentadecane, and heptadecane in *E. coli* (Schirmer et al., 2010). Despite these advances, current biofuel technology is limited by a lack of pathways to produce short-chain *n*-alkanes. In this work, we present a proof-of-concept pathway demonstrating that it is possible to produce such short alkanes via a lipoxygenase-mediated reaction in *Yarrowia lipolytica*. Specifically, we demonstrate the first microbial production of pentane by importing a lipoxygenase-based pathway found in soybeans.

Lipoxygenase enzymes convert polyunsaturated fatty acids into an unsaturated fatty acid hydroperoxide by adding a molecular oxygen to a (Z,Z)-1,4-pentadiene structural unit located within the fatty acid (Brash, 1999; Porta and Rocha-Sosa, 2002). Pentane production by means of this pathway has been previously demonstrated in both soybeans and peanuts (Pattee et al., 1974; Sanders et al., 1975a). In particular, soybean (*Glycine max*) lipoxygenases I and II and peanut (*Arachis hypogaea*) lipoxygenase convert linoleic acid (C18:2) into a 13-hydroperoxy linoleic acid intermediate (13-HPOD). Further catalysis via a homolytic-β-scission reaction and hydride abstraction converts 13-HPOD into pentane and 13-oxo-cis-9-trans-11-tridecadienoic acid, or into *n*-hexanal and 12-oxo-cis-9-dodecenoic acid (Bate et al., 1998; Garssen et al., 1971; Pattee et al., 1974; Sanders et al., 1975c). Since no lipoxygenase pathway for short-chain alkane synthesis has been synthetically imported into a microbial system, there is discrepancy as to the enzymes required for this pathway. In particular, while it is clear that lipoxygenase enzymes mediate at least the first step to pentane (Fig. 1), hydroperoxide lyase enzymes have been implicated in *n*-hexanal formation from 13-HPOD degradation

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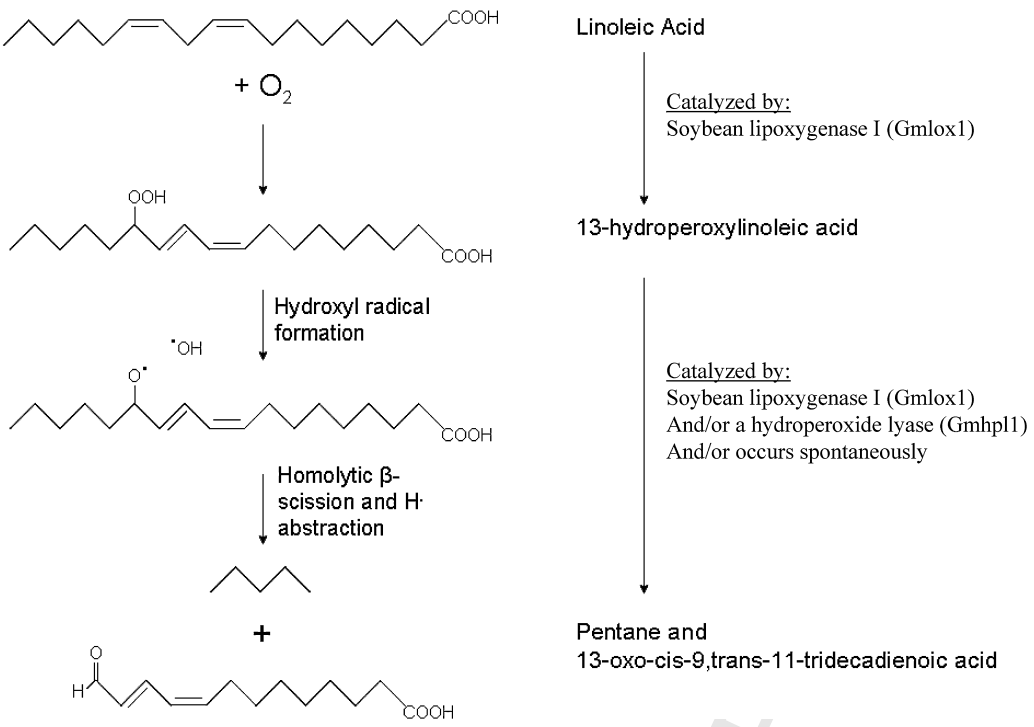

Fig. 1. Lipoxygenase-mediated conversion of linoleic acid to pentane. Soybean lipoxygenase I, Gmlox1, catalyzes the addition of molecular oxygen to the *cis* double bond within linoleic acid to form 13-hydroperoxylinoleic acid. Scission of this intermediate to pentane and a byproduct can proceed either spontaneously or through further Gmlox1 catalysis, and evidence suggests that a hydroperoxide lyase enzyme may assist this reaction.

(Andrianarison et al., 1989; Bate et al., 1998; Bourel et al., 2004; Vick and Zimmerman, 1989). Thus, it is unclear if the conversion of 13-HPOD to pentane product occurs spontaneously, is mediated by the lipoxygenase, or requires a hydroperoxide lyase enyzme. In vitro analysis has demonstrated that soybean lipoxygenase I (Minor et al., 1996), henceforth referred to as Gmlox1, is highly active under a broad range of conditions, can produce pentane from linoleic acid substrate, and does not produce *n*-hexanal – making it promising for short-chain alkane production (Garssen et al., 1971; Sanders et al., 1975b).

Biosynthetic pentane production requires a host system capable of producing linoleic acid substrate. Thus, the oleaginous yeast *Y. lipolytica* was chosen for this study based on reports that linoleic acid can account for up to 47% of total lipid extract (Beopoulos et al., 2008). Gene deletion analyses of β-oxidation enzymes and other key fatty acid enzymes in *Y. lipolytica* have established mechanisms to further increase accumulation of fatty acid substrates (Beopoulos et al., 2008, 2012; Mlickova et al., 2004; Dulermo and Nicaud, 2011; Wang et al., 1999). As a well-characterized oleaginous yeast (Barth and Gaillardin, 1997; Beopoulos et al., 2008, 2009a,b; Dujon et al., 2004; Morin et al., 2011; Sherman et al., 2006) and a proven host for high-level native and heterologous protein excretion (Ángel Domínguez et al., 1998; Madzak et al., 2004; Muller et al., 1998), *Y. lipolytica* possesses the requisite genetic toolbox for transformations (Barth and Gaillardin, 1996; Davidow et al., 1985), gene deletions (Fickers et al., 2003), and episomal and integrative expression cassettes (Fournier et al., 1993; Juretzek et al., 2001; Ledall et al., 1994; Matsuoka et al., 1993; Vanheerikhuizen et al., 1985) that enable metabolic pathway engineering approaches. Recent advances by our group to generate hybrid promoters have significantly increased *Y. lipolytica*'s transcriptional capacity to enable high-level genetic overexpressions necessary for heterologous pathways such as the lipoxygenase studied here (Blazeck and Alper, 2012; Blazeck et al., 2011, 2012).

Here we report a proof-of-concept pathway for the production of a short-chain alkane in the oleaginous yeast *Y. lipolytica*. We were able to increase pentane production through three distinct, combinatorial efforts – strongly overexpressing a codon optimized Gmlox1 gene, altering media formulation to increase fatty acid and lipid accumulation, and further increasing lipid content by removing β-oxidation degradation with a genetic knockout. We also tested and discounted the utility of the hydroperoxide lyase enzyme for pentane production. In doing so, this work describes the first ever microbial production of a short-chain alkane, and characterizes a lipoxygenase-mediated pathway toward pentane production from a linoleic acid substrate. Moreover, this work establishes *Y. lipolytica* as a model host for the production and conversion of fatty acids to more valuable metabolites such as alkane biofuels, especially with further modifications to divert fatty acids away from triacylglyceride production.

2. Materials and methods

2.1. Base strains and media

E. coli strain DH10B was used for cloning and plasmid propagation. DH10B was grown at 37 °C with constant shaking in Luria–Bertani Broth (Teknova) supplemented with 50 μg/mL of ampicillin for plasmid propagation. *Y. lipolytica* strain PO1f (ATCC # MYA-2613), a leucine and uracil auxotroph devoid of any secreted protease activity (Madzak et al., 2000), was used as the base strain for all studies. Table 1 contains a complete list of PO1f derivatives produced in this study. *Y. lipolytica* was cultivated at 30 °C with constant agitation at 180 rpm for all experiments.

YSC-LEU media consisted of 20 g/L glucose (Fisher Scientific), 0.69 g/L CSM-Leucine supplement (MP Biomedicals), and 0.67 g/L Yeast Nitrogen Base w/o amino acids (Becton, Dickinson, and Company). YSC-URA media contained 0.77 g/L CSM-Uracil in place of CSM-leucine, and YSC media contained 0.79 g/L CSM in place of CSM-Leucine. YPD media contained 10 g/L yeast extract (Fisher Scientific), 20 g/L peptone (Fisher Scientific) and 20 g/L glucose, and was often supplemented with 300 μg/mL Hygromycin B

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