The story of mycodiesel

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Highlights

A number of endophytic fungi produce volatile organic compounds with hydrocarbon-like properties.

These microbes are capable of hydrocarbon production exclusively using agricultural wastes as substrates.

These compounds have the potential to serve as both green chemicals and or fuels (Mycodiesel).

Specific examples are given in this report describing methods used to find and study hydrocarbon production by fungi.

Summary

Recently, a number of endophytic fungi have been discovered that produce volatile organic compounds (VOCs) whilst growing on agricultural waste substrates, whose chemistry is best defined as hydrocarbon and hydrocarbon-like. These compounds have potential use as both "green chemicals" and fuels. This report discusses the discovery of the first fungus proposed as a producer of "Mydodiesel". It also describes the sequence of important steps needed to domesticate these endophytes for VOC production. Also mentioned are many examples of fungi making these VOCs and some of the novel methods that have been specifically developed and used to study the fungal production of hydrocarbons. Finally, the report concludes with a discussion of commercial scale up and feasibility of this approach in helping to solve the world's need for liquid fuels.

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Introduction

Planes, trains, automobiles, boats, buses and trucks are the largest users of liquid hydrocarbon fuels worldwide. These fuels have a huge advantage in driving these devices because of their high volumetric density and relative ease of production, transport and storage. Many factors are now pushing a search for alternative sources of liquid fuels including issues of diminishing supplies of these fossil hydrocarbons and concerns over climate change brought on by increasing levels of greenhouse gases in the world's atmosphere.

Biofuels in the form of plant derived lipids and ethanol from the fermentation of sugars and starch are starting to provide some small relief in the enormous demand for fossil-based hydrocarbon fuels. eg. coal, natural gas and oil. Attempts are being made to find still other biological means to increase the pipeline of renewable liquid fuels. One consideration in this approach is based in the well- known fact that cellulosic materials constitute the world's largest source of organic carbon. There are over 1.3 billion tons of cellulosic materials available each year in the USA alone from non- food crop production [1]. Yeasts, higher plants and algae do not reuse or recycle this important source of carbon, but fungi do. The fungi capable of lignocellulose, and hemicellulose breakdown are those commonly associated with plant decay processes such as the basidiomycetes commonly associated with rotting leaves, stems and other plant parts in all parts of the world.

Other fungi are also capable of degrading plant materials and they are generally represented by a group of organisms known as endophytes [2]. These organisms, mostly fungi of the ascomycetous group, live in association with stems, roots, leaves, fruits and flowers of plants and provide no outward appearance of their presence in the plant [2]. As they invade plant tissues they remain alive but are quiescent. Their biology can be affected by the plant and it appears that their biochemistry is also regulated by plant products. Most certainly, their growth and development in the plant is limited to just a few cell divisions after plant entry. The growth restricting compounds in the plant are probably the tannins and tannin-like substances [3]. However, after the plant dies, rainwater and ground water leach the plant structures. This is an important and critical step since the water soluble inhibitors of endophyte growth are removed in by leaching. It is understood that endophytes are the first group of microbes that have access to the complete corpus of the plant with the largest weight and volume of material being the complex carbohydrates that make up and provide supportive structures of the plant [3].

The utilization of these complex carbohydrates by the biofuel industry has come via the production of ethanol starting by the preparation of sugars from polymeric carbohydrates and this is an expensive process. Ethanol, the end product of this process, is not the most desirable fuel blending substance since engine fouling and other problems have arisen including the diversion of corn from the food and feed stocks of the world. Ideally, it should be possible to find fungi that can consume complex carbohydrates while at the same time producing fuel –like hydrocarbons. Recently, a number of endophytic fungi have been discovered that produce volatile organic compounds (VOCs) whose chemistry is best defined as hydrocarbon and hydrocarbon-like. These compounds have potential use as both green chemicals and fuels. Included in this report is mention of the latest developments in this important emerging field including many examples of fungi making these VOCs and some of the novel methods that have been specifically developed and used to study the fungal production of hydrocarbons. Also mentioned are considerations for commercial scale up and feasibility of this approach in helping to solve the world's need for liquid fuels. Finally, the report concludes with a discussion of the importance of conducting actual combustion studies on fungal VOCs as they may differ from compounds in petroleum distillates [4].

Mycodiesel, the birth of a concept

While it is recognized that some fungi make one or more volatile components under culture conditions and in the niches where they live, most fungi do not produce detectable quantities of VOCs [5][Green, unpublished SGI, San Diego]. An interesting development occurred nearly 15 years ago when *Muscodor albus* was discovered and characterized as an endophytic fungus producing VOCs with impressive and potentially useful antibiotic properties [6]. This was a first in endophyte biology and was a critical development in focusing attention to the prospects that VOC producing microbes could be used in agriculture, medicine and industry. The compounds present in the VOC mixture ranged from small esters, alcohols and acids to azulene and naphthalene derivatives [6]. The bioactivity of these VOCs was broadly ranged and included both pathogenic fungi and bacteria. This fungus was used to quickly find its relatives in nature by simply using it as a selection tool by first growing it on plates and then seeding the plates with plant materials suspected of harboring other species of this fungus. Many other species of this fungus were discovered from many parts of the earth. However, one sampling done from plants in coastal Chile yielded an organism that grew in the presence of the VOCs of *M. albus* and it too produced VOCs with antimicrobial activities [7]. The organism was morphological defined as *Gliocladium roseum,* an endophyte of *Eucryphia cordifolia.* More comprehensive taxonomic studies eventually showed that the organism was actually a "peculiar" imperfect stage of the ascomycetous fungus- *Ascocoryne sarcoides* [8]. Analysis of the VOCs of this fungus yielded an array of alcohols, ketones, and hydrocarbons [9]. 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 [10, 11]. Most importantly, in the initial VOC analysis was the appearance of an extensive series of the acetic acid esters of straight chained alkanes including those of hexyl, heptyl, octyl, and sec-octyl alcohols [9]. Comparable analyses done independently also indicated the presence of the nonyl and decyl esters in the fungal VOC mixture as well, but many other VOCs from this organism have also been reported [10, 11]. It is to be noted that the backbone composition of all diesel fuels are the straight chained hydrocarbons such as hexane, heptane, octane, nonane and decane along with many other ingredients including the branched alkanes, cyclic alkanes, a plethora of benzene derivatives and polyaromatic hydrocarbons [12]. 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**. Then, other major questions arose including what other fuel –like compounds can be produced by this organism and what can be learned from genetic, fermentation and spectroscopic analyses of the organism that relates to hydrocarbon production. The answers to these questions would await further experimentation and development of new techniques for VOC analysis. In the meantime, other field collections of plants around the world have yielded still other microbes having the capability to make hydrocarbon related products as are described below.

The need for novel analytical technologies and Mycodiesel

The most commonly used method for analyzing volatile products from microorganisms is the SPME (solid phase microextraction) technique used in conjunction with gas chromatography/mass spectroscopy followed by matching mass spec data to a standard data base [6]. This method uses a fiber coated with divinylbenzene/carboxen on polydimethylsiloxane which captures volatile organics in the atmosphere of the device. The technique can provide an initial estimate of the number and identity of products in a sample, but it has some limitations relative to fiber trapping efficiencies for certain compounds and it is not a reliable and accurate technique for the quantification of VOCs. To circumvent this limitation a novel Carbotrapping system that uses an appropriately designed stainless column containing an appropriate Carbotrap (Supelco) or other carbon- based material that will effectively trap the VOCs of interest Fig. 1 [13]. Once the VOCs are trapped they are eluted in an oven under a stream of nitrogen which is directed to a liquid nitrogen trap where the gaseous products are collected (Fig.1). The column method can also be effectively used to quantify microbial VOCs, by trapping, drying, and simply obtaining weight differences before and after trapping [13]. Another major advantage of the column is that it will provide a concentrated mixture of products which will greatly aid in eventual SPME analysis [13]. Thus, armed with these and other analytical methods including NMR, and PTR (Proton Transfer Reaction) mass spectroscopy it was possible to obtain the best estimates of amounts and identities of VOCs being made by *Ascocoryne sarcoides* [10,14].

When this organism was grown on a pure cellulose substrate and analyzed by the methods described above it generally produced the greatest variety of VOCs [10]. However, sometimes the same or a different suite of VOCs appeared when other substrates were used including a glucose/ base salts medium or potato dextrose broth [10]. Some of the branched hydrocarbons (compounds with high enthalpies of combustion) detected in the VOCs of this fungus included hexane, 3-methyl-;1-heptene, 6-methyl-; hexane, 3,3-dimethyl-; nonane, 4,5-dimethyl-; undecane, 3-methyl-; and dodecane [10]. Some of the cyclic alkenes/alkanes produced were cyclohexene, 4-methyl; 1, 6 cyclodecadiene, and n-propylidenecyclohexane among others. A plethora of benzene derivatives are made by this fungus including benzene, 1,2,4,5-tetramethyl-; benzene, 2-ethenyl-1,4-dimethyl-; benzene, 2-ethyl-1,4-dimethyl-; benzene, 1-methyl-2-(1-methylethyl)-, and benzene, 1-ethyl-2-methyl- and to round out the list there are some saturated azulenes made by *A. sarcoides* representing the polyaromatic hydrocarbons on the list of compounds normally found in crude oil [10]. The remainder of the list includes alcohols, acids, some unknowns plus the esters previously mentioned but with the appearance of acetic acid, nonyl ester, 5-decen-1-ol, acetate, (E)- and pentanoic acid , pentyl ester to complete the list alkanes (represented by alkyl esters) ranging from 5 -10 carbons that are commonly found in diesel fuel [10,11]. Those molecules having fuel –like potential made by *A. sarcoides* on a cellulose medium represent a production level of 105 mg per g of biomass [10]. Thus, while the organism makes representative products in each of the major classes of compounds commonly associated with diesel fuel, some compounds are not produced most notably certain branched alkanes, certain cyclohexanes, a number of derivatized benzenes and the longer chained hydrocarbons.

Finally, it is to be noted that the proton reaction mass spectrometry (PTR) method has been developed for use in continuous on line-real time monitoring of VOC production by *A sarcoides* and other fungi [14]. This mass spectral technique can be used on-line, real time, to quantify the production of individual fungal volatiles on a continuous monitoring basis [15,16]. It is to be recognized that the PTR-MS instrument ionizes organic molecules in the gas phase through their reaction with H_3O^+, forming mostly protonated molecules (MH^+, where M is the neutral organic molecule) which can then be detected by a standard quadrupole mass spectrometer [15]. This process can be run on air samples with or without dilution, since the primary constituents of air (nitrogen, oxygen, argon and carbon dioxide) have a proton affinity less than water and thus are not ionized and not detected. Most organic molecules (excepting alkanes) have a proton affinity greater than water and are therefore ionized and detected [15]. For instance, this would include hydrocarbons with double or triple bonds, and compounds bearing one or more oxygen atoms. A further advantage of PTR-MS is that from the known or calculated quantities, the reaction time, the amount of H_3O^+ present, and the theoretical reaction rate constant for the proton transfer reaction, the absolute concentration of constituents in a sample can be quantified [14-16]. In order to interpret the PTR –MS spectrum it is critical to have knowledge of the ion signals generated by the molecules of interest. This means that knowledge of the organic constituents in the head space of the culture are known and what specific ions that they will generated by each compound. This kind of information can be generated by the Carbotrap/SPME techniques described above and by examining the PTR – MS of known compounds. At this point, the ion signals in the PTR spectrum can be assigned to specific compounds known to be present in the mixture after appropriate standards have been run. Because the PTR-MS can be run in real time, it will continuously produce data on the concentrations of specific ions of interest over the entire course of a fermentation period [14,16]. A study of volatile compound production by *A. sacroides* (on a minimal medium plus salts) over a 16 day growth cycle was undertaken. Gas phase compounds were measured continuously by PTR-MS and periodically with gas chromatography-mass spectrometry (GC-MS) using head space solid phase microextraction (SPME). The SPME technique revealed some compounds that were not detectable with the PTR-MS, e.g. 4-methyl heptane. The PTR-MS showed the volatile production was dominated by ethanol and acetaldehyde, though the concentration of the remainder of volatiles reached 2,000 ppbv. Notable compounds of fuel interest included nonanal, 1-octen-3-ol, 1-butanol, 3-methyl- and benzaldehyde.[13]

Now, most recently, a PTR-TOF mass spectrometer has been used to identify and quantify compounds in the gas phase of *Hypoxylon* sp. [Knighton and Strobel, unpublished]. This emerging technique holds enormous promise as a means of accurately identifying and at the same time quantifying compounds in the gas phase in microbial fermentations. Again, initially, some information on the nature of the compounds present, as obtained by SPME analysis, is desirable in order to acquire identifiers for specific ions generated by the PTR-TOF.

Carbon balance experiments

The yield of potential fuel products made by any hydrocarbon- producing organism while growing on an agricultural waste substrate is a critically important question. A system to gain this information has been devised and consists of an appropriate bioreactor containing a known amount of substrate for the test fungus, water and a proper sterile air flow plus the test microbe. After a few days of incubation, the output gas flow of the system is connected with a four way nexus valve and monitored. One line leads to a carbon dioxide monitor, one line is passed to a furnace (oven) which burns all volatiles into carbon dioxide which can be monitored, and finally a line leads to a Carbotrap which removes all hydrocarbons prior to being directed to the oven and finally the carbon dioxide monitor (Fig. 2). The information from the monitor is being fed to a computer and the information is constantly monitored (Fig.2.) Thus, with this system it is possible to monitor the total metabolic carbon dioxide, along with the production of hydrocarbons on a continuous basis and obtain data on the conversion rates of the biomass to products of interest (Knighton, Booth and Strobel, unpublished). Although the system has not been applied to *A. sarcoides*, it has been used to monitor other VOC producing fungi and it represents a critical device for evaluating VOC production by microbes and accurately assessing conversion rates and the economics of hydrocarbon production (Knighton, Booth, Strobel, Blatt, unpublished).

Genetic analyses of *A. sarcoides*

It can generally be stated that the genetic pathways needed for the production of fuel-like compounds of *A. sarcoides* are unknown and thus the lack of genetic tools makes traditional reverse genetics difficult [17]. This fungus has been genomically characterized using transcriptomic and metabolomic data in order to describe the genes involved in cellulose degradation and provide hypotheses for the 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 RNAs in gene regulation.

Thus, this is the most thoroughly annotated and transcriptionally profiled fungal endophyte genome currently available [17]. Some hints for the formation of certain products could be found in the analysis. For instance, it is known that lipoxygenases are involved in the formation of C8 alcohols and ketones in fungi via the breakdown of linoleic acid. There are 5 of these enzymes in the *A. sarcoides* genome and two are correlated with C8 production, thus accounting for some of the C8 compounds in the VOC mixture [17]. Understanding of the other products in the VOC mixture of *A. sarcoides* must await further developments in the genetics, and enzymology of the biosynthetic pathways involved in product formation. As an example, Metacyc, a repository of metabolic pathways, contains 8,869 compounds linked to 1,908 known pathways, but this represents less than 1% of the compounds estimated to be produced by micro-organisms [17]. An integrated omics approach could provide a relatively simple means of exploring the biosynthetic potential of uncharacterized non-model organisms [17].

Other hydrocarbon producers

The discovery of other hydrocarbon producing fungi has just begun. Authenticated *Gliocladium roseum* cultures, (from a National Collection- ATCC) were capable of producing complex hydrocarbons under microaerophilic conditions. The compounds detected included: hexane, benzene, heptane, hexane 3,4 –dimethyl, 1- octene, nonane, 3-methyl, dodecane, tridecane among others [18]. Co- culturing with *E. coli* enhanced hydrocarbon production [18]. Recently, a number of *Nodulisporium* sp isolates have been obtained that make a plethora of VOCs including many with fuel – potential and a recent review and other papers cover these developments [19-22]. Many of these organisms have been sequenced and some annotation has been accomplished [23]. Literally, hundreds of genes for both plant cell wall and polymer degradation have been observed along with hundreds of genes for secondary metabolite production. Each of these organisms produces 1,8 cineole as one of its main volatile products. This compound itself has enormous potential as a fuel additive with amounts up to 80% cineole being compatible with gasoline resulting in an octane rating of 95 [20]. Other isolates of this organism make a number of ketones [22]. A wide range of other useful compounds are also made by these organisms including terpenoids, cyclohexanes, benzene derivatives, esters, ketones, straight chained and branched hydrocarbons[19-22]. Another *Nodulisporium* sp. that has been examined makes azulenes, cycloalkanes along with an array of smaller molecular weight alcohols, acids, esters and aldehydes [3].

Economics, combustion green chemicals and Mycodiesel

As can be seen from above, a logical course in the study of hydrocarbon production by these endophytic fungi is first- acquisition of the microbe, second- microbe identification, third- identification of VOC ingredients, fourth- generation of gene sequence information (annotation) all of which is followed by considerations of scale up including genetic mutation/selection techniques or genetic manipulation. It is my opinion that the utility of these hydrocarbon producing organisms will first be realized through the production of “green chemicals” used by the chemical industry for a variety of industrial, medicinal, and household purposes. As an example, the value of cineole for current uses in medicines, flavorings, and lubricants, depending on purity, is between $145 Gal to $500 Gal. However, as a fuel cineole will be to be priced in the range of $3.00 Gal. This means that all costs of production will need to be scaled to this level, but it would appear that “fungal cineole” would first be sold as a green chemical because of its current pricing on the market. The same rationale would apply to all other fungal VOCs whether they be esters, hydrocarbons, alcohols, azulenes or naphthalenes. Since all of the endophytes presently being studied for hydrocarbon production can grow on and produce their products on agricultural wastes, the economics of fuel production by these organisms takes on an entirely different set of considerations vs. yeast and alcohol production via standard fermentation technologies that use sugar or starch. The conversion rates (dry weight basis of agricultural wastes) for hydrocarbon production by the *Nodulisporium* sp. is in the range of 1-2% using the carbon balance experiment as described above. This number can be increased to 10 % conversion by simply properly manipulating the fermenting conditions which makes the entire process economically feasible since the cost of the agricultural wastes are relatively low, generally varying from $4 - 80 per ton depending upon the source and location. Still, a major point of consideration relative to **Mycodiesel** is that some products that are most efficiently produced in novel biomass conversion strategies may be different from the compounds in petroleum distillate fuels and may have poorly known combustion characteristics [23]. An example of this includes the fungal ketones [23]. Furthermore, at the same time as the chemistry of the bio- fuel stream is beginning to change, advanced clean, efficient combustion strategies are emerging, and newly designed engines are often very sensitive to fuel chemistry [23]. Novel fuels may hinder the operation of advanced engines, or they may in fact be enabling: coordinated efforts towards biofuel-engine co-development are needed to target this problem [23]. Thus, besides strategies needed to meet the economic means of converting cellulosic agricultural wastes to fungal VOCs, there must also be major concern over the engines into which the resulting products will be placed. In this regard, combustion studies on open chained ketones have already been conducted at the Sandia National Lab [23]. Gladden et al report that low-temperature oxidation of open-chain ketones such as di-isopropyl ketone (DIPK) displays significant chain-propagating formation of OH radicals, and DIPK shows low-temperature heat release in HCCI (homogeneous-charge compression ignition) operation above 1.8 bar intake pressure, and, unusually, retains significant sensitivity to temperature even under highly boosted conditions. Consequently, DIPK is a promising fuel for high-load HCCI and CPO may be a useful as a knock-resistant spark-73 ignition (SI) fuel. Future work may elucidate the chemical reasons for the unusual pressure and temperature dependence of the DIPK autoignition and further explore the possible uses of cyclic ketones in SI engines [23]. Thus, it appears that Mycodiesel products may initially find their way to the market as simple fuel additives.

Conclusions

Some endophytic microorganisms have recently been shown to produce a wide range of hydrocarbons. These compounds are either identical or closely related to those found in diesel fuels. Novel techniques have been borrowed from existing technologies or invented to trap and quantify these compounds. Genetic studies, on several of these organisms, have revealed that they possess a plethora of genes coding for enzymes capable of degrading complex carbohydrates. Genetic systems responsible for hydrocarbon production by these organisms remain largely unknown. Since the organisms possess the capability to utilize agricultural wastes the economics of hydrocarbon production bodes well for the eventual domestication of one or more of these organisms. It appears that, in general, the endophytes studied thus far, are better able to sustain hydrocarbon production in non- standard fermentation conditions. Studies are underway to understand the ideal conditions for hydrocarbon production and to produce “super producing” strains of the organisms. However, until production levels are maximized, these organisms will probably initially best serve as producers of “green chemicals and, or fuel additives.

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****This is a nice body of work on the logic and methods used to study, at all levels, fungal hydrocarbon production. It reaffirms that the search for these organisms needs to progress and it should be done with all aspects in mind –including the final engine testing methodologies.**

***of special interest**

**** of outstanding interest**

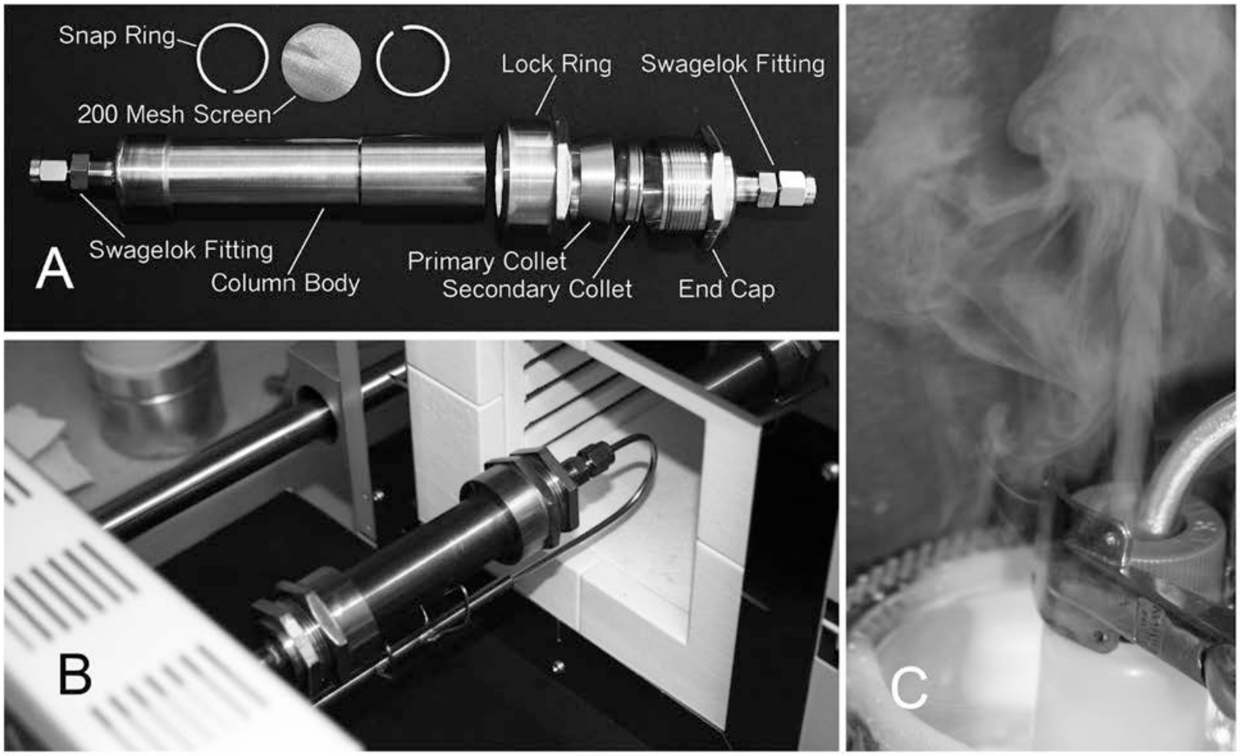

Figure 1. Details of the Carbotrapping system showing the assemblage of the stainless steel column (A), the specially designed oven shown here accepting the assembled column for degassing (B) and the liquid nitrogen trap collecting the fungal VOCs (C).

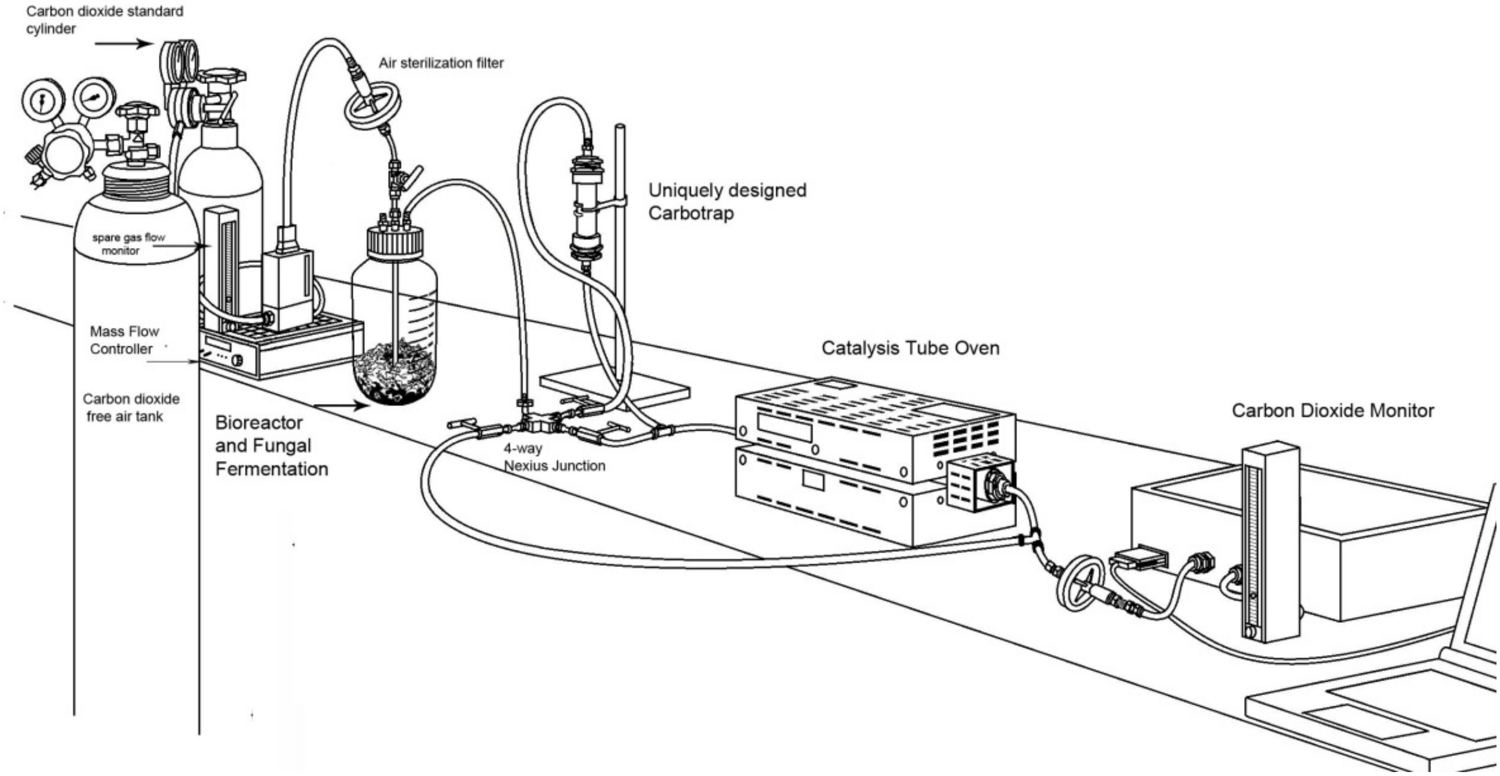

Figure 2. The physical set up of the carbon balance experiment as described in the text of this report. Note the bioreactor containing the fungus used to generate VOCs. The 4 –way nexus valve is periodically adjusted to allow VOC flow directly to the catalysis oven or first through the Carbotrap and then through to the oven. The difference measurements are then indicative of the hydrocarbon production of the organism as recorded by the computer monitor on the far right of the illustration.