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Biosynthesis of Monomers for Plastics from Renewable Oils

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Abstract: Omega-hydroxyfatty acids are excellent monomers for synthesizing a unique family of polyethylene-like biobased plastics. However, ω-hydroxyfatty acids are difficult and expensive to prepare by traditional organic synthesis, precluding their use in commodity materials. Here we report the engineering of a strain of the diploid yeast *Candida tropicalis* to produce commercially viable yields of ω-hydroxyfatty acids. To develop the strain we identified and eliminated 16 genes encoding 6 cytochrome P450s, 4 fatty alcohol oxidases, and 6 alcohol dehydrogenases from the *C. tropicalis* genome. We also show that fatty acids with different chain lengths and degrees of unsaturation can be more efficiently oxidized by expressing different P450s within this strain background. Biocatalysis using engineered *C. tropicalis* is thus a potentially attractive biocatalytic platform for producing commodity chemicals from renewable resources.

Introduction

Poly(ω-hydroxyfatty acids) have the potential to perform as functionally equivalent to versatile plastics like polyethylene while providing other attributes.[1,2] The monomers required to make these polymers are also valuable in chemical products such as lubricants, adhesives, cosmetic ingredients,[3,4] and anticancer therapeutics.[5,6] Most work on bioplastics from renewable sources has focused on polyhydroxyalkanoates and polylactic acid.[7,8] However, these materials suffer from significant performance deficits that have created difficult challenges to their adoption as general replacements for petroleum-based plastics. Poly(ω-hydroxyfatty acids) appear to overcome the functional limitations of other bioplastics, but the monomers have not been available with economics required for their conversion to commodity plastics.[9] For example, cross-metathesis of ethene and erucic acid provides 13-tetradecenoic acid, which is subsequently hydroformylated and then hydrogenated to give ω-hydroxyhexadecanoic acid.[10,11] Also, alkyl-branched ω-hydroxy fatty acids were prepared by hydroformylation (e.g., linoleic acid) followed by hydrogenation of the carbonyl group.[12] The use of other fatty acid derived monomers for preparation of polyethylene-like polyesters has been explored.[13] Quinzler and Mecking[14] converted methyl oleate, by exposure to carbon monoxide and methanol catalyzed by $Pd(OAc)_2$/1,2-bis[(di*tert*-butylphosphino)methyl]benzene methanesulfonic acid, to dimethyl-1,19-nonadecanedioate. Reduction of the former gave nonadecane-1,19-diol. Copolymerization of dimethyl-1,19-nonadecanedioate with nonadecane-1,19-diol catalyzed by titanium alkoxides gave polyethylene-like polyesters. Warwel et al.[15] synthesized polyethylene-like polyesters by first preparing α,ω-alkylene dialkenoates that were polymerized by acyclic diene metathesis (ADMET) polymerization.

In seeking a biological route to production we turned our attention to cytochrome P450s, a powerful and versatile family of enzymes capable of specifically introducing chemical functionality into parts of molecules with low reactivity. Several P450 enzymes are known to hydroxylate aliphatic fatty acids at the omega position.[16−19] Of particular interest to us were the P450s of *Candida tropicalis*:[20] a strain of this yeast (H5343) has been engineered to transform fatty acids into α,ω-diacids

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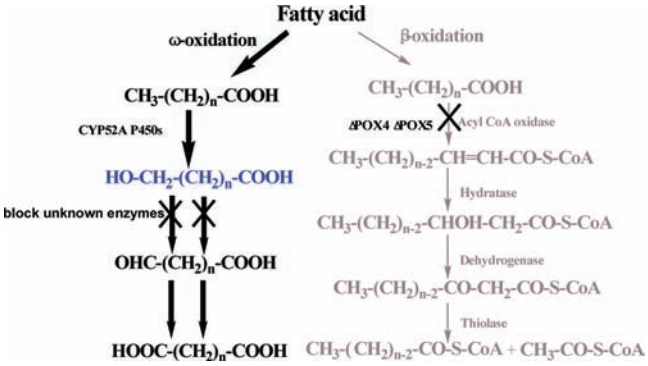

Figure 1. Two pathways for oxidation of fatty acids; the beta oxidation pathway can be blocked by deletion of both copies of the gene encoding the acyl CoA oxidase that catalyzes the first step in the pathway (POX4 and POX5).[21]

commercially,[21] by eliminating the β-oxidation pathway (Figure 1). The key requirement to convert this strain into one capable of producing ω-hydroxyfatty acids is the elimination of enzymes that oxidize the alcohol group (Figure 1); the identity of these enzymes was not known when we began this work.

Fatty alcohol oxidase and inducible wild-type P450 enzymes are generally thought to catalyze the further oxidation of ω-hydroxyfatty acids to α,ω-dicarboxylic acids in *C. tropicalis*.[20,22] However, during the course of this work it was discovered that alcohol dehydrogenases can contribute to the oxidation of ω-hydroxyfatty acids to α,ω-dicarboxylic acids.

Although *C. tropicalis* is an attractive organism for bioprocessing, the relative dearth of established molecular tools to engineer this diploid organism presents serious obstacles to its genetic manipulation. Here we describe the strain and fermentation engineering used to eliminate 16 genes, including cytochrome P450s, fatty alcohol oxidases, and alcohol dehydrogenases, to produce strains of *C. tropicalis* capable of converting a range of fatty acids to their corresponding ω-hydroxy fatty acids. A strain that reintegrates a select P450 results in the biotransformation of methyl tetradecanoate to 14-hydroxytetradecanoic acid with high yield and purity. Furthermore, when different P450s are integrated into the genome of this strain, different oxidation profiles are obtained from fatty acids with various chain lengths and degrees of unsaturation.

Materials and Methods

Full methods are available in the Supporting Information.

Results and Discussion

C. tropicalis converts fatty acids in fermentation media to the corresponding α,ω-dicarboxylic acids, but these are further oxidized to acetyl CoA by the β-oxidation pathway (Figure 1). Commercially useful levels of α,ω-dicarboxylic acids can be produced from *n*-alkanes and fatty acids by strains where the first step in β-oxidation is blocked by disruption of the POX4 and POX5 allelic gene pairs encoding acyl coenzyme A oxidases.[21,23] *C. tropicalis* strain H5343 in which β-oxidation is blocked and which can therefore produce high levels of α,ω-dicarboxylic acids from fatty acids, is thus an attractive starting point for engineering a strain to produce ω-hydroxy fatty acids from renewable sources. Creation of such a strain requires the additional disruption of the enzymes that catalyze the transformation of ω-hydroxy fatty acids to α,ω-dicarboxylic acids.

We initially believed that the enzymes responsible for fatty alcohol oxidation in *C. tropicalis* were inducible cytochrome P450s and fatty alcohol oxidases (FAOs).[20,22] Therefore, we began our strain construction by deleting from strain H5343 2 allelic pairs of P450s, CYP52A13/CYP52A14, and CYP52A17/CYP52A18: these genes are transcribed during growth on fatty acids,[24] and their encoded enzymes are known to oxidize fatty acids to dicarboxylic acids.[20] Genes were deleted by synthesizing 5′ and 3′ sequences from the target gene and placing between them two frt sites flanking genes encoding flp recombinase and nourseothricin resistance.[25] The construct was transformed into *C. tropicalis*, chromosomal integrants were selected on nourseothricin, the site of integration was confirmed by PCR, and the resistance gene was removed by induction of the recombinase, allowing the use of the same strategy for an unlimited number of genes (more details in the Supporting Information and Supplementary Figure 1). Genotypes of strains produced in this study are provided in the Supporting Information and shown graphically in Figure 2. Strain DP174, in which the four P450 genes had been deleted, was no longer capable of significant fatty acid oxidation (Figure 3A). However, when we grew DP174 in the presence of an ω-hydroxyfatty acid, it oxidized that substrate to the diacid almost as rapidly as the starting strain (Figure 3B), indicating that these P450 genes are responsible for oxidation of the fatty acids to ω-hydroxyfatty acids but are not required for subsequent oxidation to the diacids.

To eliminate ω-hydroxyfatty acid oxidation we deleted a further P450 (CYP52A12) that is also induced in *C. tropicalis* upon growth on fatty acids[24] as well as the three known fatty alcohol oxidase genes: an allelic pair and a single gene. We also reasoned that because *C. tropicalis* is diploid, there was probably an allelic copy of both CYP52A12 and the unpaired fatty alcohol oxidase (FAO1). We therefore also created constructs to eliminate these two unknown genes, as described in the Supporting Information and Supplementary Figure 2. However, we were disappointed to find that strain DP283, lacking 6 CYP450s and 4 FAOs, was almost as active as the starting strain at oxidizing ω-hydroxy fatty acids (Figure 3C).

We next turned our attention to alcohol dehydrogenase (ADH) genes. Although no alcohol dehydrogenases had been sequenced from *C. tropicalis*, it has been reported that alcohol dehydrogenases expressed in the mammalian liver catalyze the metabolism of long chain ω-hydroxyfatty acids to their corresponding fatty acid aldehydes[26] and that *Saccharomyces cerevisiae* ADH I and II can oxidize long chain alcohols to their corresponding aldehydes.[27] On the basis of these reports, we performed an in vitro analysis to determine whether a yeast alcohol dehydrogenase from *S. cerevisiae* could oxidize ω-hydroxyfatty acids. LC-MS analysis of reaction products showed that this enzyme catalyzed the conversion of ~25 mol % 12-hydroxydodecanoic acid and ~50 mol % 16-hydroxyhexadecanoic acid to their corresponding fatty aldehydes during 1 h incubations (Figure

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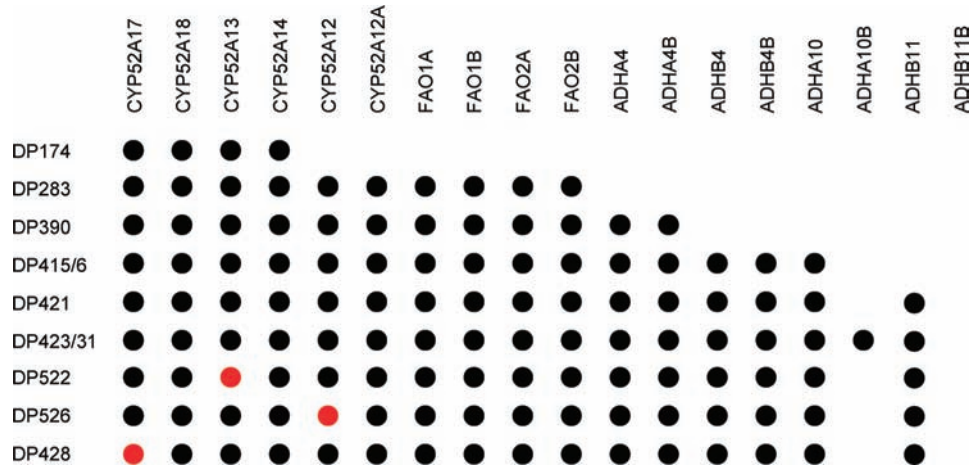

Figure 2. Genotypes of strains where black dots indicate deleted genes, red dots indicate deleted wild-type versions of the genes, with a synthetic copy integrated under control of the ICL promoter.

3D). Because alcohol dehydrogenases are highly expressed enzymes in yeast,[28] we reasoned that alcohol dehydrogenases could be responsible for some of the ω-hydroxyfatty acid oxidation in *C. tropicalis*.

To identify *C. tropicalis* ADH genes we synthesized degenerate oligonucleotide primers based on known sequences of ADHs from *Candida albicans*,[29,30] used these primers to PCR amplify gene fragments out of *C. tropicalis* genomic DNA, and cloned the amplicons into *E. coli*. We sequenced 96 clones, and about one-third of the clones (32 clones) were highly homologous to *C. albicans* ADHs. From these, 5 unique sequences were identified: ADH-A10 (GU056283), ADH-B11 (GU056287), ADH-B2 (GU056285), ADH-A4 (GU056282), and ADH-B4 (GU056286). Translation of the ADH-B2 sequence showed it to contain two in frame stop codons; it is therefore almost certainly a pseudogene. The remaining 4 sequences ADH-A4 and ADH-B4 had the most similar amino acid sequences (87%). In contrast, ADH-A4 was 94% identical to *C. albicans* ADH1A and ADH-B4 was 89% identical to *C. albicans* ADH2A. We therefore concluded that each of the four sequences ADH-A10, ADH-B11, ADH-A4, and ADH-B4 probably represented different *C. tropicalis* ADH genes rather than allelic pairs and thus each probably had an allelic partner whose sequence was too similar to have been distinguished in the initial set of 32 sequences.

To eliminate both alleles of each of these 4 alcohol dehydrogenase genes we synthesized a pair of nested knockout constructs for each gene as described in the Supporting Information and shown in Supplementary Figures 1 and 2. Following each deletion we tested the ability of the resulting strain to oxidize ω-hydroxy fatty acids to α,ω-dicarboxylic acids. As we deleted each of the *C. tropicalis* ADH genes, we tested the strains for their ability to oxidize 12-hydroxydodecanoic acid. Figure 3E shows that strain DP390, in which the first pair of ADH alleles had been eliminated, oxidized 12-hydroxydodecanoic acid more slowly than DP174 and the starting strain. Progressive deletion of additional ADHs further decreased the rate of oxidation of 12-hydroxydodecanoic acid (Figure 3E, strain genotypes are shown in Figure 2). Strains DP431, in which the second allele of ADH-A10 was deleted, appeared much less healthy than the other deletions with smaller colonies and much slower growth times.

We therefore selected strain DP421, lacking both alleles of two of the ADHs (A4 and B4) and one allele of each of the other 2 (A10 and B11), for further development, as it appeared as healthy as the starting strain. In shake flasks, strain DP421 oxidized 12-hydroxydodecanoic acid, 14-hydroxytetradecanoic acid, and 16-hydroxyhexadecanoic acid to 12%, 27%, and 11%, respectively, of the levels observed after 8 h of incubation time by strain H5343, and almost no oxidation of 18-hydroxy-9-*cis*-octadecenoic acid (ω-OH-C18:1) was detected (Figure 3F).

Deletion of genes to create strain DP421 greatly reduced the oxidation of ω-hydroxyfatty acids, but elimination of P450 genes also blocked the ω-hydroxylation of fatty acids by this strain. To restore fatty acid ω-hydroxylation activity, we integrated different P450s back into the genome under control of the isocitrate lyase (ICL) promoter; in *C. tropicalis* this promoter is induced by growth with fatty acids, acetate, or ethanol.[31] We used Gene Designer software[32] to design codon-optimized versions of CYP52A17 and CYP52A13 that were synthesized and independently integrated into strain DP421 under control of the ICL promoter to generate strains DP428 and DP522, respectively (Figure 2). Both DP428 and DP522 oxidized methyl tetradecanoate in shake flasks to primarily 14-hydroxytetradecanoic acid. In contrast, the starting strain (H5343) yielded primarily 1,14-tetradecanedioic acid, while strains lacking P450s did not oxidize the substrate at all (Figure 4A).

The aim of this work was to develop a general biological route to production of ω-hydroxyfatty acids. Thus, information was needed on the degree to which the oxidation pathway of engineered strains was substrate specific. To accomplish this, the in vivo activities and selectivities of strains DP428 or DP522, carrying the P450s CYP52A17 and CYP52A13, respectively, were assessed by feeding to cultures substrate fatty acids (C18:1 and C18:2) or fatty acid methyl esters (C14:0 to C18:0) with different carbon chain lengths and unsaturation levels (Figure

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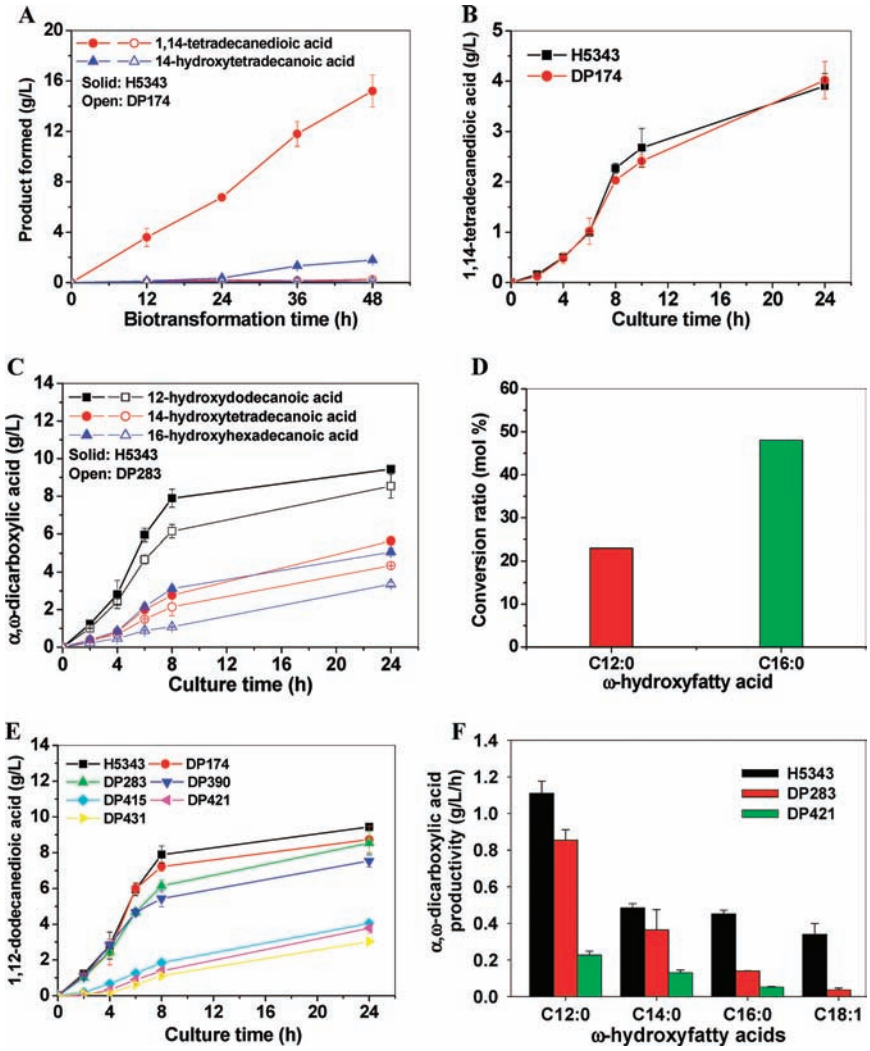

Figure 3. Oxidation of fatty acids and ω-hydroxyfatty acids by engineered strains of *C. tropicalis*. (A) Oxidation of methyl tetradecanoate to 14-hydroxytetradecanoic acid and 1,14-tetradecanedioic acid by *C. tropicalis* strains H5343 and DP174. (B) Oxidation of 14-hydroxytetradecanoic acid to 1,14-tetradecanedioic acid by *C. tropicalis* strains H5343 and DP174. (C) Oxidation of 12-hydroxydodecanoic acid, 14-hydroxytetradecanoic acid, and 16-hydroxyhexadecanoic acid to the corresponding α,ω-dicarboxylic acids by *C. tropicalis* strains H5343 and DP283. (D) Oxidation of 12-hydroxydodecanoic acid and 16-hydroxyhexadecanoic acid to their corresponding fatty aldehydes by purified *S. cerevisiae* ADH protein. (E) Oxidation of 12-hydroxydodecanoic acid to 1,12-dodecanedioic acid by *C. tropicalis* strains lacking P450, FAO, and ADH genes. (F) Oxidation of 12-hydroxydodecanoic acid, 14-hydroxytetradecanoic acid, 16-hydroxyhexadecanoic acid, and 18-hydroxy-9-*cis*-octadecenoic acid by strains H5343, DP283, and DP421. Oxidation of fatty acids or hydroxyfatty acids by *C. tropicalis* strains (parts A, B, C, E, and F): strains were precultured to $A_{600\ nm} \approx 5.0$ in shake flasks; bioconversion was initiated by addition of substrate and products measured (8 h incubation time for part F). Oxidation of hydroxyfatty acids by purified *S. cerevisiae* ADH protein was performed in an aqueous solution media containing 0.1 M Tris-HCl buffer (pH 9.0) with 166 μM NAD. In all cases products were acidified, extracted into diethyl ether, and analyzed by LC-MS. The error bars represent the range from two independent experiments.

4B). Both CYP52A17 and CYP52A13 were capable of efficient ω-hydroxylation of tetradecanoic acid with little dicarboxylic acid formation. However, with longer chain-saturated and unsaturated fatty acids, the two strains harboring CYP52A17 and CYP52A13 differed significantly in the efficiency of substrate conversion, indicating that at least some substrate specificity of the pathway resides in the P450 catalyzing the initial oxidation. We also observed that strain DP522 generally yielded a higher ratio of hydroxyfatty acid to dicarboxylic acid. This difference was particularly marked with *cis*-9-octadecenoic acid (C18:1), where DP522 (CYP52A13) produced ω-hydroxyfatty acid and the dicarboxylic acid at a ratio of ~5:2, while DP428 (CYP52A17) produced these same products at a ratio of ~1:7.5. These results clearly demonstrate that some ω-hydroxyfatty acid oxidation is catalyzed by the P450 itself. It therefore appears that manipulation of substrates transformed and products made by *C. tropicalis* can be accomplished by introduction into a DP421 background of the appropriate P450, either from a wild-type gene from another organism or by introducing genes encoding artificially engineered enzymes.[33,34]

Finally, we wished to determine whether the *C. tropicalis* platform that we developed could produce commercially useful yields of ω-hydroxyfatty acids. By moving from shake flasks to a fermentor and optimizing growth and transformation conditions, DP428 (carrying CYP52A17) produced yields of over 160 g/L of 14-hydroxytetradecanoic acid, with less than 5% formation of the corresponding dicarboxylic acid. Due to the higher rate of ester hydrolysis relative to ω-hydroxlation at early stages of the fermentation, the concentration of tetradecanoic acid increases with time for the first 80 h and, thereafter,

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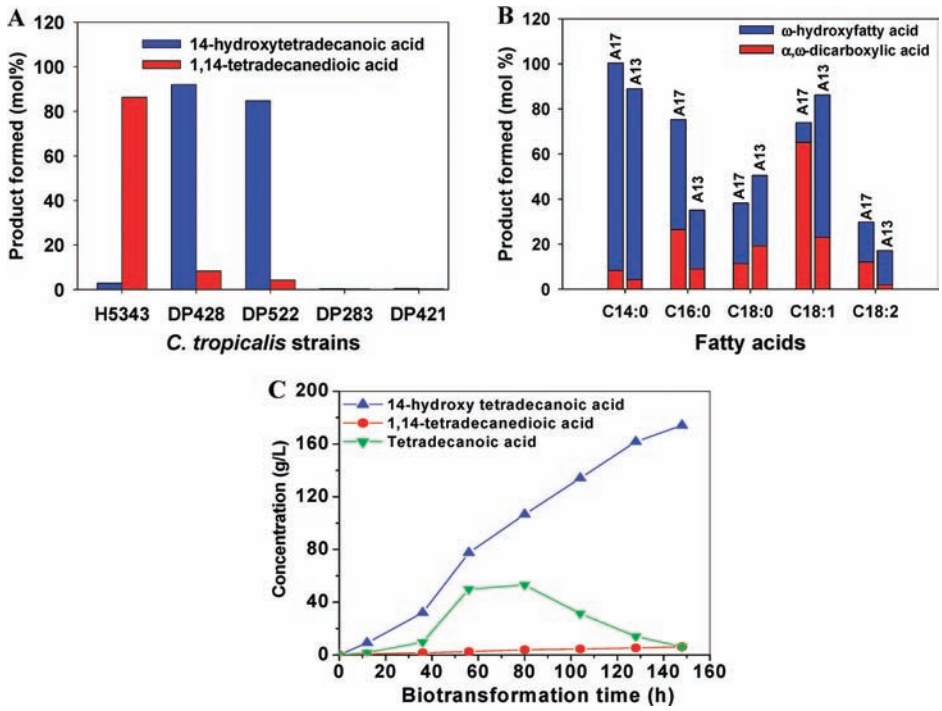

Figure 4. Production of ω-hydroxyfatty acids by engineered *C. tropicalis*. Genotypes are as shown in Figure 2. (A) Conversion of methyl tetradecanoate (30 g/L) to 14-hydroxytetradecanoic acid and 1,14-tetradecanedioic acid by engineered strains of *C. tropicalis*. (B) Oxidation of fatty acid substates (40 g/L) with different chain lengths and degrees of unsaturation by DP428 (CYP52A17) and DP522 (CYP52A13). (C) Conversion of methyl tetradecanoate (200 g/L) to 14-hydroxytetradecanoic acid by strain DP428 in a fermentor.

decreases in concentration (Figure 4C). A total 174 g/L of 14-hydroxytetradecanoic acid and 6.1 g/L of 1,14-tetradecanedioic acid were obtained with addition of 200 g/L methyl tetradecanoate after 148 h of biotransformation (Figure 4C).

Conclusion

C. tropicalis is an attractive organism for biotransformations: it grows robustly and tolerates high concentrations of substrates which it takes up readily. We created a strain of *C. tropicalis* capable of producing high levels of ω-hydroxyfatty acids by engineering a 90% reduction in the activity of the endogenous pathways that normally metabolize these compounds to diacids. The strain engineering was achieved by identifying target sequences using PCR with degenerate primers based on known *C. tropicalis* and *C. albicans* genes, then eliminating these genes with a 2-step genome modification method that allows indefinite reuse of the same selective marker.[25]

In preparation for weaning ourselves from a petrochemical society in which the infrastructure for extraction of energy from fossil fuels effectively subsidizes the production of plastics, it is important to develop alternatives that can be synthesized from renewable materials without compromising performance.

The strains described in this report provide the foundation for development of low-cost industrial processes that substitute for chemical approaches to synthesize ω-hydroxy fatty acids. Posssible future modifications include genomic integration of genes for secreted lipases into *C. tropicalis* to allow the direct conversion of natural triglyceride oils to fatty acid substrates and, ultimately, introduction of pathways for the overproduction of fatty acids by *C. tropicalis* itself. Through further P450 engineering and fermentation optimization we expect to achieve high levels of product formation, thereby providing a low-cost source of ω-hydroxyfatty acids for a variety of uses. Of particular interest to us is the conversion of ω-hydroxy fatty acids into a family of biobased plastics. Previous work on poly(ω-hydroxypentadecanoate) from a monomer that is just one methylene longer than 14-hydroxytetradecanoic acid has shown that plastics from these monomers are hard, tough materials with an elastic modulus and yield parameters intermediate between low- and high-density polyethylene.[2] Polymers from ω-hydroxyfatty acids also present appealing recycling options: conversion of poly(ω-hydroxyfatty acids) to their corresponding alkyl ester monomer units by either a chemical or a biological process would result in a biofuel similar in structure to biodiesels such as Soy Gold.

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Supporting Information Available: Full experimental details for gene deletions from *C. tropicalis* genome, identification of ADH genes, expression of synthetic P450, shake-flask oxidation and fermentation procedure, analytical methods, ω-hydroxyfatty acids and α,ω-dicarboxylic acids preparation; list of *C. tropicalis* strain used and gene sequences of FAO1B, ADH-A4, ADH-A10, ADH-B2, ADH-B4, and ADH-B11; DNA constructs for modifying *C. tropicalis* genome. This material is available free of charge via the Internet at http://pubs.acs.org.

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