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The use of endophytic fungi for the conversion of agricultural wastes to hydrocarbons

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A novel and very promising area in the search for renewable biofuels and green chemicals is the discovery of microorganisms that produce fuel-related hydrocarbons (Mycodiesel) and other useful products. This is in contrast to yeast fermentation that utilizes expensive sugars or starch to produce ethanol which is a proven and useful source of fuel, but for many reasons it is not ideal. Recently, a number of endophytic fungi have been isolated and described that make compounds such as mono- terpenoids, alkanes, cyclohexanes, cyclopentanes, and alkyl alcohols/ketones, benzenes and polyaromatic hydrocarbons. Many of these compounds are either identical to or are closely related to those specific classes of molecules that are found in diesel. Most importantly, these organisms make hydrocarbons while utilizing cellulosic and hemicellulosic polymers found in all plant-based agricultural wastes. Also discussed are the shortcomings, strategies and methods being used to further develop these technologies.

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As the world's liquid fossil fuel sources are ever diminishing, the search for alternative sources of liquid fuels is becoming increasingly important. One immediate source has been the use of yeasts to ferment sugars into ethanol. Because of the requirement that yeasts have for simple sugars to produce ethanol, the process is expensive and the alcohol produced is not the most desirable for most of the world's existing internal combustion engines since energy density is poor and fouling of engines is common. In addition, agricultural products that would have normally gone to food and feed have been diverted to energy production driving up costs of the former.

It would seem that microorganisms must exist whose metabolism to some degree is directed toward hydrocarbon production. One of the most effective ways to search for unusually metabolically active microorganisms, producing a wide diversity of secondary metabolites, is to look for such organisms in unique ecological niches. Certainly, one niche that is relatively unexplored is the living tissues of higher plants. The microbes living in these niches are known as endophytes and most commonly the greatest abundance of microbes isolated from these niches are fungi [1]. One obvious reason to search for endophytes is the fact that they, being already present in plant tissues, are the first to bring about tissue degradation upon the demise of their host plant. As such, most endophytic microbes possess a battery of hydrolytic enzymes whose substrates are complex carbohydrates. Most recently it has been discovered that some of these endophytic fungi do, in fact, produce fuel related substances [2]. These new sources of fuel-like compounds are renewable and compatible with existing engine infrastructure, which is based on drop-in fuel technology. In addition, these microorganisms also possess the capabilities of growing and producing gaseous products on agricultural and other cellulosic wastes rather than valuable agricultural products that may also serve as food and feed sources.

The discovery of these microorganisms has been facilitated by the fact that certain endophytic fungi produce volatile products in culture that possess antimicrobial activities. These fungi, especially *Muscodor* spp. can be used as selection tools to eliminate unwanted microbes in the isolation process since many hydrocarbon producing fungi are not sensitive to *Muscodor* spp. volatiles [3]. Some reviews on the bioactivity of endophytic microorganisms producing such volatile products have appeared [4-5]. However, uniquely, the main theme of this report is a discussion of the endophytic fungi producing fuel-related products and the potential that these

organisms have for being genetically improved and ultimately domesticated to make economical amounts of important products.

Endophytic fungi

Endophytic fungi are found throughout nature as they inhabit the interstitial spaces of plant tissues and cause no apparent outward evidence of their presence in the plant [1]. Apparently, the relationship existing between the microbe and the plant can vary from symbiotic to borderline pathogenic. Endophytes, at this point, represent a small fraction of the nearly 500,000 known fungal species and it is estimated that nearly 1,000,000 additional fungi remain to be discovered. They are associated with the roots, stems, leaves, flowers and fruits of plants and usually, upon sampling, one or more of these tissues will yield an endophyte. The enormous vastness of the plant kingdom (ca. 300,000 species) is only outweighed by the sheer diversity and numbers of the microbial floral species existing in these niches which is a great deal more. Ecosystems exhibiting the greatest plant diversity also seemingly exhibit the greatest abundance and diversity of endophytic populations [6]. Rainforests have been targeted for numerous searches for endophytic microbes as they claim over 60% of the world's terrestrial biodiversity, yet endophytes also exist in oceanic species such as kelps and the roots of mangroves and other marine associated plant species. Ultimately, biological diversity implies chemical diversity as constant chemical innovation is required in such highly competitive environments. It is also worthy to note that finding taxonomic novelty among organisms living as endophytes is extremely likely [6]. One must then use both molecular as well as morphological characters to best characterize the organism.

Culturing and analyzing fungal cultures

Most endophytes can be readily cultured on standard laboratory media with potato dextrose broth being one of the favorites [6]. Submerged culture as well as surface culture on agar broths can be made with these organisms for eventual chemical analyses [6]. These organisms will generally also grow on specialty media made with minimal salts, a nitrogen source and some appropriate carbohydrate polymer such as cellulose or hemicellulose [6].

Quite unlike the large number of techniques and instrumentation that are available for standard natural products chemistry, there is a relative dearth of tools available to do accurate measurements on volatile organic compounds (VOCs) that are hydrocarbon based from microbial fermentation. Thus, since the production of hydrocarbons by fungi is a relatively new observation the methods for analyzing these compounds have been borrowed from other applications. One of the most frequently used methods, in fungal studies, for a getting qualitative assessment of VOCs being produced by fungi is the SPME (solid phase micro-extraction) method [2]. However, quantification of the VOCs is extremely important especially for reasons relating to the potential commercialization of the products. A relatively new technique employing proton transfer reaction mass spectroscopy PTR-MS has been successfully employed to do direct real time monitoring of VOC production by fungal cultures[7]. Likewise, NMR (nuclear magnetic resonance) techniques have been adapted to monitor hydrocarbon production by fungal cultures [8]. The newly developed carbon-trap technology used in concert with carbon dioxide monitoring has been proven used in getting carbon –balance data from fungal cultures [9]. In this manner experimental set-ups are designed to study the efficiencies of conversion of cellulosic substrates to hydrocarbons .

What is Diesel?

Since this discussion relates to fungal products that are identical or are related to substances found in Diesel it is worthwhile to be reminded Diesel fuel is obtained by heating crude oil to 250-350 C for fractional distillation purposes. The product contains a series of compounds ranging from about C-5 to C-21. Analyses of diesel fuels obtained in different parts of the world reveals that, each while unique, all contain representatives of four major classes of compounds for purposes of this discussion. These are represented by Class 1-which are the straight chain and branched alkanes (pentane, hexane, heptane, octane etc and branched hydrocarbons such as 3-methyl nonane and many others). Class 2 compounds are the cyclic alkanes and their derivatives exemplified by cyclohexane, cyclopentane and a number of cycloalkanes decorated with various hydrocarbon side chains. Class 3 compounds are represented by a large number of benzene derivatives having from 0 to all carbon atoms in the ring possessing a hydrocarbon side chain. Finally, class 4 is a large group of substances that qualify as poly- aromatic hydrocarbons which

are represented by well hydrogenated naphthalene and azulene-like substances [10]. In addition, other miscellaneous heterocyclic substances are also found in diesel [10].

Some Specific Examples of Hydrocarbon Producing Endophytes

While a number of reports have appeared in the literature concerning the production of various volatile compounds by fungi in general, it appears that until relatively recently, it seems that no one has made the connection between fungal VOC production and the potential that fungi may have for fuel production [11]. Many studies on fungal volatiles have been done on microbes that invade moist walls or are products of the fungal induced decay of wood [12]. Now, a much wider range of fungi have been discovered that make compounds having potential as fuels.

A. Gliocladium roseum (Ascocoryne sarcoides) and Mycodiesel

An endophytic fungus, *Gliocladium roseum* (NRRL 50072) was isolated from *Eucryphia cordifolia* growing on the Pacific coast slopes of central Chile [11]. The organisms produced a series of hydrocarbons and hydrocarbon derivatives on oatmeal-based agar under microaerophilic conditions as analyzed by SPME-GC/MS. Most strikingly in the VOC mixture was a series of the acetic acid esters of straight chained compounds including those of hexyl, heptyl, octyl, sec-octyl and decyl alcohols [8,11,13]. It turns out that this was the first clue that an endophytic fungus had the metabolic capacity to produce compounds with the structural potential of being converted into the main ingredients of the Class 1 alkanes that are found in all diesel fuels and the VOCs were collectively termed- Mycodiesel. However, various straight chained (free form) have also been reported from this fungus including such compounds as heptane, octane, 2-pentene, dodecane [8,13]. Also found in the culture head space of this fungus are a number of cycloalkanes including 1-methyl cyclohexene, cyclodecane and cyclodecene and these cycloalkanes are representative of the Class 2 hydrocarbons as discussed above as well as some branched chained hydrocarbons such as hexane, 3-methyl, 1,4-hexadiene, 3-ethyl, hexane, 3,3-dimethyl, nonane, 4,5-dimethyl, and undecane, 3-methyl [8,13].

Quantification of volatile organic compounds produced by NRRL 50072, grown on oatmeal agar, as measured by proton transfer mass spectrometry (PTR-MS), resulted in a

level of detectable organic substances in the air space of the oatmeal agar medium in the order of 80 ppmv (parts per million by volume) in a 18 day old culture. Scaling the PTR-MS profile the acetic acid heptyl ester was quantified at 500 ppbv and subsequently the amount of each compound in the GC/MS profile could be estimated and all yielded a total value of ca. 4.0 ppmv. Measurements made by PTR-MS demonstrated its value in estimating the production capability of volatiles by microorganisms [11].

Since *A. sarcooides* produces potential-biofuel metabolites when grown on a cellulose-based medium the genetic pathways needed for this production are unknown and the lack of genetic tools makes traditional reverse genetics difficult [14]. Therefore, the genomic characterization of *A. sarcooides* using transcriptomic and metabolomic data has been done [14]. The data were used to establish a hypothetical base for biofuel production pathways. In total, almost 80 biosynthetic clusters were identified, including several previously found only in plants. Additionally, many transcriptionally active regions outside of genes showed condition specific expression, offering more evidence for the role of long non-coding RNA in gene regulation. The analyses and datasets, now completed on *A. sarcooides*, contribute to the study of cellulose degradation and biofuel production and *provide* the genomic foundation for the study of a model endophyte system involving this fungus. The workers in this group have provided the highest quality fungal genomes and the only thoroughly annotated and transcriptionally profiled fungal endophyte genome currently available [14]. The analyses and datasets contribute to the study of cellulose degradation and biofuel production and provide the genomic foundation for the study of a model endophyte system.

Nevertheless, other workers have acquired *Gliocladium* spp. isolates from a National Collection and proceeded to examine them for hydrocarbon production [15]. It is to be noted that the authentic *Gliocladium* spp., that they acquired is most likely the imperfect stage of either *Nectria* spp. or *Hypocrea* spp. and thus, not necessarily related to the NRRL 50072. They also observed that the *Gliocladium* spp. isolates produced C6 –C19 hydrocarbons directly from cellulosic biomass and they showed the presence of Class 1 and Class 3 hydrocarbons from these fungi. Compounds such as hexane, heptane, 1-octene, hexane 3,4 dimethyl, nonane, 3-methyl along with dodecane, tridecane, hexadecane, nonadecane and benzene were found in the gas phase of the cultures. However the production of

hydrocarbons was nearly doubled when the fungi were co-cultured with *E. coli*. No precise quantitative data on hydrocarbon production by these fungi was offered by these authors

B. *Nodulisporium* sp. (*Hypoxylon* sp.) making hydrocarbons

An endophytic fungal strain of *Hypoxylon* sp. (CI-4A) was isolated from *Persea indica* an evergreen tree native to the Canary Islands where it grows not in abundance but is found on several islands including Tenerife in the Laurisilva [7]. This organism was isolated as its imperfect stage (*Nodulisporium* sp.) from a small stem in the crown of the tree and it readily grows in the presence of the VOCs of *M. albus* which should facilitate its isolation from other plant sources. When grown on PDA –Petri plates, the VOCs produced by this fungus were primarily 1,8-cineole and 1-methyl-1,4-cyclohexadiene. Not only these but many of the compounds made by this organism are of interest because of their high energy densities and thus the potential they might have as Mycodiesel fuels [7].

Six day old cultures of *Hypoxylon* sp. displayed maximal VOC- antimicrobial activity against *Botrytis cinerea*, *Phytophthora cinnamomi*, *Cercospora beticola*, and *Sclerotinia sclerotiorum* suggesting that the VOCs may play some role in the biology of the fungus and its survival in its host plant. This is also the case with most of the other hydrocarbon-VOC producing endophytes, that is the volatile fraction of the fungus has antimicrobial activities. In fact, this discovery has implications in developing methodology for strain improvement via mutation /selection techniques [7]. Continuous direct on-line quantification of VOCs produced by this fungus was measured by proton transfer mass spectrometry (PTR-MS) covering a 12 day range with optimum VOC production occurring at 6 days at 145 ppmv with a rate of production of 7.65 ppmv/hr [7]. The production of 1,8-cineole from a fungal source is of significant interest given the fact that this compound does not have any previously known biological sources aside from plant tissue, and has thus far limited the compound's availability for fuel purposes.

Biosynthesis of 1,8-cineole, in plant tissues involves its conversion from geranyl pyrophosphate by 1,8-cineole cyclase (cineole synthase), whose activity is inhibited by cysteine- and histidine-directed reagents but protected by substrate-metal ion complexes, with the ether oxygen atom of this oxygen-containing terpene being solely derived from water [7]. However, molecular biological studies on *Hypoxylon* sp. have indicated that its

cineole synthase is not identical to the one in plants [Schaible and Strobel, unpublished]. Nevertheless, an understanding of these individual pathways and their derivation from a common pathway involving production of geranyl pyrophosphate from mevalonate (MVA pathway) agrees with the idea that *Hypoxylon sp.* may be conditioned for biosynthesis of monoterpenes and subsequent manipulation of these pathways could lead to their optimum production on a mass commercial scale.

Previous studies on 1,8 cineole have shown prevention of phase separation when used as an additive in ethanol-gasoline fuel blends [16]. Furthermore, when fuels comprised of a gasoline/eucalyptus oil mixture (with 1,8-cineole as the major fuel component up to 80% or more) there was an improved octane number and reduced carbon monoxide exhaust [17]. Thus, 1,8- cineole is a worthy target molecule for study and scale up and it has amazing potential for replacing fossil based hydrocarbons as a fuel additive.

There may be factors controlling 1,8 cineole and other hydrocarbon molecule production in this fungus based on the observation that serial transfer of the fungus on PDA resulted in a dramatic reduction of VOC production including 1,8 cineole. Then, when the attenuated organism was placed on certain plant parts and plant extracts the VOC production resumed to normal as measured by PTR-MS. This observation is suggestive of one of more regulatory mechanisms involved in hydrocarbon production that seems to be influenced by the host plant. Recently, the CPM (cineole production modulator) has been isolated, characterized and shown to be a para substituted benzene. The CPM is found in higher plants and expresses activity in the μ molar range [18].

It is also known that the gene expression for hydrocarbon production of this organism can be influenced by compounds known to act as epigenetic modifiers. Thus, there is a possibility for the appearance of new products and/or the increased or decreased production of other VOCs already known from this organism. For instance, it is realized that the complete analyses of fungal genomes in recent times has indicated that many putative biosynthetic gene clusters are located in the distal regions of the chromosomes and exist in a heterochromatin state with the constitutive genes often transcriptionally controlled by epigenetic regulation such as histone deacetylation and DNA methylation [19]. Several studies have demonstrated that the inhibition of histone deacetylase activity, through gene disruption or use of epigenetic modulators, leads to the transcriptional activation of gene

clusters resulting in enhanced production of secondary metabolites [19,20]. Fungi treated with DNA methyl transferase- and histone deacetylase inhibitors exhibited natural product profiles with enhanced chemical diversity demonstrating that the small-molecule epigenetic modifiers are effective tools for rationally controlling the native expression of fungal biosynthetic pathways and generating biomolecules not previously associated with the organism [20,21]. Thus, *Nodulisporium* sp. was exposed to the epigenetic modulators- histone deacetylase- and a DNA methyltransferase inhibitor -SAHA (suberoylanilide hydroxamic acid) as well as 5-azacytidine (AZA), respectively. Subsequently, the organism displayed striking cultural changes including variations in pigmentation, growth rates and odor, in addition to significant differences in the bioactivities of its VOCs [21]. Analyses (GC/MS) of the VOCs produced by the modulated fungus showed considerable variation with the emergence of several compounds not previously observed in this fungus, particularly an array of tentatively identified terpenes such as α -thujene, sabinene, δ -4-carene, γ - terpinene, α -terpinolene and β -selinene, in addition to several primary and secondary alkanes, alkenes, organic acids and derivatives of benzene (Table 1)[20]. Results of this epigenetic study have implications in understanding why there may be a wide range of VOCs found in various isolates of the same fungus in nature as well as the critical importance that epigenetic factors, possibly associated with plants, may play in VOC production by endophytes [21].

C. Other endophytes making fuel related products

There are an increasing number of endophytic microbes that have been isolated and screened for VOC production. A few newly isolated and characterized ones are *Daldinia* sp. (a *Nodulisporium* sp.)(EC-12) which was isolated as an endophyte of *Myroxylon balsamum* found in the upper Napo region of the Ecuadorian Amazon [22]. This organism produced (VOCs) that have both fuel and biological potential. Under microaerophilic growth environments, the organism produced 1, 4-cyclohexadiene, 1-methyl-, 1-4 pentadiene and cyclohexene, 1-methyl-4-(1-methylethenyl)- along with some alcohols and terpenoids of interest as potential fuels.

Likewise an endophytic *Nodulisporium* sp. (*Hypoxylon* sp.) has been obtained from *Thelypteris angustifolia* (Broadleaf Leaf Maiden Fern) in a rainforest region of Central

America [23]. The endophyte produced VOCs that have both fuel and biological potential. When grown on PDA the organism uniquely produced a series of ketones including: acetone; 2-pentanone; 3-hexanone, 4-methyl; 3, hexanone, 2, 4 –dimethyl; 2- hexanone, 4-methyl and 5- hepten, 2- one and these account for about 25 % of the total VOCs. The most abundant identified VOC was 1,8 cineole which is commonly detected in this group of organisms. Other prominent VOCs produced by this endophyte included 1- butanol, 2 methyl and phenylethyl alcohol. Also, of interest was the presence of cyclohexane, propyl which is a common ingredient of diesel fuel. From this work it is becoming increasingly apparent that each isolate of this endophytic organism- *Nodulisporium* spp, including the *Daldina* spp., *Hypoxyton* spp. and *Annulohypoxyton* spp. teleomorphs, seems to produce its own unique set of VOCs with 1,8 cineole as a product common to all.

An unusual *Phomopsis* sp. was isolated and identified as an endophyte of *Odontoglossum* sp. (Orchidaceae), growing in rain forests of South America [24]. This fungus produces a mixture of volatile organic compounds (VOCs) with sabinene (monoterpene), which turns out to be previously known only as a plant product, was identified by the gas chromatography/mass spectrometry under cultural conditions. Other than sabinene, the most abundant compounds recorded in *Phomopsis* culture were 1-butanol, 3-methyl, benzeneethanol, 1-propanol, 2-methyl, benzene methyl, (+ -) and gymnomitrene.

Larrea tridentata (creosote bush) is a pungent aromatic plant (creosote bush) growing the desert regions of Southwestern USA. This plant was studied for its associated endophytes since it produces a plethora of volatile hydrocarbons that are flammable with the rationale that its associated endophytes may also produce identical or related substances. An endophytic fungus (*Phoma* sp.) was isolated from the roots, stems and flowers of this plant [25]. This fungus produced a unique mixture of volatile organic compounds (VOCs) including a series of sesquiterpenoids, some alcohols, and several reduced naphthalene derivatives. Trans-caryophyllene, a product in the fungal VOCs, was also noted in the VOCs of this desert plant. It would appear that this is only one case out of many that may exist in nature in which a microbial endophyte may mimic the biochemistry of its host in order to survive the conditions of a stressful environment. While both the host and the endophyte do produce at least one hydrocarbon in common, namely trans- caryophyllene, the most abundant fungal product is cis- caryophyllene or humulene. The authors point out that the

myriad of VOCs, such as the alcohols, and other reduced products of this organism have potential as bio-fuels [25].

Fungi as fuel producers

While the discovery of microbes that make fuel –related products is the first step in the process of advancing a new fuel source technology, so too are a number of other steps. Equally important are the economics of hydrocarbon production, engine –fungal hydrocarbon compatibility, and the potential for other high end uses of the fungal products. The economics are driven by considerations of all cost inputs versus value of the products. In this case, the input costs for fungal food substrates are minimal generally given the low cost of agricultural wastes. The carbon-balance after fermentation will relate the ability of any organism to convert a substrate to all carbon based products including carbon dioxide and the products of interest. In the case of *Nodulisporium* sp., the conversion to hydrocarbons has been noted as 1-2 % of the weight of the starting materials. Obviously this was not adequate, so physical changes were made in the manner that the fermentation was performed and the conversion rate now approaches 9-10 %. The process is now economically feasible from the standpoint of acquiring green chemicals such as cineole and cyclohexanes from fungal fermentation. With strain improvement and the development of new technologies for culturing and trapping hydrocarbons the processes will become more efficient allowing certain fungal products to be economically feasible as a fuel additive.

Also of note is an extremely important point made in a recent report by the US- DoE- Sandia lab indicating that any proposed fungal fuel products need to be thoroughly studied for engine compatibility [26]. The report also acknowledges that the approach of finding and thoroughly studying the biology of these hydrocarbon producing microbes has merit. In fact, the Sandia lab has sequenced 4 of the cineole producing endophytes and done extensive annotation work on them. Each organism is equipped with hundreds of enzymes that can digest complex carbohydrates. Each also produces cineole as one of its chief volatile ingredients. In this regard, initial studies on the ignition chemistry of cineole shows significant formation of OH indicating the possibility of low-temperature heat release in compression-ignition engines. Direct testing in internal combustion engines has shown that it can serve as a fuel additive up to 70% with gasoline and up to 40 % in diesel [16,17].

The pursuit of the new hydrocarbon fuel technology is being driven by Endophytics of Bozeman, Mt., who in cooperation with Katzen International of Cincinnati Ohio just publicly announced a their cooperative efforts in studying and designing facilities that represent the next step in making the cineole technology a reality. Endophytics also announced that the primary patent for the fungal production of cineole was granted by the US- PTO and other patents are pending.

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