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Biosynthesis of hydrocarbons and volatile organic compounds by fungi: bioengineering potential

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Abstract

Recent advances in the biological production of fuels have relied on the optimization of pathways involving genes from diverse organisms. Several recent articles have highlighted the potential to expand the pool of useful genes by looking to filamentous fungi. This review highlights the enzymes and organisms used for the production of a variety of fuel types and commodity chemicals with a focus on the usefulness and promise of those from filamentous fungi.

Keywords

Biofuels; Filamentous fungi; Terpene Cyclase; Polketide Synthase; Volatile Organic Compounds; Hydrocarbons

Introduction

The production of biofuels at scales necessary to meet demand and compete with fossil fuel prices remains a tremendous hurdle (Chu and Majumdar 2012). The rational manipulation of metabolic pathways and the selection of the most active enzymes from an ever-increasing pool of candidates have dramatically increased yields (reviewed in Peralta-Yahya et al 2012). The sources of the enzymes that are being used in these pathways derive from organisms that span the tree of life, however, the kingdoms of Bacteria and Plantae are most highly represented. Much work in recent years has demonstrated the ability of filamentous fungi to produce hydrocarbons and commodity chemicals and this group represents a largely untapped source of enzymatic potential.

Filamentous fungi have been observed to produce volatile alcohols, alkanes and terpenoids for many years (Murahashi 1938; Freeman, G. G. 1949; Or et al. 1966). These molecules are often described as Volatile Organic Compounds (VOCs) and have been explored for diverse applications. Notably, VOCs have been suggested as a means to identify the presence of fungi in indoor environments (Samson 1985). VOC production from a variety of common indoor fungi in the genera *Aspergillus, Fusarium* and *Penicillium* has been characterized for this purpose (Larsen and Frisvad 1995; Fiedler et al. 2001; Wihlborg 2008; Lancker et al.

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2008; Schuchardt and Kruse 2009; Polizzi et al. 2012). The suggested application of fungal VOCs as biofuels was made relatively recently (Strobel et al. 2008). A sampling of isolates in the genus *Ascocoryne* revealed a series of C8 compounds as well as C6 to C9 alkanes and branched alcohols that could be a gasoline surrogate (Griffin et al. 2010). Similarly, many fungi have been shown to produce many volatile terpene molecules, which are being used as jet or diesel fuels (Fig. 1)(Peralta-Yahya et al. 2011; Tess Mends and Yu 2012; Riyaz-Ul-Hassan et al. 2013).

Described herein are applications where the application of fungal enzymes shows great potential. For three major fuel types (gasoline, jet fuel and diesel) biosynthetic strategies and enzymes that have been successfully applied are discussed and the potential role for filamentous fungi is highlighted.

Gasoline surrogates

The current biological surrogates for gasoline are mostly short-chain alcohols, notably ethanol. However, longer chain alcohols that more closely mimic the properties of petroleum-derived fuel have also been biosynthetically explored (Fig. 1)(International Energy Agency 2011). Significant effort has gone into the production of these molecules via the α-ketoacid elongation pathway, whereby amino acid biosynthesis pathways are re-routed to decarboxylate, rather than aminate, amino acid precursors. In the first demonstration of this production, Atsumi and colleagues made C3-C4 linear and C4-C5 branched primary alcohols as well as C8 phenylethanol. They then optimized the production of 1-butanol by surveying decarboxylases from three bacteria (*Megasphaera elsdenii*, *Streptomyces coelicolor* and *Clostridium acetobutylicum*) to find the enzyme that produced the highest titers (Atsumi et al. 2008b; Atsumi et al. 2008a). Further extensions of these methods have led to a C8 linear alcohol that closely mimics the energy density and hydrophobicity of gasoline (Marcheschi et al. 2012).

Filamentous fungi have been observed to produce alcohols of these chain lengths, from ethanol to decan-4-ol, including several different isomers for each of the C5-C7 alcohols (Table 1)(reviewed in Korpi et al. 2009). Where known, these alcohols derive from amino acid degradation via the Ehrlich pathway (Schoondermark-Stolk et al. 2006), though it remains unclear how several of the alcohols with longer chains are produced. In the case of the C8 1-octen-3-ol, often referred to as the characteristic mushroom odor, the mechanism of synthesis has been shown to occur via the peroxidation and cleavage of linoleic acid (Tressl et al. 1982; Wurzenberger and Grosch 1984b; Wurzenberger and Grosch 1984a). Other C8 alcohols and ketones were produced by the *Aspergillus* lipoperoxidase PpoC in a lysate of *E. coli* (Brodhun et al. 2010). Homologs of PpoC were associated with the production of alkenes and alkanes by transcriptomic studies, though it remains unclear if what other enzymes may also have been involved (Gianoulis et al. 2012). This suggests that fungi may have enzymes capable of reducing the alcohols and ketones to hydrocarbons. Though the breakdown of linoleic acid is unlikely to be as energy efficient as α-ketoacid elongation for the production of long-chain alcohols, these downstream fungal enzymes may be of significant use for the conversion of alcohols to alkenes and alkanes.

Other mechanisms for the production of alkenes and alkanes thus far have been successful only for longer products, more suitable for use as jet or diesel fuel surrogates. However, some fungal enzymes may be useful in generating shorter alkenes. Specifically, recent work has discovered a nine-carbon alkene from an endophytic fungus isolate of *Nigrograna mackinnonii* that is likely to be polyketide-derived which approaches a chain length useful for gasoline applications (Shaw et al. 2015b).

Jet Fuel surrogates

Significant advances have been made in the biological production of hydrocarbons with chain lengths suitable for use as surrogates of jet fuel, most notably those derived from terpene biosynthesis. Terpenes are produced by the condensation of phosphorylated isoprene monomers that can then be cyclized and modified to form diverse natural products with myriad uses (Gershenzon and Dudareva 2007). Mono-and sequiterpenes, from the condensation of two and three isoprene units, respectively, are the most common isoprene polymers used in the context of biofuels. Monoterpenes alone have been suggested as a jet fuel surrogate after chemical dimerization (Harvey et al. 2010); however, other modifications such as hydrogenation give properties more suitable as a diesel additive (Tracy et al. 2009). A fuel from a mixture of mono and sesquiterpenes were recently used in a test flight (Amyris 2012), however sequiterpenes are more often used as a diesel fuel. Monoterpenes are also desirable for a variety of other uses including as pharmaceuticals (Lambert et al. 2001), flavorings and fragrances (Werf et al. 1997; Carrau et al. 2005; Kirby and Keasling 2009).

Eukaryotes and bacteria typically form the precursors to terpenes via different pathways, however the mevalonate pathway, which is mostly found in eukaryotes, has been entirely reconstituted in *E. coli* for engineering purposes (Martin et al. 2003). Since then, the pathway has been extensively optimized, including by adding more active versions of several enzymes (*Staphylococcus aureus* 3-hydroxy-3-methylglutaryl (HMG)-CoA Synthase and *Enterococcus faecalis* HMG-CoA Reductase) (Renninger et al. 2010), the identification and expression of only the catalytic domain of HMG-CoA Reductase to remove its feedback inhibition (Ohto et al. 2009; Asadollahi et al. 2010), the substitution of a modified Acetyl-CoA synthetase from *Salmonella enterica* to relieve glucose repression (Shiba et al. 2007) and the transfer of the entire pathway to the mitochondrion (Farhi et al. 2011). Further improvement in product titers are now limited by the terpene cyclase, the last step of the process that generates a specific molecule or set of molecules. What are needed now are novel and more efficient terpene cyclases.

Many of the terpene cyclases currently used for synthetic biology applications come from plants. For example, for the production of the monoterpene dimer jet fuel described above, three enzymes were tested from among the 11 that were known to produce β-pinene, all of which came from plants (Degenhardt et al. 2009; Sarria et al. 2014). Several other dimerization strategies have been reported that have utilized both α and β pinene (Chen and Forbus Jr 1990; Booth and Phillips Jr 1998; Chapaton et al. 2004; Zou et al. 2012). Filamentous fungi have been observed to produce both α and β pinene, though α is considerably more common (Table 1).

Indeed, terpenes are among the most numerous volatile products observed from fungal cultures, including both *Ascomycetes* and *Basidiomycetes*, and with especially high structural diversity from marine-derived fungi (Ebel 2010). However, the difficulty to identify terpenes by methods such as GC/MS often makes further study challenging. For example, in Griffin et al 2010 strains of *Ascocoryne* produced as many as 39 unique sesquiterpenes, nearly as many as all other classes of VOCs combined (Griffin et al. 2010). However, comparing these unknown products to other observations is challenging without column and temperature-independent values, such as retention indices, which are not universally reported.

A major barrier to the use of these fungal terpene cyclases for biosynthetic applications is the correlation of products with a particular gene. The first gene-product association for a monoterpene was recently reported for the oxygenated terpenoid 1,8-cineole. Several sesquiterpene synthases have been correlated with products from the basidiomycete *Coprinus cinereus*, including germacrene A, α-muurolene, δ-cadinene and α-cuprenene (Agger et al. 2009). Though no gene has yet to be associated with the jet fuel precursor pinene the genomes of several fungi that produce α and β pinene have been sequenced (Nierman et al. 2005; Cuomo et al. 2007; Shaw et al. 2015a; Shaw et al. 2015b).

Terpene cyclases are readily predictable from genome sequences by their highly conserved catalytic core motifs, however the overall sequence similarity is strikingly small, making product prediction difficult (Lesburg et al. 1997; Starks et al. 1997). In known structures bacterial and fungal cyclases tend to be more compact than their plant counterparts, lacking several domains that appear to have evolved only in the plant lineage (Kampranis et al. 2007; Nakano et al. 2011; Köksal et al. 2011; Liu et al. 2014). This suggests that the use of fungal or bacterial enzymes in overexpression strains might be more efficient than their plant counterparts; in the very few cases where enzymatic efficiencies can be compared this appears to hold true (Felicetti and Cane 2004; Shaw et al. 2015a). For example, the k_{cat} of the *Hypoxylon sp.* 1,8-cineole synthase is roughly 5-fold faster than from the bacterium *Streptomyces lividans* or the plant *Salvia fruticosa* (Kampranis et al. 2007; Nakano et al. 2011; Shaw et al. 2015a).

Diesel surrogates

There are a variety of molecule types that have been suggested for use as diesel surrogates, including hydrogenated mono and sesquiterpenes and alkanes/alkenes. Where tested, each has been shown to mimic diesel in several respects including cetane number and energy density (Peralta-Yahya et al. 2011). Terpene-based diesel surrogates include chemically hydrogenated forms of monoterpenes such as limonene, and sesquiterpenes such as farnesene and bisabolene. Similar to the example of β-pinene production described above, optimal bisabolene titers were obtained by testing six different plant cyclases to identify the most active from the Giant Fir tree *Abies grandis* (Peralta-Yahya et al. 2011). Many different filamentous fungi have been observed to produce bisabolene, in both the *Ascomycetes* and *Basidiomycetes* phyla (Müller et al. 2013; Zhang et al. 2014) though no genes have been associated with the synthesis of the molecule.

Even more structurally similar to existing diesel fuel blends are long-chain alkanes and alkenes, which have been demonstrated to be produced by bacteria through the modification of fatty acid precursors. Alkanes have been produced by reducing fatty acids of varying lengths to an aldehyde and then decarbonylating to leave an n-1 alkane of sizes 13-17 (Winters et al. 1969; Schirmer et al. 2010; Wang et al. 2013). Alkenes have been produced by two mechanisms: a P450-type enzyme was shown to decarboxylate a free fatty acid leaving an n-1 terminal alkene (Rude et al. 2011) and two fatty-acid precursors are merged and then reduced in a process known as head-to-head condensation (Beller et al. 2010; Sukovich et al. 2010). None of these mechanisms have been observed in eukaryotes though they have been hypothesized to be widely present (Cheesbrough and Kolattukudy 1988; Dennis 1992; Kunst and Samuels 2003).

Fungi have been observed to produce alkanes and alkenes for decades, though no mechanism for their production has been elucidated (Or et al. 1966; Walker and Cooney 1973). Spores from several species have been shown to contain n-alkanes, with 27, 29 and 35 carbons dominating (Or et al. 1966). Their odd-chain lengths are consistent with the n-1 decarboxylation or decarbonylation mechanisms described above. Even-chain n-alkanes, such as the n-16 observed from *Hormoconis resinae* (*Cladosporium resinae*) do not clearly fit into a mechanism that has been described in bacteria (Walker and Cooney 1973). More recent work has observed a variety of even-chain alkanes/alkenes from members of the genus *Ascocoryne* (Griffin et al. 2010), however these were later correlated with the breakdown of linoleic acid (Gianoulis et al. 2012) which could not explain the n-16 alkanes of *H. resinae.* Further work will be necessary to discover how fungi are able to produce this important class of molecules.

Conclusions

There is a pressing need for “drop-in” surrogates for the three primary transportation fuels: gasoline, jet fuel and diesel. In the case of gasoline, short chain alcohols predominate, though longer chains that more closely mimic the properties of gasoline continue to be explored. In the case of jet fuel, dimerized monoterpenes and mixtures of hydrogenated mono and sesquiterpenes have been used in a jet engine (Amyris 2012). In the case of diesel, hydrogenated sesquiterpenes and long-chain alkanes and alkenes show promise (Peralta-Yahya et al. 2011). In each of these cases filamentous fungi are known to produce similar if not the same molecules and may lend useful properties to these biosynthetic pathways.

Notably lacking are polyketide-derived biofuels. In theory, the ability to rationally design PKSs could produce biofuels with whatever degree of saturation and branching are required for the application (Menzella et al. 2005), however the heterologous expression of PKSs is only beginning to be implemented (Pfeifer and Khosla 2001; Ma et al. 2009; Yuzawa et al. 2012). The substrate molecule for polyketide biosynthesis is the same as for terpenes, acetyl-CoA, so engineering efforts that have increased the amount and availability of acetyl-CoA would benefit PKS enzymes as well. The commonly-used heterologous host, *S. cerevisiae,* would be particularly useful for PKS-based fuels as it does not contain endogenous PKSs, thereby reducing the effort to eliminate off-target pathways (Tsunematsu et al. 2013). A

potential source for finding such enzymes is within the many diverse and unstudied filamentous fungi.

The major hurdle in the utilization of fungal enzymes in these synthetic biology applications is the identification of the genes responsible for the production of the many interesting molecules that are reported from fungi. Decreasing costs of DNA sequencing have made the larger genome sizes of fungi less of an impediment, though developing genetic tools necessary for discovering these genes remains a bottleneck and methods that are broadly and expediently applied to the many unstudied organisms are needed (Li and Vederas 2009). Decreasing costs of DNA synthesis have alleviated some of this concern, as many genes of interest can be synthesized into vectors for testing in heterologous hosts. Particularly useful in this regard is the terpene production pathway in *E. coli* developed by the Keasling Lab, which allows for many unknown terpene cyclases of fungi to be expressed and their products quickly characterized (Martin et al. 2003; Shaw et al. 2015a). A similarly universal system for PKS genes would be immensely useful, though promoting the correct folding and phosphopantetheinylation of these large enzymes will likely be a challenge.

The efficiencies of the production enzymes are not the only hurdle to the widespread adoption of biofuels. The biofuel titer is often tempered by the toxicity of the products (Brennan et al. 2012), and the ease of purification has a significant effect on the overall efficiency (Stephanopoulos 2007). The carbon efficiency of the system must also be considered; acetate-derived biosyntheses including terpenes and polyketides, have a maximum theoretical carbon efficiency of only 66% (Rontein et al. 2002). Moreover, the carbon inputs are important beyond the scope of the fuel production; the use of food-crop feedstocks have a significant impact on the environment and economy, leading to recent policy suggestions to end biofuel subsidies (Searchinger and Heimlich 2015).

The recent gains in the biological production of fuel molecules and commodity chemicals are owed to the significant modification of chasis organisms and the assemblage of genes from several different species. The successes of these systems makes it increasingly unlikely that we will discover a single organism that represents a “consolidated bioprocess,” i.e. that uses the desired feedstocks and produces the desired molecules at a scale competitive with these hybrid organisms. Moreover, the success of synthetic biology applications will likely grow as the pool of enzymes increases with more organisms being sequenced and effectively annotated. Filamentous fungi represent a relatively untapped pool of such enzymes, with potential for many different applications.

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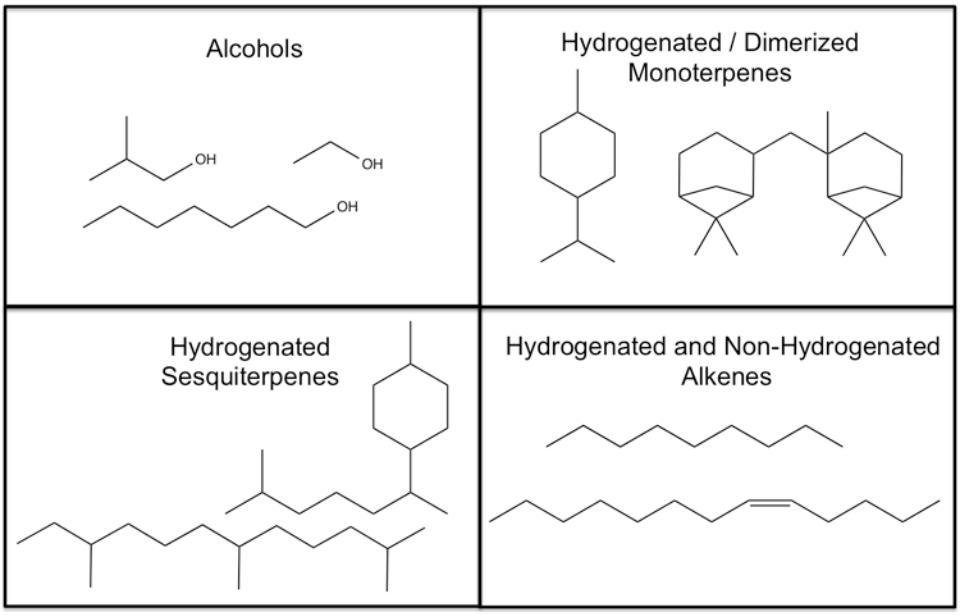

Figure 1. Biofuel molecule classes

Table 1

Fungal observations of molecules of interest.

Fuel Surrogate	Number of Carbons	Fuel molecule	References	Taxa of filamentous fungi observed to produce that molecule	References
Gasoline	3	propanol	(Atsumi et al 2008b; Shen and Liao 2008; Atsumi and Liao 2008; Ammar et al 2013)	*Penicillium spp., Tuber spp., Aspergillus spp., Rhizopus stolonifer, Ascocoryne sarcoides*	(Börjesson et al 1990; Mauriello et al 2004; Splivallo et al 2007; Griffin et al 2010; Beck et al 2012)
	4	isobutanol	(Hazelwood et al 2008; Atsumi et al 2008a; Atsumi et al 2009; Atsumi et al 2010; Smith et al 2010; Chen et al 2011; Higashide et al 2011; Li et al 2012; Brat et al 2012; Lee et al 2012; Urano et al 2014)	*Ascocoryne spp., Tuber spp., Muscodor crispans, Phoma Sp.*	(Díaz et al 2002; Mauriello et al 2004; Splivallo et al 2007; Strobel et al 2008; Griffin et al 2010; Mitchell et al 2010; Strobel et al 2011)
	4	1-butanol	(Atsumi et al 2008a; Lan and Liao 2011; Gulevich et al 2012; Chen et al 2013)	*Ascocoryne sarcoides, Trichoderma viride*	(Griffin et al 2010; Hung et al 2013)
	5	3-methyl-1-butanol	(Atsumi et al 2008b; Connor et al 2010; Shen et al 2011; Li et al 2012; Lu et al 2012; Park et al 2014)	*Ascocoryne spp., Muscodor spp., Aspergillus spp., Boletus variegatus, Penicillium spp., Tuber spp., Trichoderma viride, Phoma Sp., Rhizopus Stolonifer, Laccaria Bicolor, Paxillus involutus, Armillaria mellea, Pholiota squarrosa, Verticillium longisporum, Stropharia rugosoannulata,*	(Stotzky and Schenck 1976; Börjesson et al 1990; Strobel et al 2001; Díaz et al 2002; Mauriello et al 2004; Meruva et al 2004; Splivallo et al 2007; Griffin et al 2010; Mitchell et al 2010; Strobel et al 2011; Hung et al 2013; Müller et al 2013; Heddergott et al 2014)
	7	1-heptanol	(Marcheschi et al 2012)	*Ascocoryne sarcoides, Aspergillus spp.*	(Griffin et al 2010; Beck et al 2012)
	8	2-phenyl ethanol	(Etschmann et al 2002; Atsumi et al 2008b; Kim et al 2014)	*Ascocoryne spp., Phellinus sp., Tuber spp., Phoma sp.*	(Stotzky and Schenck 1976; Díaz et al 2002; Mauriello et al 2004; Splivallo et al 2007; Griffin et al 2010; Strobel et al 2011)
	8	1-octanol	(Marcheschi et al 2012)	*Trichoderma viridae, Penicillium glabrum, Rhizopus stolonifer, Aspergillus flavus, Ascocoryne cylichnium*	(Stotzky and Schenck 1976; Chitarra et al 2004; Meruva et al 2004; Splivallo et al 2007; Griffin et al 2010; Beck et al 2012; Hung et al 2013)
Jet fuel	10	α-pinene	(Bokinsky et al 2011; Yang et al 2013; Sarria et al 2014; Scullin et al)	*Fusarium graminearum, Tuber magnatum, Nigrograna mackinnonii, Hypoxylon spp., Annulohypoxylon spp., Aspergillus fumigatus*	(Gioacchini et al 2008; Heddergott et al 2014; Buśko et al 2014; Shaw et al 2015b)
	10	β-pinene	(Harvey et al 2010; Scullin et al)	*Nigrograna mackinnonii, Aspergillus fumigatus*	Shaw and Spakowicz 2015 (Heddergott et al 2014)
Diesel fuel	10	limonene	(Alonso-Gutierrez et al 2013; Davies et al 2014)	*Hypolyon spp., Annulohypoxylon spp., Tuber spp., Trichoderma viride, Nigrograna mackinnonii, Aspergillus fumigatus*	(Mauriello et al 2004; Gioacchini et al 2008; Hung et al 2013; Heddergott et al 2014; Shaw et al 2015b) Shaw and Spakowicz 2015,

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Fuel Surrogate	Number of Carbons	Fuel molecule	References	Taxa of filamentous fungi observed to produce that molecule	References
	15	α-bisabolene	(Peralta-Yahya et al 2011; Phelan et al 2014; Davies et al 2014)	*Armillaria Mellea, Aspergillus fumigatus*	(Müller et al 2013; Heddergott et al 2014)
	15	β-bisabolene	(Peralta-Yahya et al 2011; Phelan et al 2014; Davies et al 2014)	*Trichoderma Harzianum, Laccaria Bicolor, Paxillus Involutus, Paxillus Involutus, Armillaria Mellea, Pholiota Squarrosa, Stropharia Rugosoannulata, Trichoderma Viride, Aspergillus fumigatus*	(Müller et al 2013; Heddergott et al 2014; Zhang et al 2014)
	15	α-farnesene	(Wang et al 2011; Zhu et al 2014)	*Laccaria Bicolor, Paxillus Involutus, Armillaria Mellea, Pholiota Squarrosa, Stropharia Rugosoannulata, Trichoderma Viride, Aspergillus fumigatus*	(Müller et al 2013; Heddergott et al 2014)