Bioprospecting—fuels from fungi

Author: Gary Strobel

The final publication is available at Springer via <http://dx.doi.org/10.1007/s10529-015-1773-9>.

Strobel, Gary Allan. "Bioprospecting—fuels from Fungi." *Biotechnology Letters* 37, no. 5 (February 4, 2015): 973–982. doi:[10.1007/s10529-015-1773-9](http://dx.doi.org/10.1007/s10529-015-1773-9).

Made available through Montana State University's [ScholarWorks](http://scholarworks.montana.edu)
scholarworks.montana.edu

Bioprospecting—fuels from fungi

Gary Allan Strobel

Abstract

The world has a continuing demand and utility for liquid fuels to power its societies. The utilization of crude oil based fuels is leading to a dramatic increase in the CO₂ content of the atmosphere which is being related to a dangerously warming earth. Having liquid fuels that are derived from biological sources is one solution to this growing problem since the carbon being utilized is only from recycled sources. Presently, the microbes, having the greatest impact on the world's economies, producing liquid fuel are various yeasts producing ethanol. Other microbial sources need to be sought since ethanol is not the most desirable fuel and yeasts require simple sugars to carry out the fermentation processes. Recently, several endophytic fungi have been described that make hydrocarbons with fuel potential (Mycodiesel). Among others the compounds found in the volatile phases of these cultures include alkanes, branched alkanes, cyclohexanes, cyclopentanes, and alkyl alcohols/ketones, benzenes and polyaromatic hydrocarbons. Most importantly, generally these organisms make hydrocarbons while utilizing complex carbohydrates found in all plant-based agricultural wastes. Also discussed in this review is a rationale for finding hydrocarbon producing endophytes as well as examples of other promising hydrocarbon producers-Nodulisporium spp. which make 1,8-cineole and families of other hydrocarbons. Extremely favorable results of engine and fuel testing experiments recently completed on cineole and other products of Nodulisporium sp. are also presented. Finally, there is a brief discussion on the main limiting steps in the domestication of these fungi.

Introduction

The recognition that climate change may have consequences for human activities has caused public concerns in many economic, political and scientific arenas. Apparent warming has been suggested to be directly associated with a non-historic rise in the levels of CO₂ and other heat trapping gases in the atmosphere (UN-GAP report 2014). The increased levels of environmental CO₂ is directly related to the consumption of fossil fuels in all sectors of the world that support mobilized-industrialized societies (UN-GAP report 2014). One of the major fossil fuels has utility in many forms, but all originate with crude petroleum oil. Finding substitutes for liquid oil-based fuels, that

represent recycled carbon, rather than mined or drilled carbon (fossil) is a major challenge to the academic and business communities. Thus, this potentially dire situation represents the main impetus for an effort to find and learn more about microbes as a source of hydrocarbons which represents a non-fossil source of liquid fuels.

The most classical fungus (yeast) now serving mankind as a source of fuel (ethanol) is *Saccharomyces cerevisiae*. The organism requires simple sugars in order to grow and make ethanol. If sugar is readily available, and the fermentation process is efficient, then the process is economical. Unfortunately, most would agree, that ethanol is not the most desirable fuel since it has low energy content, and can foul engines. Nevertheless, it seems possible that other microbes exist in nature producing metabolites that have fuel potential and can yield such products whilst growing on complex waste carbohydrates such as corn stover, beet pulp, dead leaves or sugarcane bagasse (Strobel 2014b). Ideally, compounds having fuel potential would necessarily be heavily reduced, have relatively small molecular weight, have high octane ranking and a high enthalpy of combustion (Mallette et al. 2014). Traditionally, it appears as if there has been no concerted search for such organisms much less any understanding of how they might be assayed, or domesticated for energy production.

It is important, in launching a search for microbes having the potential to produce fuel-related hydrocarbons that a scientifically-based rationale be developed. Thus, it seems logical to carefully examine the various explanations as to how crude oil became deposited in the earth's sediments in the first place. It is well recognized the majority of the earth's crude oil has its origins in plant materials (Russell 1960). A commonly held idea on the origin of crude oil is based on the high temperatures, high reducing conditions and high pressures of the earth to transform the large amounts of complex carbohydrates and lignin found in dead plants into oil (Russell 1960). While many of these physicochemical transforming reactions undoubtedly have taken place in the earth, it appears that there are also alternative hypotheses in understanding how oil has been formed. This would especially apply to origins of the carbon backbones of each of the major classes of compounds found in crude oil. One of these is based in a plethora of processes involving the microbiological degradation of plant materials with

the concomitant formation of hydrocarbons (Strobel et al. 2013; Strobel 2014a, b).

It stands to reason that those microorganisms, deemed endophytes, are the first to bring about the breakdown of a plant once it has died since they are already present in its tissues. They exist in the interstitial tissues of the roots, stems, leaves, fruits and flowers of plants (Bacon and White 2000). They do not produce any signs or symptoms associated with their presence and they commonly make a myriad of biologically active products (Strobel and Daisy 2003). Also, it is the case that in any given plant, the roots are the most likely place where endophytes may be successfully isolated. However, under tropical rainforest conditions, endophytes can commonly be isolated from all of the major plant organs (G. Strobel unpublished work). Likewise, many of the endophytic genera isolated from terrestrial plants have their counterparts associated with aquatic plant forms (Sandberg et al. 2014). Furthermore, when a plant dies, the endophytes are the first microbes on site to begin the processes of degradation (Strobel et al. 2013).

Thus, hypothetically, it would seem logical the most logical place to find hydrocarbon producing endophytic microbes would be in geographical areas of the world in which oil has been found or is likely to be found that still retain all or much of the same environmental conditions as they did originally (Strobel 2014b). In other words, the search should be concentrated in areas having high plant diversity along with a warm and damp ecosystem. Basically, this description translates into those oil-bearing areas of the world that are in or associated with tropical/temperate rainforests such as the Amazonian basin, SE Asia, the Pacific Coastal areas and forests bordering the Gulf of Mexico. Alternative avenues in producing microbial hydrocarbons involve synthetic biological or genetic engineering approaches, but this review will not discuss or cover these alternatives.

While some reviews have appeared on hydrocarbon production by fungi, this review represents an update on recent discoveries and progress made on microbes known to make fuel related compounds while at the same time presenting the reader with a brief description on how the initial discovery was made (Strobel 2014a, b). This review does not incorporate recent information on the production of lipids (triacyl- and diacylglycerols by algae, fungi or higher plants) or other alcohols such as isobutanol which are known

fuel sources. Also discussed are the expectations, limitations, shortfalls and challenges awaiting to make fungal hydrocarbon technology a reality.

Mycodiesel

While it is recognized that some fungi make one or more volatile organic compounds (VOC) under laboratory culture conditions and in the niches where they live, most fungi do not produce detectable quantities of VOCs (Strobel et al. 2011). The discovery and characterization of *Muscodora albus* was central to the finding of other fungi producing volatile compounds with a fuel potential (Strobel et al. 2001, Strobel 2011). Since 2001, many other isolates of *Muscodora* have been found and given species designations and each of these fungi make volatile compounds possessing a wide range of bioactivities (antimicrobial, insecticidal, nematocidal) representing a wide range of chemistries (Strobel 2011). This fungus was used to facilitate the recovery of some of its relatives in nature by using it as a selection tool. *M. albus* was first grown on plates and, then, plant materials suspected of harboring other species of this fungus were subjected to the VOCs of the *M. albus* (Mitchell et al. 2008; Strobel 2011). Many other species of this fungus were discovered from wide ranging parts of the earth including India, China and Thailand. However, a totally serendipitous surprise came from some samples obtained from stems of *Eucryphia cordifolia* sampled in coastal temperate rainforests of Chile. These samples yielded an organism that grew in the presence of the VOCs of *M. albus* and it too produced VOCs with antimicrobial activities (Stinson et al. 2003). The organism was morphologically defined as *Gliocladium roseum*, but comprehensive taxonomic studies eventually showed that the organism was a “peculiar” imperfect stage of the ascomycetous fungus-*Ascocoryne sarcoides* (Strobel et al. 2010).

The most important aspect of this fungus was its ability to make an extensive series of the acetate esters of straight chained alkanes including those of hexyl, heptyl, octyl, and sec-octyl alcohols (Strobel et al. 2008). It was immediately recognized that the alkyl alcohol side chains of these acetate esters represent the basic free alkanes of diesel as they specifically have been found in every diesel sample that has been analyzed (G. Strobel—personal observations). Thus,

breaking of the ester bond followed by a reduction of the alcohol to a methyl group would result in an alkane which is identical to that found in diesel. Further work, done independently of the original report, also has indicated the presence of nonyl and decyl esters in the mixture of fungal VOCs (Griffin et al. 2010). While diesel fuels contain a series of straight chained hydrocarbons there also exists branched alkanes, cyclic alkanes, and a myriad of benzene derivatives along with many polyaromatic hydrocarbons (Griffin et al. 2010; Mallette et al. 2014). In addition, analysis of the VOCs of this fungus yielded an array of alcohols, ketones, esters and various other hydrocarbons including derivatized benzenes, cycloalkanes, and cycloalkenes from a cellulose substrate (Mallette et al. 2014). Reassessment of the capability of the organism to produce VOCs yielded an even greater array of VOCs depending upon the substrate on which the organism was grown as well as some components that had and had not been originally reported (Griffin et al. 2010; Mallette et al. 2014). Thus, a complete series of lower mass straight-chained alkane derivatives being produced by an endophytic fungus suggested, for the first time, that microbes might have the capability to produce the basic carbon- skeletons that make up diesel fuel and the VOC mixture of this fungus was dubbed—Mycodiesel (Strobel et al. 2008). Immediately, these initial findings were instrumental in promoting a wealth of physiological, genetic, biochemical and fermentation based studies on this organism and a search for other organisms making similar products (Mann 2014; Ahamed and Ahring 2011).

Ascocoryne sarcoides genetics

After the initial report appeared on Mycodiesel, the Scott Strobel laboratory at Yale University has made a major effort to completely sequence and annotate the genome of *A. sarcoides* (Gianoulis et al. 2012). It has turned out to be the highest quality genome of an endophytic fungus known to date. At least 80 biosynthetic clusters were identified including several previously only found in plants. The authors found many transcriptionally active regions outside of genes suggesting a role of long non-coding RNA in gene regulation. Also, while the analyses has contributed to the understanding of complex carbohydrate degradation by this organism, the lack of information on the biochemical pathways needed for the production of

hydrocarbons made traditional reverse genetics quite difficult. Nevertheless, putative genes involved in the production of fungal hydrocarbons and hydrocarbon derivatives included the identification of 19 polyketide synthetase clusters, and 60 genes with homology to putative alcohol dehydrogenases. Other work on another isolate of *A. sarcoides* has independently confirmed some of these findings (Mann 2014). Finally, the Yale authors used coupled transcriptomics and metabolomics to generate a pathway prediction for the generation of the several octane derivatives commonly found in the VOCs of *A. sarcoides* (Gianoulis et al. 2012). The pathway begins with linoleic acid (octadecadienoic acid) followed by breakdown into a wide range of compounds including 1-octen-3-ol, 2-octen-1-ol, 2-octenal and 3-octanone as per *Escherichia. coli* lysates (Gianoulis et al. 2012). Each of these compounds with the exception of 2-octenal has been recorded in the VOCs of *A. sarcoides* (Griffin et al. 2010). Other final fungal C-8 products include 1-octene, octane and 1,3-octadiene. The original hypothesis for the production of C-8 compounds from linoleic acid involves two enzymes: a lipxygenase and a lyase. However, for reasons unknown, an active lyase has yet to be successfully purified from this fungi. It is to be noted that there is a wide range of products of interest in the fungal VOC mixture including branched hydrocarbons, benzene derivatives and cycloalkanes (Griffin et al. 2010; Mallette et al. 2014). A major challenge exists to metabolically account for each one of these compounds since unique enzymes and pathways are obviously involved. Once this is known it will be possible to go back into the genome and make genetic/pathway assignments.

Ascocoryne sarcoides fermentation

While an understanding of fungal genetics and biochemistry is a critically important aspect of making fungal produced hydrocarbons eventually a commercial reality, so too is an understanding of those factors that govern all of these processes at the fermentation level (Mallette et al. 2014). It turns out that substrate, growth stage, pH, temperature and medium composition are statistically significant factors that dictate the quality and quantity of hydrocarbons produced by *A. sarcoides* (Mallette et al. 2014). Gasoline range,

aviation range, and diesel range organics were detected in all culture media most of which were cellulose based. A large range of hydrocarbon products were observed when the organism was grown on pure cellulose as well as potato/dextrose broth including alkanes, branched alkanes, aromatics, poly-aromatic hydrocarbons, esters, acids, ketones, alcohols, and a plethora of unidentified volatiles. The greatest yield was 105 mg of total product per gram of pure cellulose. In the late stationary stage more oxygenated compounds appeared with longer carbon chain length and fewer fuel-related compounds (Mallette et al. 2014). It appears that control of the composition of fuel-like compounds can be made by changing the culturing parameters of the fungus (Mallette et al. 2014). Ultimately, it appears that many challenges still exist in understanding VOC production in *A. sarcoides* and much more work will be needed to make the hydrocarbon products of this fungus a commercial reality.

A search for other hydrocarbon-producing fungi

In the past few years a search for other hydrocarbon producing fungi has resulted in the discovery of a number of isolates of *Nodulisporium* sp. (Ascomyceteous fungus) having been found in such locations as the Canary Islands, the Ecuadorian Amazon, the Pacific Northwest, the jungles of Thailand and Nicaragua, South Australia, Pacific coastal Colombia and the wetlands of Florida. Each of these isolates makes a plethora of VOCs including many with fuel potential and there is a recent review and other papers cover these developments (Strobel 2014a, b; Tomsheck et al. 2010). The genomes of many of these organisms have been sequenced and some annotation has been accomplished (Gladden et al. 2013). Literally, hundreds of genes for both plant cell wall and polymer degradation have been observed along with hundreds of genes for secondary metabolite production. Many of these *Nodulisporium* spp. isolates produce 1,8-cineole as one of their main VOCs [Fig. 1]. However, while many of the VOC analyses of *Nodulisporium* spp. have appeared in the literature, it seems as if there is a common theme and that is each isolate makes a widely different gas profile analysis from the one first reported (Tomsheck et al. 2010; Hassan et al. 2013;

Fig. 1 The structure of 1,8-cineole

Mann 2014; Mends et al. 2012; Gladden et al. 2013). Cineole appears in the gas phase of each isolate but, in some cases, the list of other metabolites varies enormously. A wide range of other useful compounds are also made by various *Nodulisporium* spp. including terpenoids, cyclohexanes, benzene derivatives, esters, ketones, straight-chained and branched hydrocarbons. The wide variation in VOCs being produced by *Nodulisporium* spp. can be even more generally enhanced by considering that this organism is under epigenetic control (Hassan et al. 2012). Those agents that generally interfere with this mechanism, such as the histone deacetylase and a DNA methyltransferase inhibitor—suberoylanilide hydroxamic acid (SAHA), as well as 5-azacytidine (AZA), do have an effect on the quality and quantity of VOCs being emitted by this organism (Hassan et al. 2012). Also affected are the cultural characteristics of the organism including pigmentation, and mycelial growth patterns (Hassan et al. 2012). This observation greatly adds to the potential biochemical resource that might be realized not only in this organism but possibly many other microbes as well (Hassan et al. 2012).

Still other factors seem to be involved in the biochemical regulation of hydrocarbon production in yet another isolate of this organism. Initial observations of the fungal isolate Ti-13 of *Nodulisporium* sp. suggested that reversible attenuation of the organism via removal from the host and at least five successive transfers in pure culture resulted in a 50 % decrease in cineole production unrelated to an overall alteration in fungal growth (Nigg et al. 2014). A compound (CPM₁) was obtained from *Betula pendula* (silver birch) that increases the production of 1,8-cineole by an attenuated Ti-13 strain to its original level as measured by a novel bioassay method employing a 1,8-cineole-sensitive fungus - *Sclerotinia sclerotiorum* (Nigg et al. 2014). The host plant (*Cassia fistula*) produces similar compounds possessing this activity.

Bioactivity assays with structurally similar compounds such as ferulic acid, gallic acid and others suggested that the CPM₁ does not act as a simple precursor to the biosynthesis of 1,8-cineole. These *para*-substituted compounds also reversed the attenuation of cineole biosynthesis in *Nodulisporium* sp. NMR and HPLC/electrospray ionization mass spectrometry indicated that the CPM₁ is a *para*-substituted benzene with alkyl and carboxyl substituents (Fig. 2).

A new isolate of *Nodulisporium* sp. with a very peculiar VOC content was obtained from a cypress knee (*Taxodium distichum*) in a swamp in central Florida. It was cultured on potato dextrose agar and the gas phase analyzed by the SPME fiber technique (G. Strobel and MT Mends unpublished work). As expected, cineole was a major ingredient but also present was a list of unexpected highly-derivatized cycloheptenes and a cycloheptane that are energy-rich compounds (Table 1). In addition, there were a number of other terpenes including caryophylenes, and thujopsene along with naphthalene- and azulene-related compounds. The list is rounded out with the presence of alcohols plus 2-hexanone, 4-methyl-. A closer look will undoubtedly reveal that many of these volatiles have fuel potential. These results only beg the question as to what other VOCs are being made by isolates of this organism waiting to be discovered.

The majority of endophytic fungi that are isolated from all plant sources turn out to be ascomycetes. Most recently, however, a VOC producing basidiomycete did appear as an endophyte from *Cannabis sativa*. Analysis of its gas phase, as performed by the methods described, revealed an interesting surprise (Strobel et al. 2001). Some of the main ingredients were *alpha*-bergamotene, and *beta*-bisabolene (Table 2). These are compounds with enormous fuel potential (Peralta-Yahya et al. 2011). The biosynthetic pathway for bisabolene has just been engineered into *E. coli* (Peralta-Yahya et al. 2011). The basidiomycetes are the most important complex carbohydrate-digesting organisms on the planet. Now the prospect exists that it is reasonable to expect that many other basidiomycetes may also have pathways for hydrocarbon production and they too should be examined for this potential. Most of these organisms will not be found as endophytes but as saprophytes living on organic debris in the forests of the world.

Fig. 2 NMR data for the CPM₁. **a** A proposed structure for the CPM₁ based on NMR data. **b** Assignments and HMBC correlations for the CPM₁. **c** The ¹H NMR spectra of the CPM₁. Negative peaks at 3.2, 4.6 and 4.8 ppm represent partially suppressed solvent resonances (Nigg et al. 2014)

Fuel and engine testing

The discovery of microbes making fuel-related hydrocarbons is only the first step in realizing the commercial potential of these compounds. Thus, as wisely suggested by the authors of a recent DOE report (Gladden et al. 2013), it is critical that any newly proposed compound, having fuel potential, be fully evaluated for its engine compatibility, fuel mixing potential, burning characteristics and a myriad of other properties as a fuel compound. In this regard, cineole, which has been demonstrated to be made by many *Nodulisporium* spp. fits this description (Fig. 1). Minus the bridging oxygen atom in its structure, the molecule is a branched cyclohexane of the type commonly found in diesel. It has an enormous potential as a fuel additive with amounts up to 80 % (v/v) cineole being compatible with gasoline resulting in an octane rating of 95 (Barton and Tjandra 1989; Sugito and Takeda 1981). More comprehensive tests with cineole showed that blending it (10 % v/v) with ultra-low diesel improved the cold filter plugging point, the cetane number and oxidative stability in comparison to the diesel itself (Windy-Boy et al. 2014). There were also

significant reductions in exhaust emissions with regard to total particulate matter, and total hydrocarbons and carbon monoxide (Windy-Boy et al. 2014). In addition, Jet-A when blended with 20 % cineole resulted in no significant changes in its fuel properties or its distillation characteristics. Other chemical tests on cineole including cloud point, flash point, kinematic viscosity, copper strip corrosion tests and distillation properties were each compatible with acceptable fuel performance properties. The initial testing results definitely show that cineole has real potential as a fuel additive to many of our most commonly used liquid-based fuels including diesel, Jet-A, and gasoline. In fact, there is enough interest in cineole as a fuel that Virgin and Airbus have announced a program for the acquisition of cineole (eucalyptol) for use in jet engines (Leach 2012).

Another isolate of this organism makes a number of ketones including 2-pentanone, 3-hexanone 4-methyl, 3-hexanone 2-4-dimethyl, and 2-hexanone 4-methyl along with several alcohols, esters and isobutyric acid (Hassan et al. 2013). Fundamental chemistry studies and engine measurements on ketones uncovered some unusual and beneficial characteristics in their ignition

Table 1 The production of VOCs by *Nodulisporium* sp. isolated as an endophyte from *Taxodium distichum* from a swampland in Florida

Retention time (min)	Relative area (ng)	Compound	Molecular weight (g/mol)	Quality* (%)
8.37	79	1-Propanol, 2-methyl-	74	90
8.82	132	2-Hexanone, 4-methyl-	114	90
10.81	85	D-Limonene	136	95
11.17	6,153	1,8-Cineole	154	99
11.41	1,999	1-Butanol, 3-methyl-	88	72
14.13	313	3-Heptanone, 5-ethyl-4-methyl-	156	72
18.32	146	cis-(-)-2,4a,5,6,9a-Hexahydro-3,5,5,9-tetramethyl(1H) benzocycloheptene	204	89
19.43	122	Himachala-2,4-diene	204	87
20.20	257	Tricyclo[5.4.0.0(2,8)]undec-9-ene, 2,6,6,9-tetramethyl-(1R,2S,7R,8R)-	204	93
20.78	84	10 s,11 s-Himachala-3(12),4-diene	204	93
21.61	178	Caryophyllene	204	97
22.71	1,516	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethylidene)-, (4aR-trans)-	204	79
23.10	89	(+)-Epi-bicyclosquiphellandrene	204	89
23.20	276	1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-octahydro-3,5,5-trimethyl-9-methylene-, (4aS-cis)-	204	96
23.37	139	.alpha.-Caryophyllene	204	97
23.52	234	Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-, (-)-	204	87
23.94	339	1H-3a,7-Methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3R-(3 α ,3a β ,7 β ,8a α)]-	204	83
24.26	236	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	204	93
24.42	882	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1 α ,7 α ,8a β)]-	204	99
24.65	264	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR-trans)-	204	74
25.37	651	Epizonarene	204	94
25.56	155	Thujopsene	204	70
28.94	70	Phenylethyl alcohol	122	97
34.63	4,055	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1 α ,7 α ,8a β)]-	204	92
—	16,793	19 Unknowns	—	70
Overall area	35,261			

The VOCs were analyzed by the SPME fiber technique after the organism was grown on PDA for 10 days (Strobel GA and Mends MT, unpublished work, 2014)

* Quality is defined as the relative fit of the data to that of an authentic standard compound in the data base used as a match

chemistry. The cyclic ketones, exemplified by cyclopentanone, are resistant to autoignition and show low reactivity in HCCI (homogeneous charge compression ignition) engines (Gladden et al. 2013). Further testing of these ketones and their fuel potential is underway.

Pressing issues for the future

While the discovery of microbes making hydrocarbons is a necessary first step in the development and commercialization of these products it is by no means

Table 2 The production of VOCs by *Schizophyllum commune* isolated as an endophyte from *Cannabis sativa*. The VOCs were analyzed by the SPME fiber technique after the organism

was grown on PDA for 10 days (SR Hassan, GA Strobel, unpublished work, 2013)

Compound	Retention time (min)	Conc. (µg/mL)	Quality* (%)	MW
2-Propanone	2.52	52.48	86	58
2-Butanone	3.56	43.86	80	72
Acetic acid, 2-methylpropyl ester	5.72	63.78	72	116
1-Propanol, 2-methyl-	7.86	115.11	91	74
1-Butanol, 3-methyl-	10.84	51.02	78	88
<i>alpha</i> -Bergamotene	23.27	78.47	87	204
<i>beta</i> -Bisabolene	24.09	209.24	95	204
2-(Acetylmethyl)-(+)-3-Carene	24.42	119.42	72	192
Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	25.20	55.72	94	202
Diepi-alpha-cedren I	32.67	68.00	89	204

* Quality is defined as the relative fit of the data to that of an authentic standard compound in the data base used as a match

an end unto itself. It is just the beginning. As mentioned above, testing the engine performance properties and other fuel-characteristic qualities of the fungal product is also a crucial step in making a fuel performance microbial hydrocarbon (s).

The economics hydrocarbon production begins with the cost and availability of the feed stock for the microbe. Thus far, it has turned out that the majority of the VOC producing-endophytic microbes (*A. sarcoides*, *Nodulisporium* sp., *Phoma* sp., *Phomopsis* sp.) have the capability of utilizing complex carbohydrates (organic agricultural and forestry wastes) whilst simultaneously making hydrocarbons (Strobel 2014a, b). Thus, the cost of this resource is minimal i.e. \$25 per ton of sugar beet pulp on site.

However, the main hurdle in the commercialization process is the yield of the product which allows it to be compatible with the economics of other crude-oil based fuels or ethanol. This problem is multi-fold and begins with the acquisition of data from carbon balance experiments that will give one an estimate of the successful conversion of the food stock into the hydrocarbon product (Strobel 2014b). In the case of *Nodulisporium* sp.-CI-4, this value is in the range of 1–2 % i.e. carbon in the feedstock vs carbon recovered as cineole and other trappable hydrocarbon products (Strobel 2014b). This number is close to acceptable if one were making cineole as a green chemical but not as a fuel-related compound valued in the range of \$3–\$7 per US gallon.

The solution to the problem is multi-fold beginning with the manner and conditions in which the fermentation process proceeds. Generally, the best conditions are not used as standard practice with yeast fermentation. It appears to be the case that the endophytic microbes, making hydrocarbons, prefer solid state conditions for fermentation (Strobel 2014a, b). Then, once the mycelium is established, the fermentation can be maintained by continually feeding the mycelium that is already established on the agricultural waste. In this case, the VOCs are constantly being swept from the fermentation vessel. Thus, there is little to no concern that either major or minor products in fermentation will be accumulated to a point that they will inhibit enzymatic process in VOC production or even kill the source microbe. In this manner, the process can be extended for many weeks as the microbe continues to breakdown and utilize the carbohydrates in the biomass. Other parameters could also be adjusted to better accommodate and optimize the process including regulation of pH, temperature and constant removal of secondary products.

The factor likely to greatly influence the production of cineole and related products in *Nodulisporium* sp. CI-4 is the development and utilization of a mutant that is facilitated for hydrocarbon production. While such a mutant has not been developed, it seems possible that random mutants via the use various agents including chemicals and UV radiation could be generated. This seems likely since it has already

established that both epigenetic and plant secondary products are influencing the genetic regulation of cineole production in this organism (Hassan et al. 2012; Nigg et al. 2014). In order to acquire mutants of this organism that are producing increased amounts of cineole, a selection mechanism will be needed. It seems possible to employ the unique fact that *S. sclerotiorum* is extremely sensitive to this compound as a means to select cineole-facilitated mutants (Tomscheck et al. 2010; Nigg et al. 2014).

Conclusions

Some, recently isolated and studied, endophytic fungi make and release products that are fuel-related hydrocarbons. Many of the products have been identified by spectroscopic means and still many others in the VOC stream of these organisms remain chemically unidentified. Some of these fungi have been completely sequenced and partially annotated with one in particular—*A. sarcoides* being the best example yet of an endophyte that has been completely annotated as well as possible with existing molecular information in the literature.

Most of the studied VOC-producing fungi are ascomycetes but this report provides some promise that basidiomycetes also can make promising fuel-related compounds (Table 2). Thus, it would appear that many more fungi and bacteria are yet to be found that make promising fuel-related hydrocarbons. In addition, many of the VOC producing fungi can grow and thrive on agricultural wastes whilst making and releasing VOCs (Gladden et al. 2013). In fact, *A. sarcoides* is capable of VOC production while utilizing pure cellulose as its carbon source (Malette et al. 2014). The production of VOCs is also related to the environment in which the organism is grown (Malette et al. 2014). Hydrocarbon production, in the organisms studied, is under metabolic control including epigenetic as well as by induction mechanisms which suggests that these control mechanisms maybe overcome by proper mutation and selection techniques (Hassan et al. 2012; Nigg et al. 2014). Ultimately, the potential of domesticating any of the VOC producing fungi will be dependent upon the type of fermentation system that is used as well as developing strong and stable mutants of the organism that are facilitated for the production of one or more products of importance.

Acknowledgments The author wishes to acknowledge the financial support of the NSF via a grant to Dr. Brent Peyton of the MSU Department of Chemical and Biological Engineering and the DoE to Dr. Gary Strobel. Technical and financial support were also provided from Endophytics LLC of 920 Technology Blvd. Suite 201, Bozeman, MT.

References

- Ahamed A, Ahring BK (2011) Production of hydrocarbon compounds by endophytic fungi *Gliocladium* sp. grown on cellulose. *Bioresour Technol* 102:9718–9722
- Bacon CW, White JF (2000) Microbial endophytes. Marcel Dekker Inc., New York
- Barton A, Tjandra J (1989) Eucalyptus oil as a cosolvent in water-ethanol-gasoline mixtures. *Fuel* 68:11–17
- Gianoulis TA, Griffen MA, Spakaowicz DJ, Dunican B, Alpha CJ, Sboner A, Sismour M, Kodira C, Egholm M, Church G, Gerstein MB, Strobel S (2012) Genomic analysis of the hydrocarbon-producing, cellulolytic, endophytic fungus *Ascocoryne sarcoides*. *PLoS Genet* 8(3):e1002558. doi:10.1371/journal.pgen.1002558
- Gladden AM, Taatjes CA, Gao C, O'Bryan G, Powell AJ, Scheer AM, Turner K, Wu W, Yu ET (2013) Tailoring next-generation biofuels and their combustion in next-generation engines. Sandia rep 2013–10094
- Griffin MA, Spakowicz DJ, Gianoulis TA, Strobel SA (2010) Volatile organic compound production by organisms in the genus *Ascocoryne* and a re-evaluation of myco-diesel production by NRRL 50072. *Microbiology* 156:3814–3829
- Hassan R, Strobel GA, Booth E, Knighton B, Floerchinger C, Sears J (2012) Modulation of volatile organic compound formation in the mycodiesel producing endophyte—*Hypoxylon* sp. CI-4. *Microbiology* 158:465–473
- Hassan SR, Strobel GA, Geary B, Sears J (2013) An endophytic *Nodulisporium* sp. from Central America producing volatile organic compounds with both biological and fuel potential. *J Microbiol Biotechnol* 23:29–35
- Leach A (2012) Airbus and Virgin join forces to develop fuel from eucalyptus trees. Supply management (web site)
- Malette N, Pankratz E, Busse S, Strobel GA, Carlson R, Peyton B (2014) Evaluation of cellulose as a substrate for hydrocarbon-fuel production by *Ascocoryne sarcoides* (NRRL 50072). *J Sustain Bioener Syst* 4:33–49
- Mann R (2014) PhD thesis—Genomic and metabolomic diversity of the native bioprotectant endophytes, *Nodulisporium* and *Ascocoryne*. Latrobe University, Melbourne
- Mends MT, Yu E, Strobel GA, Hassan SRU, Booth E, Geary B, Sears J, Taatjes CA, Hadi M (2012) An endophytic *Nodulisporium* sp. producing volatile organic compounds having bioactivity and fuel potential. *J Petrol Environ Biotechnol*. doi:10.4172/2157-7463.1000117
- Mitchell AM, Strobel GA, Hess WM, Vargas PN, Ezra D (2008) *Muscodor crispans*, a novel endophyte from *Ananas ananassoides* in the Bolivian Amazon. *Fungal Divers* 31:37–43
- Nigg J, Strobel GA, Knighton B, Hilmer J, Geary B, Hassan SR, Harper J, Valenti D, Wang Y (2014) Functionalize *para*-substituted benzene as 1,8-cineole production modulators

- (CPMs) in an endophytic *Nodulisporium* sp. Microbiology 160:1772–1782
- Peralta-Yahya PP, Quellet M, Chan R, Mukopadhyay A, Keasling J, Lee TS (2011) Identification and microbial production of a terpene—based advanced biofuel. Nat Commun 2:483. doi:10.1038/ncomms1494
- Russell WL (1960) Principles of petroleum geology. McGraw-Hill Co, N.Y.
- Sandberg DC, Battista L, Arnold E (2014) Fungal endophytes of aquatic macrophytes; diverse host—generalists characterized by tissue preferences and geographic structure. Microb Ecol 67:735–747
- Stinson M, Ezra D, Hess WM, Sears J, Strobel GA (2003) An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. Plant Sci 165:913–922
- Strobel GA (2014a) The story of mycodiesel. Curr Opin Microbiol 19:52–58
- Strobel GA (2014b) Methods of discovery and techniques to study endophytic fungi producing fuel-related hydrocarbons. Nat Prod Rep 39:259–272
- Strobel GA, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491–502
- Strobel GA (2011) *Muscodor albus* and its biological promise. Phytochem Rev 10:165–172
- Strobel GA, Dirksie E, Sears J, Markworth C (2001) Volatile antimicrobials from a novel endophytic fungus. Microbiology 147:2943–2950
- Strobel GA, Knighton B, Kluck K, Ren Y, Livinghouse T, Griffin M, Spakowicz D, Sears J (2008) The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum* (NRRL 50072). Microbiology 154:3319–3328
- Strobel GA, Tomsheck A, Geary B, Spakowicz D, Strobel S, Mattner S, Mann R (2010) Endophytic strain NRRL 50072 producing volatile organics is a species of *Ascocoryne*. Mycology 1:187–194
- Strobel GA, Singh SK, Riyaz-Ul-Hassan R, Mitchell A, Geary B, Sears J (2011) An endophytic/pathogenic *Phoma* sp. from creosote bush producing biologically active volatile compounds having fuel potential. FEMS Lett 320:87–94
- Strobel GA, Booth E, Schaible G, Mends MT, Sears J, Geary B (2013) The Paleobiosphere a novel device for the in vivo testing of hydrocarbon production-utilizing microorganisms. Biotechnol Lett 35:539–552
- Sugito K, Takeda S (1981). Japan Patent 4,297,109
- Tomsheck A, Strobel GA, Booth E, Geary B, Spakowicz D, Knighton B, Floerchinger C, Sears J (2010) *Hypoxylon* sp. an endophyte of *Persea indica* producing 1,8-cineole and other bioactive volatile with fuel potential. Microb Ecol 60:903–914
- UN-GAP (2014) The emissions GAP report (2014), A United Nations Environment Programme; online
- Windy-Boy J, Maglinao R, Richardson K (2014) Engine performance, exhaust emissions, and fuel properties of cineole blends. MSU Bio-energy center—special report for the State of Montana. Energy Planning and Renewable Energy Program, Department of Environmental Quality Energy and Pollution Prevention Bureau, Helena