2019

Novel Fuel-producing Fungi and Methodologies for Increasing Fuel Production

Yuemin Wang Kovaleff
University of Central Florida

NOVEL FUEL-PRODUCING FUNGI AND METHODOLOGIES FOR INCREASING FUEL PRODUCTION

by

YUEMIN WANG KOVALEFF
M.S. University of Central Florida, 2015
B.S. Southwest University, 2012

A dissertation submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy
in the Department of Chemistry
in the College of Science
at the University of Central Florida
Orlando, Florida

Spring Term
2019

Major Professor: James Harper

ABSTRACT

An endophytic fungus *Hypoxylon sp*. (BS15) has recently been isolated and found to produce volatile organic compounds (VOCs) that have potential relevance as hydrocarbon fuels. In the work described here, the traditional refined carbohydrate (e.g., sucrose) diet source was replaced by simple sugars produced using a solvent free green chemistry mechanocatalytic method involving ball milling in the solid. BS15 is able to grow on this degraded cellulose as well as the more traditional potato dextrose broth. The volatile compounds produced from both media were largely the same.

Unfortunately, it is observed that long term in vitro growth of BS15 results in diminished VOC production. The VOC production was partially restored by cultivating BS15 in growth media containing finely ground woody tissue from the original host plant (*Taxodium distichum*). Extracts from this woody tissue were made by sequentially extracting with dichloromethane, methanol, and water with a goal of isolating VOC production modulators. Both the dichloromethane and water extracts place on bio-mimicking filter paper were found to modulate VOC production, while the methanol extract had no significant impact. Surprisingly, the woody tissue remaining after exhaustive extraction also acted as a VOC production modulator when combined with the growth media, with noticeable changes in the production of four compounds. This woody tissue also induced production of two compounds not observed in the original BS15 extract, and their changes are inheritable. Remarkably filter paper had the same modulating effect [i]as exhaustively extracted woody tissue, suggesting the modulation was partially due to cellulose degradation products.

Extraction of the maximum amounts of VOCs is desirable and here a comparison of solid phase extraction (SPE) and solid phase micro-extraction (SPME) techniques is made. This comparison involves two endophytes, BS15C and *Streptomyces ambofaciens* (SA 40053). The SPE technique is more effective in retaining compounds having lower vapor pressures and higher boiling points with nearly three to five times more VOC mass obtained versus SPME.

Keywords: Volatile organic compounds, solid phase extraction, solid phase micro-extraction, fungi, biofuels, Hypoxylon sp., VOC production

ACKNOWLEDGMENT

First and foremost, I would like to give my special heartfelt thanks to Dr. James Harper, my academic advisor, for everything he has done for me throughout the six years of my Ph.D. journey at the University of Central Florida. All of my research achievements and experiences would not have been possible without his guidance, support, encouragement, and feedback. Dr. Harper is not only my academic advisor, but also a role model, a coach, and a significant mentor in my career. I will carry the knowledge I have acquired from the experience of working with him and learning from him for the rest of my career.

I would also like to express my appreciation to all my committee members: Dr. Andrew Frazer, Dr. Melanie Beazley, Dr. Candice Bridge, and Dr. Richard Blair for their assistance, opinions and time throughout my dissertation. I would like to recognize group members I have worked alongside in Dr. Harper's lab: Shannon Sestile, Domenic Valenti, Dr. Jacob Powell, Giovanna Pope, and Alexander Elliott.

My deepest gratitude goes to my husband and parents for their unconditional love and support during my time as a graduate student at UCF. My parents have sacrificed a lot to help me pursue my dream of living and studying in the United States. I still vividly remember how confused and uncertain about my future I first felt when I came to Orlando six years ago. My husband has been my biggest cheerleader, and has continuously been offering me endless emotional support and encouragement. Thank you all so much for always believing in me. I will continue to strive for excellence in everything I do to keep you proud of my successes in the future!

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CHAPTER 1: INTRODUCTION

1.1 Types of fossil fuels-an overview

For over a century, fossil fuels have powered modern life. Fossil fuels such as coal, natural gas and oil are currently some of the most reliable sources of energy in the world. They are also extensively used as raw materials for a wide scope of secondary products, ranging from diesel and gasoline to chemical and pharmaceutical products. Fossil fuels are expected to dominate the global energy market for the next several decades. In fact, The World Energy Outlook (WEO) 2017 claims that the world's demand for oils will remain robust up until mid-2030.[1] However, as every energy source has some undesirable characteristics, widespread use of fossil fuels leads to the production of greenhouse gases (carbon dioxide, nitrogen oxide, etc.), as well as other significant pollutants, contributing to global warming.[2,3,4,5] Fossil fuels are not classified as a renewable resource as they take millions of years to produce. Given our current consumption of fossil fuel and their rapid depletion, it is imperative that alternative energy sources be developed that are environmentally friendly, scientifically practical, and technologically promising.

1.2 Endophytic fungi producing volatile organic compounds (VOCS)

Endophytes are microorganisms, usually fungi and bacteria that live inside the host plant without producing signs of their presence or causing apparent disease symptoms. They occur in the tissue of the stems, leaves, and roots. The relationship between endophytes and their host plants varies from symbiotic to pathogenic.[6] Research relating to endophytic fungi has drawn considerable attention due to their production of a remarkable variety of natural products.[7,8,9,10] Although there has been significant focus on endophytes, they remain relatively understudied.

In the past few decades, much of the interest in fungal metabolites has been on natural products, especially bioactive compounds.[11] However, numerous microbes also produce volatile organic compounds (VOCs), and recently some work has explored the feasibility of employing VOCs from microbes as fuels. In the work described herein, VOCs are defined as compounds having sufficient volatility to be mobile in gas chromatography. Only fungi producing components having a boiling point range similar to that of fossil fuels have been considered to have fuel potential. One of the most significant microbes discovered to produce fuel was *Saccharomyces Cerevisiae.*[12] This fungus has the ability to produce ethanol under fermentation as long as there is glucose in the media as the carbon source. Unfortunately, ethanol is not the optimal fuel alternative due to its low energy content and compatibility issues with engines. Ideally, compounds having fuel relevance should have relatively small molecular weight, have a high octane ranking, and a high enthalpy of combustion.[13] It is likely to speculate that other microbes existing that can also yield significant amounts of fuel related VOCs, and can produce those VOCs while growing on carbohydrate based media. In 2001, Strobel *et al.* discovered *Muscodor albus* and characterized the VOCs produced by this fungus. Since the discovery of this microbe, many other *Muscodor* species have been also isolated around the world and reported to produce VOCs with fuel potential and a plethora of bioactivities.[14]

Increasingly certain endophytes are being reported that can produce VOCs with potential usefulness as fuels or fuel additives.[15] For instance, Griffin *et al.* isolated *Ascocoryne* genus and demonstrated that the VOCs produced consist of ketones, esters, alcohols, both short and long chain alkanes, and sesquiterpenes.[16] More interestingly, a subsequent reassessment of this organism yielded even more VOCs that not originally reported, including cycloalkanes and benzene derivatives.[17] This study demonstrated that the profile of VOCs produced by the same

organism can also vary depending on the substrate and environment from which the organism was isolated. Among all of the VOCs with fuel potential characterized and reported, 1,8-cineole is a compound of special interest because it is known that a 70/30 (v/v) mixture of petrol/1,8-cineole has performance characteristics similar to petrol with less carbon monoxide emissions.[18,19,20] In 2010, a *Hypoxylon sp*. designated CI-4 was reported as the first non-plant source to produce 1,8-cineole (hereinafter referred to as cineole).[21] Other Hypoxylon sp. Isolated from different sources have also been found to produce cineole.[22] However, the profile of VOCs from these of this species vary enormously. It was observed that the cineole production from CI-4 gradually decreased over a period of time when the organism was removed from the host plant. This decrease suggested the presence of one of more cineole production modulator compounds in the host plant. Nigg *et al.* isolated and characterized a modulator in an endophytic *Nodulisporium* species, the imperfect stage of *Hypoxylon*.[23] This modulator was able to restore cineole biosynthesis, and it is likely that similar outcomes can be obtained in other endophytic fungi where production of valuable products decrease over time. All of these prior studies provide new insight into why such a wide range of VOCs are found in the same organism.

1.3 Methodology

In the work described herein, the methods used to extract volatile organic compounds from fungi cultivated on potato dextrose broth are solid phase micro-extraction (SPME), solid phase extraction (SPE) and liquid liquid-liquid extraction (LLE). Gas chromatography /mass

spectrometry (GC/MS) analysis is employed to identify the VOCs. More detailed descriptions of each technique are summarized in 1.3.1 and 1.3.2.

1.3.1 GC/MS

In chapter two and chapter four, an Agilent 6850 was used with a 5975CVC MS detector a Restek Rxi-5HT capillary column (30 m x 0.25 mm, film thickness 0.25 μm). The carrier gas was ultra-high purity helium with a one cm^3/min constant flow rate and initial column head pressure of 77 kPa. The injector split was set to 250 °C at a 20:1 split ratio with 1 μL volume per injection. The column oven temperature was programmed at 45 °C initial temperature hold for one minute and a 10 °C /min ramp to 100 °C and hold for 5 minutes followed by a 5 °C /min ramp to 200 °C and hold 5 min. The detector was set at a constant 280 °C and set to scan 30-350 m/z. Data acquisition and processing were performed on Agilent MSD ChemStation software. In chapter three, the instrument used was a Finnigan TraceGC ultra with Trace DSQ detector and a Restek Rtx-225 capillary column (cyanopropyl-methyl/phenyl-methyl polysiloxane, 50/50, 30 m x 0.25 mm, film thickness 0.25 μm). The carrier gas was ultra-high purity helium with a 1.5 cm^3/min constant flow rate and initial column head pressure of 77 kPa. The injector was set to 250 °C with a 1 μL injection volume using splitless injection mode to facilitate quantitative analysis. The column oven temperature was initially 45 °C and held for one minute followed by a 10 °C /min ramp to 100 °C where the temperature was held for 5 minutes. Finally, the temperature was increased by a 5 °C /min to 200 °C and held for 7 min. The detector was set at 280 °C and set to scan 50-650 m/z. Data acquisition and processing were performed on Xcalibur software. Identification of the compounds was made via library comparison using National Institute of Standards and Technology (NIST) database.

1.3.2 Solid phase extraction (SPE)

Since its introduction in the mid-1970s, SPE has become one of the most popular techniques in a variety of fields to separate ultra-trace and trace amounts of organic and inorganic compounds.[24] This technique is rapid, simple and recoveries and yields are higher than traditional liquid-liquid extractions, making it ideal for the pre-treatment of various matrices. e.g. water and blood. The sorbents used for SPE are packed into disks or cartridges and are commercially available from a wide variety of suppliers. The cartridges used herein were purchased from Thermo Scientific and Silicycle. The sorbent inside these cartridges was C18 modified silica, which is a highly retentive alkyl-bonded phases. This phase retains most organic analytes, ranging from nonpolar to moderately polar compounds from aqueous samples. SPE processing of a liquid sample usually involves the following steps: Firstly, methanol is utilized to wet the C18 sorbent, then water is used to rinse the column to completely remove all methanol. The solution containing the analytes of interest is then passed through the column under vacuum, and most analytes are retained on the sorbent. The column is then washed with water several times to remove sugars and other less retained compounds and is subsequently dried by drawing air through the column for at least 30 minutes. Lastly, the column is eluted by organic solvent (e.g. acetonitrile) to yield a solution with target analytes before proceeding with the GC/MS analysis.

1.3.3 Solid phase micro-extraction (SPME)

SPME has been widely accepted and utilized in extracting volatile to nonvolatile compounds in a wide variety of matrices after it was introduced by two Canadian scientists in 1990.[25] Apart from having many of the advantages SPE has, SPME is also a convenient and

solvent free technique that doesn't plug or block the pores in the sorbent.[26] By coupling the SPME sampling technique with high-performance liquid chromatography (HPLC) or GC/MS methods, it provides a quick and efficient analysis for volatile organic compounds.[27,28,29] The SPME technique uses a fused silica fiber coated with a thin layer of selective coating to extract organic compounds directly from samples. The fiber used in chapter four is a 2 cm StableFlex fiber coated with Divinylbezene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDTS), which is ideal for analyzing trace amounts of organic compounds within the molecular weight range of 40 to 275 Daltons. There are usually two steps for SPME procedures. The first step is to expose the coated fiber directly to the sample for a period of time. In the second step, the fiber saturated with the target analytes was desorbed, separated and quantified in HPLC system or GC/MS by placing the fiber into the injector, which is heated to desorb analytes.

1.4 Scope of current dissertation research

In the work described herein, an endophytic fungus *Hypoxylon sp.* was isolated from a bald cypress tree (*Taxodium distichum*) in central Florida, USA. This organism produces volatile organic compounds (VOCs) having fuel potentials, most notably 1,8-cineole, 1-pentanol, phenylethyl alcohol, 1-methyl-1, 4-cyclohexadiene. At present, most of the fungi that produce VOCs having fuel potentials discovered so far have been isolated from the plants outside the United States (U.S.), and it's of great importance to acquire organisms from U.S. sources in order to avoid bio-piracy concerns and legal challenges.

In chapter two, simple sugars produced from a solvent-free mechanocatalytic degradation of cellulose were evaluated for suitability as a carbon source for growing fungi that produce volatile organic compounds. Endophytic fungi *Hypoxylon sp.* (CI-4) and *Hypoxylon sp.* (BS15)

known to produce volatiles having potential value as fuels were initially evaluated. Growth was obtained on a medium containing the degraded cellulose as the sole carbon source and the volatile compounds produced were largely the same as those produced from a conventional dextrose/starch diet. The degraded cellulose medium supports growth of BS15 and approximately the same quantity of volatile compounds was produced as from conventional diets. Although the major products from BS15 grown on degraded cellulose were identical to those from dextrose, minor products differed. Extraction of volatiles from growth media was achieved using SPE in order to reduce solvent waste and more efficiently retain compounds having low vapor pressures. A comparison to more conventional liquid-liquid extraction demonstrates that, for CI-4, both methods gave similar results. Solid-phase extraction of BS15 retained a significantly larger variety of volatile compounds than liquid-liquid extraction. These advances positon the coupling of solvent-free cellulose conversion and endophyte metabolism as a viable strategy for the production of important hydrocarbons.

Chapter three mainly focuses on restoring VOCs production of BS15. This is necessary because VOC production decreases after prolonged in vitro growth. This restoration is achieved by growing BS15 in a growth media containing finely ground woody tissue from the original host plant (*Taxodium distichum*). In an effort to isolate VOC production modulators, extracts from this woody tissue are made by sequentially extracting with dichloromethane, methanol and water. Both the dichloromethane and water extracts are found to modulate VOC production while the methanol extract has no effect. Surprisingly, the woody tissue remaining after exhaustive extraction also is shown to act as a VOC production modulator when included in the growth media with changes observed in the production of four compounds. This woody tissue also induces production of two compounds not observed in the original BS15 extract. Filter

paper has the same modulating effect as exhaustively extracted woody tissue, suggesting the modulation is perhaps due to cellulose degradation products.

In chapter four, both the SPME and the SPE techniques are compared and contrasted qualitatively in extracting VOCs with fuel potentials from BS15 and *Streptomyces ambofaciens* broth. Prior to the research described in this dissertation, SPME has been the most commonly utilized technique to extract VOCs from fungi with fuel potentials. Although SPME is a solvent free, selective, inexpensive technique that is compatible with HPLC or GC/MS, our results demonstrate that SPE is more effective than SPME in extracting compounds with lower vapor pressure and higher boiling point. Specifically, SPE includes a significantly greater number of compounds than SPME. This work suggests that instead of using SPME, SPE should be employed to extract hydrocarbons having fuel potentials from fungi.

In summary, this dissertation reports the successfully isolation of a biofuel producing fungal species and characterization of some of the VOCs, as well as their concentrations. Insight into the mechanism of modulating the VOCs production in BS15 is also provided. Finally, the SPME and SPE techniques are compared to gain insight into how these two techniques should be applied to separate and extract fungal metabolites. These two techniques are commonly utilized in a wide variety of fields, thus this work portends additional applications.

1.5 Chapters that appear as publications

The following chapters appear as they were published in the Journal of Fungi.[30,31]

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CHAPTER 2: A SOLVENT FREE APPROACH FOR CONVERTING CELLULOSE WASTE INTO VOLATILE ORGANIC COMPOUNDS WITH ENDOPHYTIC FUNGI

Adapted with permission from: Maxwell, T.; Blair, R.G.; Wang, Y.; Kettring, A.H.; Moore, S.D.; Rex, M.; Harper, J.K. *J. Fungi* **2018**, 4, 102.

2.1 Introduction

The endophytic fungi are organisms that colonize the tissue of living plants. In most cases, this relationship is asymptomatic and may even provide benefits to plants.[1] Endophytes have been studied extensively and found to produce a remarkable variety of natural chemical products.[2] While much of the interest has focused on bioactive compounds, the production of other important compounds has also been reported. A recent noteworthy discovery is that certain endophytes can produce hydrocarbons that have the potential to be used as fuels or fuel additives[3]. These products have been compared to diesel fuel and even described as "myco-diesel" because they include compounds normally associated with diesel fuel. Over the past decade, interest in fungi producing volatile organic products with potential use as fuels has increased and several studies have identified potentially useful fungi.[4,5,6,7,8,9,10,11] Related work has also identified fungi producing volatile products, but have not focused on their potential usefulness as fuels.[12,13,14,15,16,17,18,19,20,21,22,23,24,25,26]

The availability of hydrocarbon fuels from fungi complements fuel products produced by other organisms. For example, certain algae produce aliphatic fatty acids and considerable effort has been expended into developing these into viable biofuels.[27] Likewise, yeast fermentation has been prominently utilized to convert carbohydrates from corn into ethanol for fuel.[28] In general, fungal products contain a more complex variety of volatile compounds than

either algae or yeast, including ketones, esters, alcohols and a remarkable variety of hydrocarbon products. All of these biofuels complement more conventional fuels and thus represent important pathways worthy of exploration given the current interest in developing alternative fuels. However, one of the concerns that exists when producing hydrocarbon fuel from fungi is that they require a refined carbohydrate source (e.g. sucrose) in their diet. There has been debate regarding the suitability of devoting carbohydrates to fuel production. A solution to this dilemma would be to find an alternative food source for the fungi.

Recently, a "green chemistry" mechanocatalytic method has been reported that allows cellulose waste products to be converted into simple carbohydrates.[29] This process involves ball milling performed in the solid state and is thus entirely solvent free and capable of rapidly producing large quantities of carbohydrates (see experimental). The major water soluble products from this process have been shown to be glucose, fructose and levoglucosan. No oligosaccharides larger than dimers survive the milling, even after short processing times (e.g. 30 min.).[29] This process has been successfully demonstrated using a remarkable variety of cellulose-based feedstock materials from plants (Table 2.1) and includes numerous materials normally regarded as unusable waste such as orange peels, cherry pits, coffee grounds and discarded newspaper.

Table 2.1Waste materials containing cellulose that can be converted into simple sugars.

Feedstock	Percent Hydrolyzed[a]
Cherry pit	95.7
Flint corn kernals	93.4
St. Augustine grass	92.5
Oat	90.3
Orange peel	85.0
Corn cobs	81.5
Bamboo	75.1
Cedar	74.0
Red Oak	72.4
Maple	72.0
Douglas Fir	71.1
Nannochloropsis	69.2
Aspen	68.0
Poplar	66.9
Yellow pine	65.3
Wheat	65.0
Miscanthus grass	64.7
White pine	64.4
Mixed yard waste	58.1
Switch grass	57.9
Hickory	55.9
Paper, newsprint	54.7
Flint corn stover	52.1
Banana leaf	52.0
Big blue stem grass	50.1
Little blue stem grass	48.9
Coffee grounds	45.2

[a]This value represents hydrolysis of the holocellulose present in the material.

At the present time, however, it has not been demonstrated that fungi can actually grow on the carbohydrates created from the cellulose breakdown process. The aim of this manuscript is to demonstrate that carbohydrates produced from this solvent free degradation process are a

suitable carbon source for fungal growth and that the volatile products produced from the degraded cellulose closely match compounds produced from a more conventional diet. In the following, sugars from only one cellulose source (oak) are tested. Results from other materials in Table 2.1 are expected to give similar results as it has been demonstrated that cellulose from various sources consistently break down to simple sugars.[29]

2.2 Materials and Methods

The cellulose employed in this study to create simple sugars was obtained from water oak (*Quercus nigra*) sawdust sourced from a local sawmill. The oak was dried at room temperature to a moisture content of <10% and cut into 2 cm or smaller pieces. Delaminated kaolinite (Kaopaque 10, IMERYS) was used as received.

Mechanical processing of cellulose employed 8000M and 8000D mixer mills (SPEX Certiprep, Metuchen, NJ). Two grams of a 1:1 mixture of the kaolinite clay catalyst and biomass source were processed for two hours in 65 mL vials (1.5" ID x 2.25" deep) made of 440C steel, utilizing three 0.5 inch diameter balls composed of the same material as the milling vial. Energy was applied in 30-minute intervals with 30 minutes of cooling time to minimize the effects of frictional heating. Hydrolysis of hemicellulose and cellulose (holocellulose) was monitored gravimetrically. Conversion of holocellulose to water-soluble oligosaccharides was determined by stirring 0.1 g of the reaction mixture in 30 mL of water. The production of water-soluble products was measured by filtration through a 47 mm diameter Whatman Nuclepore® track etched polycarbonate membrane filter with a pore size of 0.220 μm. The residue was dried in a 60°C oven for 12 hours and then weighed.

Potato dextrose broth and agar were purchased from Becton Dickinson. Ammonium sulfate, acetonitrile, and ethyl acetate, anhydrous magnesium sulfate, and methanol were purchased from Fisher scientific. A sample of 1,8-cineole was obtained from TCI chemicals (Portland, USA). Yeast nitrogen base was purchased from Sigma Aldrich. HyperSep C-18 solid-phase extraction columns (1 g bed weight) were purchased from Thermo Scientific. Potato dextrose agar was purchased from Microtech Scientific. All reagents were used as received.

Isolation of the *Hypoxylon* sp., BS15, was from branch clippings of a *Taxodium distichum* (bald cyprus) gathered near Orange City, Florida USA. The branches were treated with 70% ethanol, flame sterilized and then dried in a sterile laminar-flow hood. A sterile knife blade was then used to cut away the outer tissue from the clipping and a square wedge of the inner tissue was placed on water agar. This dish was incubated and any fungal hyphae observed growing from the sample were transferred onto separate plates of potato dextrose agar.

Potato dextrose broth was prepared by adding 2.4 g of potato dextrose broth to 100 mL purified water in a 500-mL Erlenmeyer flask. The flask was then sealed with aluminum foil and autoclaved for 15 minutes to sterilize. The fungi of interest (CI-4 or BS15) were then added to the sterile broth and it was resealed with foil. Cellulose broth was prepared using 250 mL of purified water 5 g degraded cellulose, 1.5 g ammonia sulfate and 1.7 g yeast nitrogen base without amino acids. In both growth media, the fungi were then left to grow for 25 days in the lab at 20–25 °C without stirring. Each broth was then vacuum filtered twice through Whatman Grade 4 filter paper to remove all particulates.

A control sample containing cellulose not subjected to the mechanocatalytic degradation process was prepared by adding 2 g of finely ground cellulose powder and 0.5 g of ammonium sulfate to 250 mL of distilled water. This medium was autoclaved for 15 minutes and, after

cooling, two separate solutions were prepared by adding CI-4 or BS15 to the liquid. This culture was allowed to grow for two weeks at 20–25°C without stirring.

For solid-phase extraction of fungal volatile compounds a C-18 cartridge was first washed with 4 mL of methanol and then with 4 mL of water. Filtered fungal broth (50 mL) was then passed through the column slowly under vacuum. The column was washed again with 4 mL of water to remove any contaminants and then dried by drawing air through the column for 15 minutes. Retained compounds were then eluted by passing acetonitrile through the column. A clear brown solution was typically recovered from this process. The eluent was then filtered with 0.22 µm syringe filter prior to analysis.

For liquid-liquid extractions, a total of 300 mL of the filtered fungal broth was shaken in a separatory funnel with 50 mL of ethyl acetate. The ethyl acetate was then separated from the water and dried over anhydrous magnesium sulfate. The solution was then filtered with 0.22 µm syringe filter prior to analysis.

The GC/MS analysis for volatile compounds was performed using a method described previously with slight modification.[30,31] An Agilent 6850 was used with a 5975CVC MS detector a Restek Rxi-5HT capillary column (30 m x 0.25 mm, film thickness 0.25 µm). The carrier gas was ultrahigh purity helium with a one cm^3/min constant flow rate and initial column head pressure of 77 kPa. The injector split was set to 250 °C at a 20:1 split ratio with 1 µL volume per injection. The column oven temperature was programmed at 45 °C initial temperature hold for one minute and a 10 °C /min ramp to 100 °C and hold for 5 minutes followed by a 5 °C /min ramp to 200 °C and hold 5 min. The detector was set at a constant 280 °C and set to scan 30-350 m/z. Data acquisition and processing were performed on Agilent MSD ChemStation software.

Identification of compounds was made via library comparison using National Institute of Standards and Technology (NIST) database.

For DNA extraction from BS15, a small sample of fungal tissue (50-100 mg) was collected into a microcentrifuge tube from the surface of a potato dextrose agar plate after one week of growth at room temperature. Tissue was lysed using a FastPrep Homogenizer (MP Biomedicals, Santa Ana, CA) by zirconia-silica bead beating in 1 mM sodium dodecyl sulfate, 5mM EDTA, 10 mM Tris-HCl, pH 8.0 with 10 µg/mL RNase A. The lysate was centrifuged, then DNA was purified from the supernatant by silica column binding in guanidinium thiocyanate.[32]

Diagnostic gene sequences used for identification by genetic bar-coding were amplified by routine polymerase chain reaction (PCR) with Taq.[33,34] Primers ITS1-F_KYO1 and ITS4_KYO1 were used to target the internally transcribed spacers (ITS1 and ITS2) and flanking portions of ribosomal RNA encoding genes (SSU, 5.8S, and LSU).[35] The protein-coding genes α-actin and β-tubulin were amplified by primers ACT-512F/ACT-783R and T1/T22 respectively.[36,37] PCR products were visualized by agarose gel electrophoresis and similarly purified by silica column binding, then sequenced commercially (GENEWIZ, Plainfield, NJ). Sequences were deposited in GenBank under accession number MH223406 (ITS), MH465497 (actin) and MH465498 (tubulin).

Ribosomal gene sequences were analyzed with a series of BioPython-based scripts.[38] First, full-length ITS sequences were extracted via ITSx and used to locally query the UNITE+INSDC fungal database by BLAST search.[39,40,41] Based on these search results, relevant taxa were selected, then a list was compiled of all unambiguous binomial species within these taxa. Corresponding UNITE records were pooled and analyzed by ITSx. For each species, a

single representative full-length ITS2 record was chosen for alignment. Relevant α-actin and β-tubulin records used in alignment were retrieved from GenBank.[42]

Phylograms were generated using MEGA software.[43] Sequences were aligned by MUSCLE algorithm, then clustered by Maximum Likelihood method with 1000 bootstrap replicates.[44,45] Both α-actin and β-tubulin sequences were treated as protein-coding during phylogenetic analyses, while ITS sequences were not. All other settings in MEGA were unchanged and no manual modifications were made during alignment or clustering. Resultant phylograms were exported and visualized via Interactive Tree of Life (iTOL) web software.[46] Nodes were pruned on the basis of relatedness to BS15 and intra-generic species richness. Alignments and phylograms were deposited in TreeBase under submission number 23089.

2.3 Results and discussion

2.3.1 Growth of the *Hypoxylon* CI-4 on degraded cellulose

As an initial test of the feasibility of using carbohydrates from mechanocatalytic cellulose degradation as a fungal diet, a *Hypoxylon* sp. was added to a growth medium consisting of the degraded cellulose as the sole carbon source (see experimental). A control sample was also prepared having the fungus on a conventional diet of potato dextrose broth. The particular *Hypoxylon* fungus used for this study (designated CI-4) was selected because it has been previously shown to produce a diverse variety of volatile organic hydrocarbons.[47,48] Both cultures exhibited similar fungal growth and were incubated for three weeks. The hydrocarbon fraction was extracted from the growth media using a solid-phase extraction process (see experimental). A gas chromatography/mass spectrometry (GC/MS) analysis exhibited a diverse range of volatile products as expected from the previous study.[47] A comparison of the volatile

compounds produced from each growth condition is illustrated in Figure 2.1 and demonstrates that the degraded cellulose material produces the same major products as a conventional carbohydrate rich diet. A notable difference, however, between the growth media is that the amounts of volatile compounds produced from the cellulose degradation products was roughly two to five times less than the same products produced from potato dextrose broth.

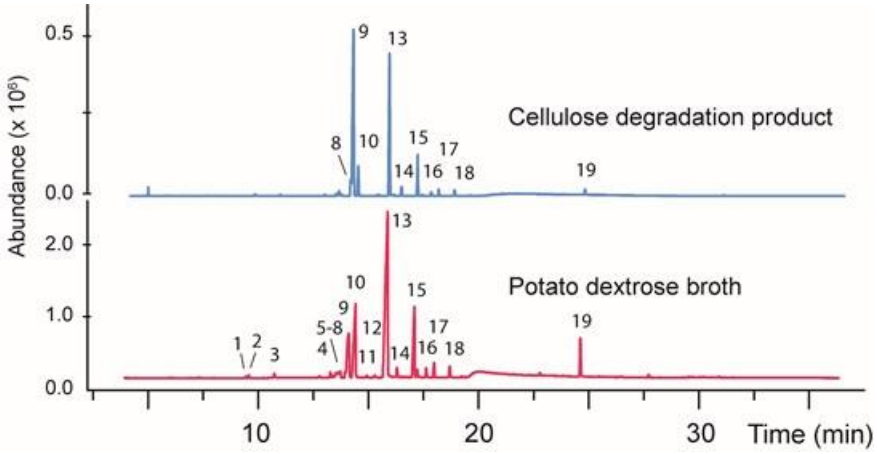

Figure 2.1 A gas chromatogram showing volatile organic products produced by the fungus CI-4 growth on a conventional media (bottom) versus a diet containing carbohydrates produced from cellulose degradation (top). Nominal masses for each numbered peak are given in Table 2.2 In each case a control sample was also analyzed consisting of the growth medium without fungi added. This solution was processed identically to the fungi containing samples. In each case, no peaks from the control samples corresponded to any of the peaks shown above.

Molecular masses and tentative identification of individual compounds from CI-4 was made by comparing the mass spectrum of each peak against data in the NIST database. Although nominal masses were obtained in all cases, most compounds were not identifiable. All results are summarized in Table 2.2 Also included in Table 2.2 are results from analysis of a second fungus (BS15, described below).

Table 2.2 A list of the volatile compounds produced by CI-4 or BS15 grown on either potato dextrose broth (PD) or degraded cellulose (DC) showing tentative compound identification where possible.

Fungus	Peak #[a]	R.T. (min.)	Area (%) PD, DC [b]	Tentative Identity[c]	Mol. mass (Da)	Qual.[d]
CI-4	1	9.58	0.1, -	Unknown	126	–
CI-4	2	9.72	0.3, -	Unknown	138	–
CI-4	3	10.88	0.5, -	Unknown	124	–
CI-4	4	12.93	0.2, -	Unknown	152	–
CI-4	5	13.43	0.8, -	Unknown	122	–
CI-4	6	13.57	0.3, -	Unknown	154	–
CI-4	7	13.68	1.4, -	3-Ethenyl-2-methylene cyclopentanecarboxylic acid,	152	50
CI-4	8	14.11	0.6, 2.8	Unknown	152	–
CI-4	9	14.25	10.6, 45.7	Unknown	150	–
CI-4	10	14.57	15.5, 6.0	Unknown	154	–
CI-4	11	15.08	0.3, -	Unknown	154	–
CI-4	12	15.44	0.3, -	Unknown	152	–
CI-4	13	16.02	43.8, 2.0	Unknown	168	–
CI-4	14	16.45	1.1, 2.0	1-Acetyl-2-(1-hydroxyethyl)-cyclohexene	168	50
CI-4	15	17.24	10.4, 7.6	Unknown	150	–
CI-4	16	17.79	1.0, 0.8	Unknown	170	–
CI-4	17	18.14	1.5, 1.2	Unknown	170	–
CI-4	18	18.84	1.1, 1.1	3-Isopropoxy 5-methyl-phenol	166	61
CI-4	19	24.78	4.0, 1.0	2,3-Dimethoxy-naphthalene	188	85
BS15	1	4.82	1.1, 2.7	Furfuryl alcohol	98	72
BS15	2	5.07	9.9, 2.4	Methyl 4-oxo-2-butenoate	114	94
BS15	3	7.85	9.3, 1.7	Benzeneacetaldehyde	120	70
BS15	4	8.22	1.6, –	4-methoxy-2,5-dimethyl-3 (2H)-furanone,	142	77
BS15	5	8.45	36.7, 26.1	2,5-furandione dihydro-3-methylene	112	55
BS15	6	8.65	– , 21.5	Levoglucosenone	126	78
BS15	7	9.65	20.9, 12.8	2-Phenyethanol	122	86
BS15	8	12.24	3.0, –	Unknown	158	–

Fungus	Peak # [a]	R.T. (min)	Area (%) PD, DC [b]	Tentative Identity[c]	Mol. Mass (Da)	Qual. [d]
BS15	9	12.34	– , 20.3	5-(Hydroxymethyl)furfural	126	91
BS15	10	13.84	17.8	Unknown	86	–
BS15	11	14.56	2.5, –	Phenylacetic acid	136	75
BS15	12	16.23	3.0, –	Unknown	138	–
BS15	13	16.45	– , 3.2	Unknown	142	–
BS15	14	18.20	3.7, –	Unknown	154	–
BS15	15	20.47	– , 8.1	Unknown	162	–
BS15	16	22.73	– , 3.2	2,4-dihydroxy-3,6-dimethyl Benzoic acid, methyl ester	196	72
Fungus	Peak #	R.T. (min)	Area (%) PD, DC [b]	Tentative Identity[c]	Mol. mass (Da)	Qual.[d]
BS15	17	25.58	– , 2.1	Dihydro-5-(2-oxocyclohexylidene) 2(3H)-furanone	180	70
BS15	18	26.63	1.7, –	Furo[3,4-f][1,3] benzodioxole-5,7-dione	192	65
BS15	19	26.78	2.9, –	Unknown	97	–
BS15	20	27.06	8.7, –	Unknown	127	–
BS15	21	28.04	1.8, –	Unknown	127	–

[a]Peak numbers correspond to the numbering shown in Figure 2.1 (CI-4) or Figure 2.4 (BS15).
[b]The labels PD and DC refer, respectively, to potato dextrose broth and degraded cellulose. Areas listed are the relative peak areas.
[c]All assignments of structure were made on the basis of the match to the NIST database.
[d]Qual. refers to the highest listed quality value for peaks that occur in both growth media or, for peaks that occur only in a single medium, to the value from that solution.

To verify that the volatile compounds produced from CI-4 grown on the degraded cellulose are the result of the presence of simple sugars rather than residual cellulose, a control containing cellulose not degraded by the mechanocatalytic process was also prepared for comparison (see Material and Methods). After two weeks of incubation on this medium, CI-4 showed no growth.

A notable difference between the compounds extracted here by solid-phase extraction and the previous study of CI-4 [47,48] is that solid-phase extraction failed to recover some of the early eluting peaks. As discussed below, a liquid-liquid extraction demonstrates these compounds are, in fact, present and the cause of their omission from the solid-phase extraction sample is currently under investigation.

2.3.2 Phylogenetic characterization of a new *Hypoxylon* sp., BS15

Recently a second fungus producing volatile organic products was isolated from a Bald Cyprus (*Taxodium distichum*) near Orange City, Florida (USA). This fungus, designated BS15, was selected for study based on the serendipitous observation that compounds having a distinctive odor were produced.

Identification of BS15 involved extracting genomic DNA, amplifying and sequencing its ribosomal internally transcribed spacer regions (ITS), and then applying an improved bioinformatics analysis based on existing methods. Detection of flanking ribosomal genes in the BS15 sequence by ITSx allowed for extraction of full-length ITS1 and ITS2 sub-sequences, a critical factor for producing alignments where gap site data is utilized in phylogenetic analyses.[49] Independent BLAST searches using these sub-sequences to query the UNITE+INSDC database returned alignments with species exclusively of the taxonomic family *Xylariaceae*. Therefore, all public sequence records pertaining to the family *Xylaraiaceae* were comprehensively screened. ITS sequences were detected by ITSx in 3443 of 3470 records from 394 unique binomial species.

A notable discrepancy regarding the naming and classification of organisms described in this work is the recent recognition of the family *Hyopxylaceae* by INSDC, whose members were previously included within *Xylariaceae*.[50] However, these records have not yet been updated in

UNITE at this time. For the present work, non-*Hypoxylaceae* species were included in alignment and clustering, but pruned from the ITS phylogram with the exception of *Xylaria hypoxylon*, presented as a rooted out-group (Figure 2.2).

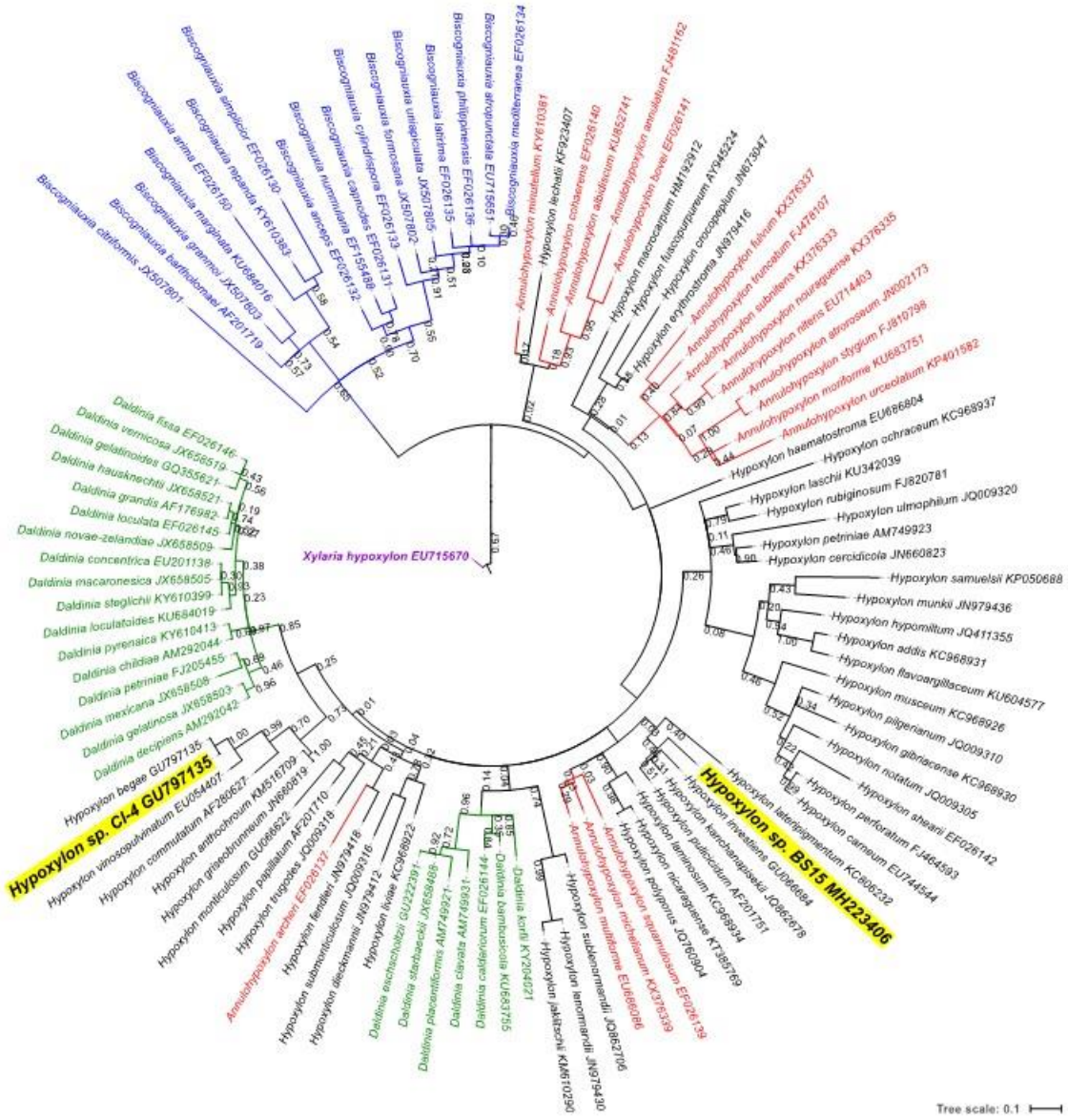

Figure 2.2 Phylogenetic reconstruction of *Hypoxylon sp*. BS15 and related organisms generated from Maximum Likelihood clustering of MUSCLE aligned ITS2 sequences. Branch lengths are drawn to scale representing the average number of nucleotide substitutions per site between sequences. 121 nodes were selected for inclusion in the present figure from 331 nodes in the original phylogram. Bootstrap values at nodes are from 1000 bootstrap iterations.

The relative richness of full-length sequence records and consistency in sequence length made ITS2 a more favorable target for multiple alignment than ITS1 for the family *Xylariaceae*. An analysis of ITSx outputs revealed bias for sequences containing the large ribosomal subunit sequence (LSU) compared with small subunit (SSU) sequences among the UNITE records for

the family *Xylariaceae*. Because detection of these flanking ribosomal sequences is required for extraction of full-length extraction of ITS sequences by ITSx, there were nearly twice the number of full-length ITS2 sequences (*n* = 2165) available for alignment compared with ITS1 (*n* = 1212). Sequence lengths were considerably less variable for ITS2 (SD = 5) than ITS1 (SD = 51).

Our taxonomic evaluations are consistent with other authors who found protein-coding genes more congruent with phenoytpic observations than non-coding ITS sequences for *Hypoxylon* and related genera.[42] Phylograms generated from ITS2 sequences were remarkably unresolved regardless of alignment and clustering methods, with several genera not clustered into monophyletic groups (e.g., *Annulohypoxylon* spp., *Daldinia* spp.) (Figure 2.2). Both α-actin and β-tubulin genetic analyses were able to fully resolve these taxa, albeit with fewer specimens (n=78) than ITS (Figure 2.3). For all three genetic markers, the fungal strain BS15 was consistently clustered among *Hypoxylon* spp. and most closely associated with *H. investiens.*

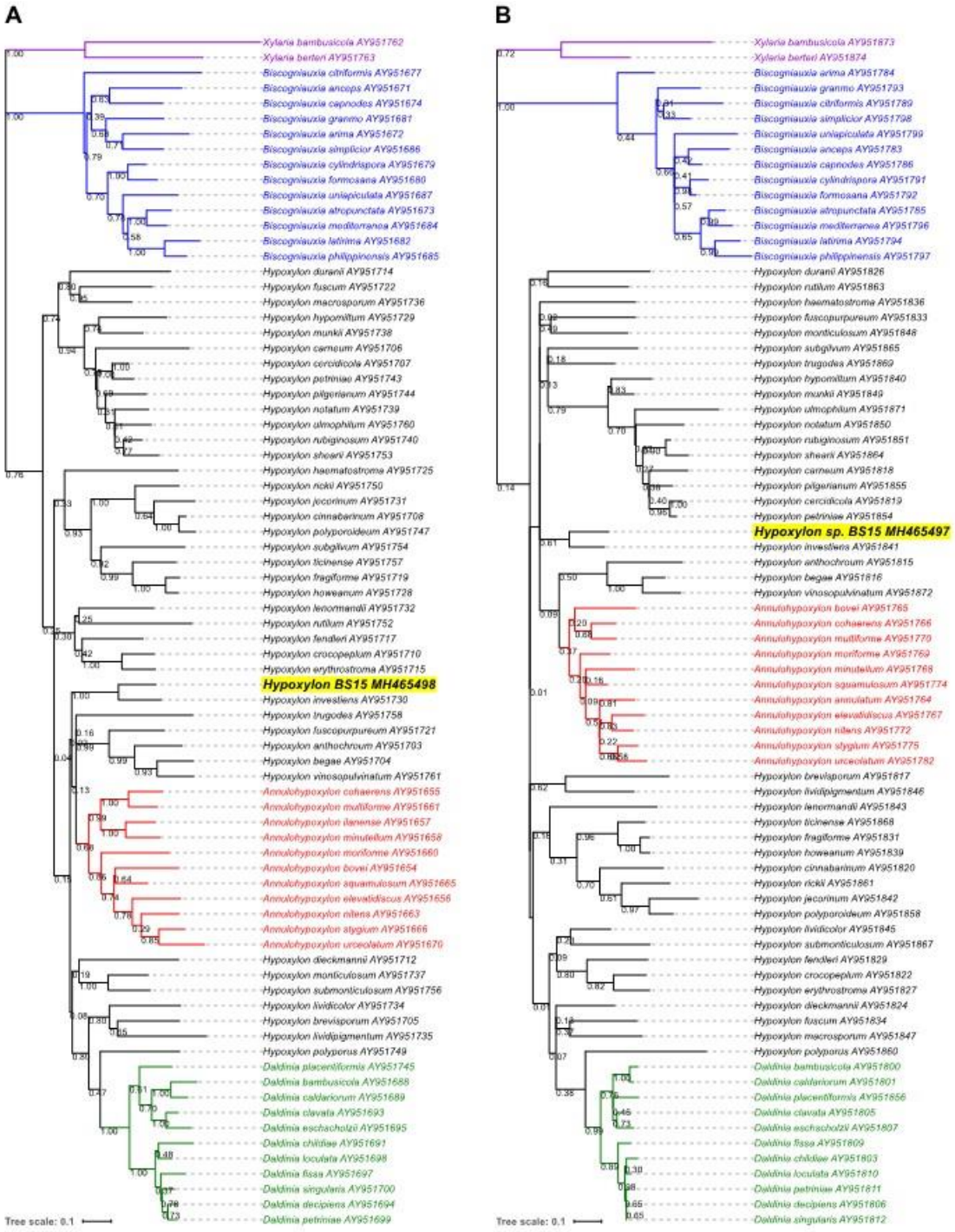

Figure 2.3 Phylogenetic reconstruction of *Hypoxylon sp.* BS15 and related organisms generated from Maximum Likelihood clustering of MUSCLE aligned protein-coding gene sequences. A total of 78 sequences were analyzed for α-actin (A) and β-tubulin (B). Branch lengths are shown to scale representing the average number of nucleotide substitutions per site between sequences. Bootstrap values at nodes are from 1000 bootstrap iterations.

2.3.3 Growth of BS15 on degraded cellulose and analysis of volatile hydrocarbons

In order to more generally evaluate the suitability of the degraded cellulose as a carbon source for fungi, BS15 was also evaluated for its ability to grow on the material. The procedure described above using two separate diets was employed with BS15 grown. The first included the degraded cellulose as the sole carbon source (see experimental) and the second contained potato dextrose broth. Both cultures exhibited strong fungal growth with mycelium covering the entire surface of the liquid media in approximately two weeks. The hydrocarbon fraction was extracted after three weeks using the solid-phase extraction process described above. A GC/MS analysis exhibited a large number of volatile products. A chromatographic comparison of the volatile compounds produced from each growth condition is illustrated in Figure 2.4 with tentative structural assignments and molecular weights listed in Table 2.2. Structures of compounds listed in Table 2.2 are illustrated in Figure 2.5. In the case of BS15, both diets produced compounds 1, 2, 3, 5, and 7 but all other products differ depending on the diet employed. Another notable difference versus CI-4 is that BS15 on the degraded cellulose diet produces approximately the same amounts of volatile products as the potato dextrose diet.

In order to verify that the volatile compounds resulting from growth of BS15 on degraded cellulose are being generate from simple sugars rather than residual cellulose, a control was prepared containing non-degraded cellulose as described above for CI-4. After two weeks of growth on this cellulose medium, no growth was observed.

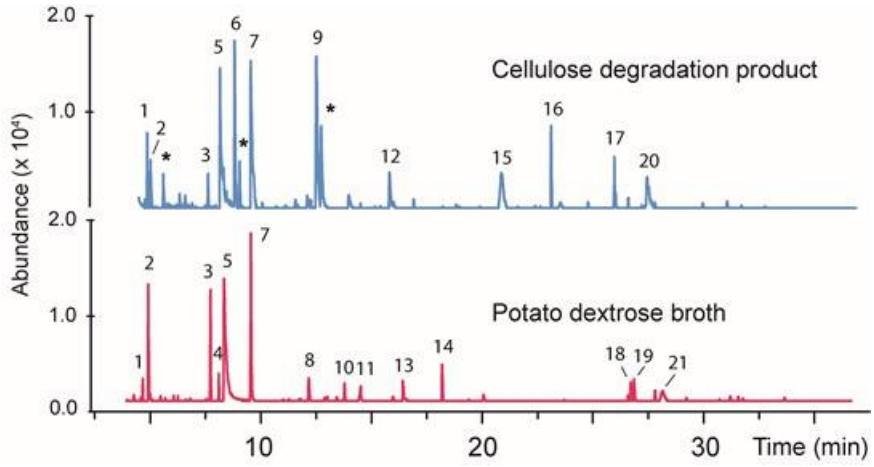

Figure 2.4 A gas chromatogram showing volatile organic products produced by the fungus BS15 growth on a conventional media (bottom) and on carbohydrates produced from degraded cellulose (top). Asterisks (*) denote volatile contaminants occurring in the degraded cellulose media as determined by analyzing a control sample with no BS15 added. Tentative identification and nominal masses of individual peaks numbered in the chromatogram are given in Table 2.2.

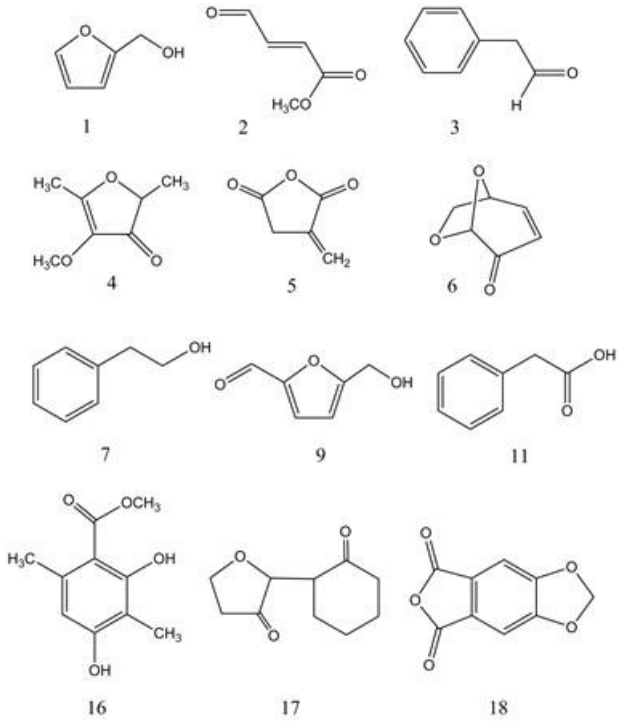

Figure 2.5 Structures of volatile compounds tentatively identified from BS15 by comparison to mass spectra in the NIST database. Compound numbers correspond to peak numbers listed in Table 2.2 and in Figure 2.4.

2.3.4 Comparing solid-phase and liquid-liquid extraction methods

All extractions of volatile compounds in this study were performed using solid-phase extraction with a C-18 stationary phase. This method is widely viewed as a "green chemistry" alternative to liquid/liquid extractions that requires two to three orders of magnitude less solvent. However, in comes with the risk of potentially extracting fewer or different compounds from the growth media. In order to verify that solid-phase extraction was effectively extracting the growth media, a direct comparison was made versus liquid/liquid extraction using ethyl acetate/water. Comparison were made for CI-4 and for BS15. For CI-4, Figure 2.6 illustrates that solid phase extraction gives very similar results while using approximately 100 times less solvent. Differences in relative amounts of certain products are notable. For example, the liquid/liquid extraction includes 1,8-cineole while this product is missing from the solid-phase extraction. At present, the cause of this difference is unknown and further study is needed. Overall, however, the majority of the compounds extracted are the same using either method and each technique extracts similar amounts as judged by the similar peak areas for signals having the same retention times.

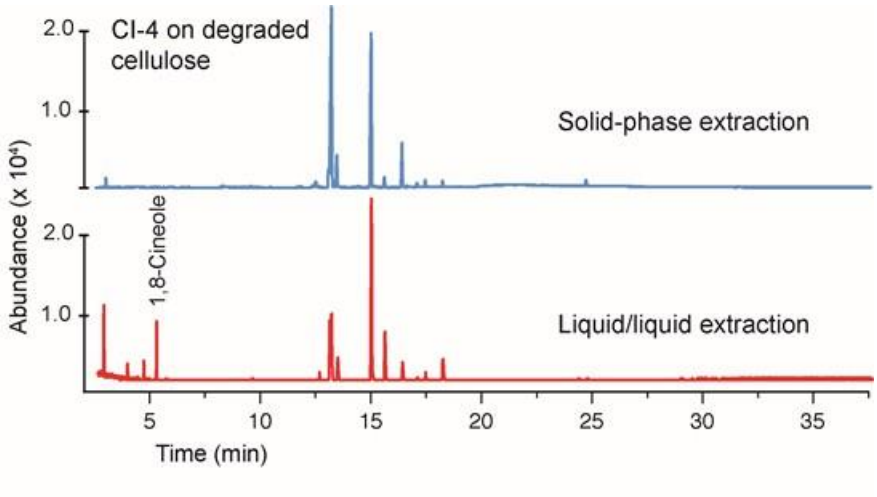

Figure 2.6 A comparison of solid-phase extraction versus liquid/liquid extraction (ethyl acetate/water). The growth media contained degraded cellulose as a carbon source. A notable difference is the presence of 1,8-cineole in the liquid/liquid extraction. A comparison of potato dextrose broth gave very similar results and therefore is not shown. The identity of 1,8-cineole was verified by comparison to an authentic standard.

A similar comparison of the effectiveness of liquid/liquid extraction vs. solid-phase extraction was performed using BS15 grown on degraded cellulose (Figure 2.7). Here the differences between methods is more pronounced with the solid-phase process extracting a more diverse range of products than does liquid/liquid extraction. The absence of cineole in Figure 2.7 is a notable omission.

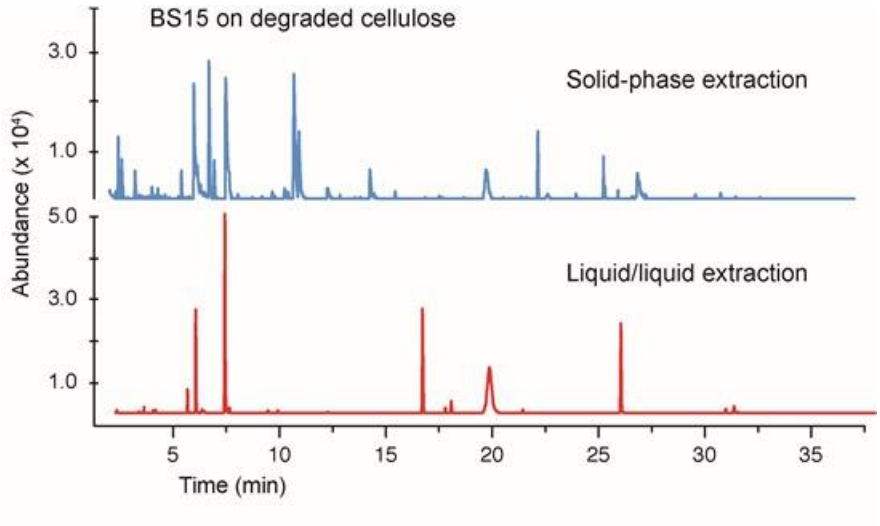

Figure 2.7 A comparison of solid-phase extraction versus liquid/liquid extraction (ethyl acetate/water). The growth media contained BS15 grown on degraded cellulose as a carbon source.

The solid-phase extraction process use herein differs from the vast majority of prior studies on volatile products from fungi which utilize solid-phase micro-extraction (SPME). This choice was made because SPME preferentially measures compounds having significant populations in the vapor phase and thus biases analysis against materials having low vapor pressures. Since compounds having potential use as fuels may have low vapor pressure, methodology was employed here that includes these compounds. Admittedly, the use of SPE represents a significant deviation from common practice in the analysis of volatiles and future work is needed to directly compare SPE and SPME to clearly identify advantages and limitations.

2.4 Conclusions

The work described herein establishes the ability of certain endophytic fungi to convert a mechanochemically degraded cellulose product into volatile organic products with potential relevance as fuels. This process has been shown to be feasible with two different *Hypoxylon* sp.

to demonstrate that the results are not limited to a single organism. One of the fungi employed (BS15) is described here for the first time and phylogenetic analysis demonstrates that it is substantially divergent from any other named species. BS15 produces a range of volatile products that differ significantly from those previously described from CI-4, emphasizing the importance of intra-generic species variation in metabolic studies. Here, the measurement of total concentration of volatile products is not reported because quantification of individual peaks in the chromatograms is not possible without standards and, at present, several products remain unknown. Future work will focus on a more complete characterization of individual compounds and measurement of total production.

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CHAPTER 3: RESTORING WANING PRODUCTION OF VOLATILE ORGANIC COMPOUNDS IN THE ENDOPHYTIC FUNGUS HYPOXYLON SP. (BS15)

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3.1 Introduction

Endophytes are microorganisms, usually fungi and bacteria, that live inside the host plant without showing signs of their presence or causing apparent diseases symptoms. The relationship between endophytes and their host plants varies from symbiotic to pathogenic.[1] Typically, tropical areas and rainforest are presumed to have the greatest diversity and abundance of endophytes due to their vast plant diversity.[2] Investigations involving endophytes are of considerable interest, in part due to their production of a remarkable variety of natural products.[3,4,5,6] Although there has been significant focus on the endphytes, they remain relatively understudied.

Recently some work has focused on fungi producing volatile organic compounds (VOCs) including some with potential usefulness as fuels or antimicrobials. For the purposes of this manuscript, VOCs are defined as compounds having sufficient volatility to be separable/mobile on gas chromatography. Table 3.1 summarizes the presently known fungi that can produce VOCs. Currently, only fungi producing components similar to fossil fuels have been considered to have fuel potential. These compounds include branched alkanes and their derivatives, substituted cyclohexanes, benzenes, alkyl alcohols, aldehydes and polycyclic aromatic hydrocarbons.[7] For instance, *Gliocladium roseum* produces more than 40 VOCs with fuel

potentials such as pentyl, hexyl, heptyl, and octyl alcohols, 3,3,5-trimethyldecane and other branched hydrocarbons.[7] Among all the VOCs with fuel potential characterized and reported, 1,8-cineole is compound of special interest because a 70/30 (v/v) mixture of petrol/1,8-cineole has performance characteristics similar to petrol with less carbon monoxide emission.[8,9,10] In 2010, a *Hypoxylon* sp. designated CI-4 was reported as the first non-plant source to produce 1,8-cineole (hereinafter referred to as cineole). More recently, other *Hypoxylon* spp. have also been found to produce cineole.[11,12]

Table 3.1 A list of fungi presently known to produce volatile organic compounds.

Species	Site isolation	Extraction method[a]	# of VOCs detected	Reference
Aspergillus fumigatus	New Jersey, USA	SPME	>10	13
A. niger	New Jersey, USA	SPME		13
A. tubingensis	New Jersey, USA	SPME		13
A. niger	Malaysia	LLE	>295	14
Fusarium armeniacum	New Jersey, USA	SPME	>10	13
F. graminearum	New Jersey, USA	SPME		13
F. oxysporum	New Jersey, USA	SPME		13
F. proliferatum	New Jersey, USA	SPME		13
F. culmorum	Belgium	SPME	>10	15
F. langsethiae	Russia	SPME	>40	16
F. sibiricum	Russia	SPME		16
F. poae	Russia	SPME		16
F. sporotrichioides	Russia	SPME		16
Metarhizium anisopliae	New Jersey, USA	SPME	>5	13
Mucor racemsus	New Jersey, USA	SPME	>10	13
Penicillium chrysogenum	New Jersey, USA	SPME	>10	13
P. citreonigrum	New Jersey, USA	SPME		13
P. commune	New Jersey, USA	SPME		13
P. corylophilum	New Jersey, USA	SPME	>10	13
P. crustosum	New Jersey, USA	SPME		13
P. glabrum	New Jersey, USA	SPME		13
P. pinophilum	New Jersey, USA	SPME	>10	13

Species	Site isolation	Extraction method[a]	# of VOCs detected	Reference
P. polonicum	New Jersey, USA	SPME	>10	13
P. sclerotiorum	New Jersey, USA	SPME	>10	13
P. steckii	New Jersey, USA	SPME		13
P. sumatrense	New Jersey, USA	SPME		13
Nodulisporium	Canary Islands, Ecuador, Thailand, Nicaragua, South Australia, Colombia and Wetlands of Florida	SPME	>40	17
Muscodor albus	Honduras, Thailand and Ecuador	SPME	>20	18
M. crispans	Bolivian amazon basin	SPME	>15	19
M. kashayum	India	SPME	>20	20
M. strobelii	India	SPME	>14	21
M. darjeelingensis	India	SPME	>20	21
M. tigerii	India	SPME	>20	23
M. suthepensis	Thailand	SPME	>25	24
M. musae	Thailand	SPME	>15	24
M. oryzae	Thailand	SPME	>15	24
M. equiseti	Thailand	SPME	>15	24
M. sutura	Colombia	SPME	>20	25
M. fengyangensis	China	SPME	>20	26
Myrothecium inunduatum	India	SPME	>30	27
Bionectria ochroleuca	India	LLE	>5	28
Ampelomyces	Japan	SPME	>5	29
Phoma	Japan	SPME	>5	29
Cladosporium	Japan	SPME	<5	29
Phomopsis	Ecuador	SPME	>10	30
Gliocladium roseum	Northern Patagonia	SPME	>40	7
Beauveria bassiana	Montana, USA	SPME	6	31
A. cylichnium	Norway, Switzerland	SPME	>10	32

Species	Site isolation	Extraction method[a]	#of VOCs detected	Reference
Schizophylum commune	Chile	SPME	10	33
Hypoxylon	Thailand, Spain	SPME	>15	34

[a]The abbreviations, LLE and SPME denote, liquid-liquid extraction and solid phase micro-extraction, respectively.

An unexpected challenge involving cineole production in CI-4 was the observation that the production gradually decrease over a period of months when the organism was removed from the plant host. This decrease suggested the presence of one of more cineole production modulator compounds in the host plant. *Nigg et al.* isolated and characterized a modulator in an endophytic *Nodulisporium* species, the imperfect stage of *Hypoxylon*.[35] This modulator was able to restore cineole biosynthesis and it is likely that similar outcomes can be obtained in other endophytic fungi where production of valuable products decrease over time. In related work, Hassan *et al.* reported that the treatment of *Hypoxylon* sp. with known epigenetic modulators not only cause phenotypic changes, but also modify the VOCs production and the bioactivity.[36] All these prior studies provide new insight regarding why such a diverse range of VOCs are found in different isolates of *Hypoxylon* spp.

Recently, a *Hypoxylon* sp. not corresponding to any named species was isolated from a bald cypress tree (*Taxodium distichum*) near Orange City, Florida USA. A complete phylogenetic characterization of this fungus, designated BS15, will be given elsewhere. BS15 was found a variety VOCs with possible relevance as fuels or antimicrobials. As with other *Hypoxylon* spp., BS15 was found to exhibited a significant decrease in VOC production over time and, motivated by the work of Nigg et al.[35] techniques for restoring VOC production were evaluated. This manuscript describes a process in which extracts from woody tissue of the plant

from which BS15 was originally isolated were added to the growth media in an effort to restore VOC production. Serial extractions of the woody tissue were performed with dichloromethane (DCM), methanol and water. In the following we describe the changes from each extract and show that the DCM and water extracts as well as the exhaustively extracted wood tissue induce production of compounds. These changes were found to be inheritable and three of the products are shown to differ from those originally produced by BS15, suggesting that the modifications represent epigenetic changes.

3.2 Materials and methods

Dichloromethane (DCM) and methanol were purchased from Fisher scientific. SiliaPrep C-18 columns were purchased from Silicycle. Potato dextrose broth (PDB) and agar were purchased from Microtech Scientific. All reagents were used as received.

The *Hypoxylon* sp. BS15 was isolated from a bald cypress tree (*Taxodium distichum*) near the Saint Johns River near Orange City, Florida USA. Initially isolation of the fungus followed the procedures of Tomsheck *et al*.[11] This involved treating branches with 70% ethanol, further sterilizing the wood in a flame and then drying in a sterile laminar-flow hood. Outer tissue was cut away using a sterile knife blade and a square section of inner tissue then placed on water agar. Any fungal hyphae growing out from the sample were transferred onto different plates of potato dextrose agar (PDA). One such sampling resulted in isolation of BS15.

Growth of BS15 sample without added modulators (i.e. extracts from *T. distichum*) was accomplished in PDB prepared by adding 2.4 g of potato dextrose broth to 100 mL purified water in a 500-mL Erlenmeyer flask. The flask was sealed with aluminum foil and autoclaved for 15 minutes to sterilize. A culture of BS15 growing on PDA was then added to the sterile broth

and it was resealed with aluminum foil and left to grow for 30 days in the lab at room temperature without stirring. The resulting broth was then vacuum filtered twice with Whatman Grade 4 filter paper to remove all particulates.

Extractions of woody tissue involved taking approximately 210 g of shredded cypress wood and extracting with 400 mL DCM for 90 minutes with stirring. This process was repeated two times. The DCM was then removed on a rotatory evaporator (Buchi Rotavapor R-205) at reduced pressure. The cypress wood was further extracted with methanol and finally with water respectively using 400 mL of each solvent. In order to simulate the extractable components in wood matrix, all three fractions were dripped onto filter papers and the filter paper allowed to completely dry. This process was repeated several times until the filter paper contained the desired mass of the extracted components. These filter papers were cut into pieces and added to 100 mL the PDB medium in 500-mL Erlenmeyer flask and this flask inoculated with BS15. As a control, filter paper containing no extractables was added the PDB/BS15 media. As a final test, the exhaustively extracted cypress wood were also added to PDB. All flasks were then sealed with aluminum foil and autoclaved for 15 minutes. After cooling BS15 was added to the sterile broth and the resulting solution allowed to grow for 30 days at room temperature without stirring.

Media used to evaluate the influence of the DCM, methanol and water extracts on BS15 was prepared by removing a sample of the fungi grown in a solution containing PDB plus *T. distichum* extracts after 30 days of growth and transferring it onto a petri dish. Serial weekly transfers onto PDA were then performed over a period of 4 weeks to ensure that all changes in VOC production ultimately observed are epigenetic changes and that exogenous contaminants from the extractable components were rigorously removed.

Isolation of VOCs involved solid phase extraction of the growth media on a C-18 stationary phase (500 mg). First, a C-18 cartridge (particle size: 40-63 μm) was washed with 5 mL of methanol and then with 5 mL of water (three times). A total of 100 mL of filtered fungal broth was then passed through the column under vacuum. The column was washed with 15 mL of water to remove polar components (e.g. salts) and the column dried by drawing air through the column for 30 min. The column was then eluted by passing 1.5 mL of methanol through the column to yield a clear brown solution. The eluent was filtered using a 0.22 μm syringe filter prior to GC/MS analysis. This solid-phase extraction methodology differs from the solid-phase microextraction (SPME) methodology usually employed when evaluating fungal VOCs. The methodology was employed in order to more efficiently retain compounds having low vapor pressure and which may be missed by SPME.

The gas chromatography/mass spectrometry (GC/MS) method used was similar to that of Strobel et al.[37] The instrument used was a Finnigan TraceGC ultra with Trace DSQ detector and a Restek Rtx-225 capillary column (cyanopropyl-methyl/phenyl-methyl polysiloxane, 50/50, 30 m x 0.25 mm, film thickness 0.25 μm). The carrier gas was ultra-high purity helium with a 1.5 cm^3/min constant flow rate and initial column head pressure of 77 kPa. The injector was set to 250 °C with a 1 μL injection volume using splitless injection mode. The column oven temperature was initially 45 °C and held for one minute followed by a 10 °C /min ramp to 100 °C where the temperature was held for 5 minutes. Finally, the temperature was increased by a 5 °C /min to 200 °C and held for 7 min. The detector was set at 280 °C and set to scan 50-650 m/z. Data acquisition and processing were performed on Xcalibur software. Identification of compounds was made via library comparison using National Institute of Standards and Technology (NIST) database. In all the GC/MS analyses describe herein, quantities of

individual compounds detected are not reported because many of the compounds are unknowns. This ambiguity prevents the construction of calibration curves required for quantitation.

3.3 Results and discussion

The endophytic fungus BS15 was selected for study based on the observation that some of the compounds produced had a distinctive odor indicating production of volatile compounds. A GC/MS analysis of the original BS15 revealed a number of VOCs (Table 3.2). Unfortunately, the production of several compounds decreased with time in the absence of the host plant (figure 3.1). The nominal masses of all compounds were obtained and five compounds tentatively identified. A more complete characterization of the compounds present will be given elsewhere. When BS15 with diminished VOC production was transferred back to PDA containing woody tissue from the host plant (i.e. finely ground *Taxodium distichum* tissue), production of most VOCs was restored, albeit to varying degrees (figure 3.1, top plot). The ability to restore VOC production in BS15 suggests the presence of a modulator compound or multiple modulators in the host plant.

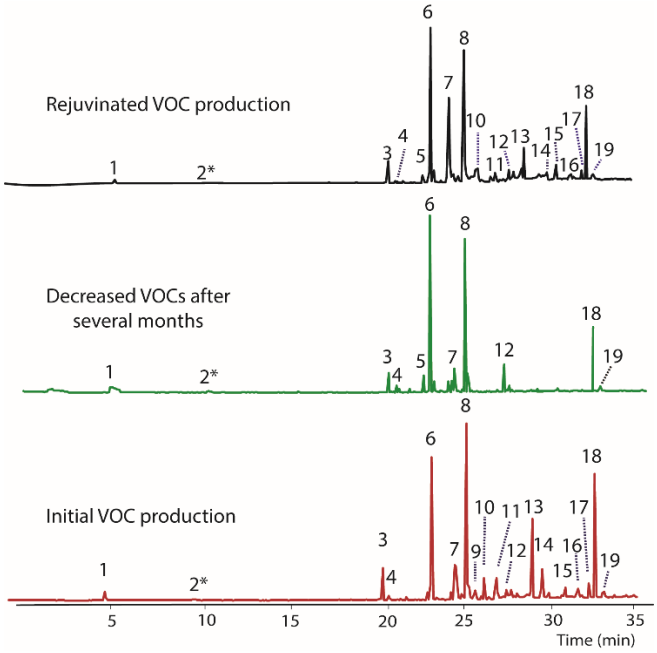

Figure 3.1 A gas chromatogram illustrating VOCs produced by BS15 showing the original production of VOCs immediately after isolation of BS15 (bottom) and decreased production after growing in the lab for several months (middle). Nominal mass of each peak and tentative identities are listed on table 2. Production of VOCs was restored to varying extents (top) by growing BS15 on PDA containing finely ground woody tissue from the *Taxodium distichum* from which the fungus was originally isolated

Table 3.2 A GC/MS analysis of the VOCs produced by BS15

Peak	R.T. (min)	Tentative Identify	Mol. Mass
1	4.75	unknown	70
2[a]	9.41	1,8-cineole	154
3	19.15	unknown	142
4	19.78	unknown	120
5	21.84	unknown	126
6[a]	22.35	Phenyl ethyl alcohol	122
7[a]	23.85	2,3-naphthalenediamine	158
8	24.51	unknown	182
9	24.75	Unknown	184
10	25.87	unknown	220
11[a]	26.58	Phenylacetic acid	136
12	27.62	Unknown	298
13[a]	28.72	Diethyl phthalate	222
14	29.52	unknown	297
15	30.72	unknown	213
16	31.47	unknown	334
17	32.15	unknown	213
18	32.63	unknown	192
19	33.27	unknown	314

[a]Assignment confidence for peaks 2, 6, 7, 10 and 13 are, respectively, 86%, 89.3%, 82.7%, 76.4% and 94%. All other peaks did not correspond to compounds in the NIST database.

3.3.1 Decreased VOC production in BS15 after extended in vitro growth

In order to investigate modulators from the host plant that restore production VOCs in BS15, serial organic solvent extraction of woody tissue was made using DCM followed by methanol and then water. Each extract was then tested for its ability to restore VOC production in BS15. Since some of the extracted compounds were insoluble in the growth media, the extracted solutions were dripped onto filter paper and then air dried. Filter paper was employed to simulate the woody matrix of the original tissue. This process was repeated until the desired mass of extract had been loaded onto the filter paper (see Materials and Methods). Growth media

(PDB) was then prepared and the filter paper impregenated with extractable compounds was included in the media. The filter paper was cut into strips of approximately 1" x ¼" to give uniform distribution in solution. In each case, a control was also prepared containing filter paper with no extract added. The exhaustively extracted wood was also evaluated by including it in the growth media. The impact of each extract on production of volatiles is discussed below.

3.3.2 Assessing the influence of DCM extract/filter paper on VOC production

A culture of BS15 grown in a PBD medium containing DCM extract/filter paper, was found to alter the VOCs produced by inducing the production of three new compounds. Specifically, the peaks labeled 20, 21 and 22 in Figure 3.2 with respective nominal masses of 112, 216 and 154, are observed only after addition of the DCM extract and thus appears to represent an epigenetic change to BS15. Surprisingly, the control containing only filter paper also induced production of compounds 21 and 22. In both cases, these changes in VOC production are inheritable and persists over several generations. Indeed, Figure 3.2 represents BS15 VOCs obtained from tissue removed from the DCM/filter paper media then plated onto PDA followed by weekly transfers onto PDA for one month and finally regrown in PDB. In other words, Figure 3.2 represents a BS15 culture three generations removed from the initial DCM/filter paper treatment. Careful inspection of the chromatograms show that the DCM extracts also increases production of peak 18 and decreases production of 6. Overall, it appears that DCM contains a modulator than alters production of peaks 6 and 18 and creates the ability to produce 20. Remarkably, the filter paper appears to be solely responsible for the production of compounds 21 and 22 as discussed below. Isolation of individual modulator compounds from the DCM extract was not performed due to insufficient mass of DCM extract.

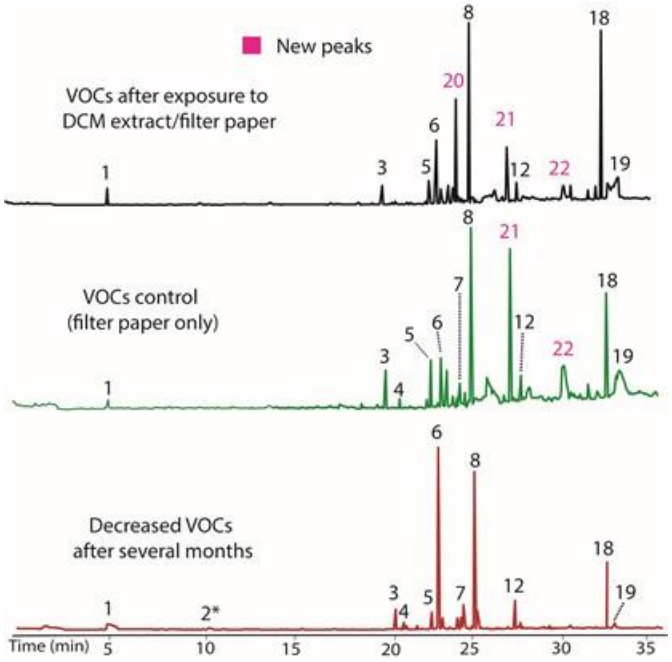

Figure 3.2 Gas chromatograms showing decreased VOCs production by BS15 after growing in vitro for several months (bottom). Adding BS15 to growth media (PDB) containing DCM extract/ filter paper induced production of new compounds 20, 21 and 22 (top) and altered the production of compounds 6 and 18. Nominal mass of each peak and their tentative identities are listed on table 3.3

Table 3.3 Compounds detected by GC/MS from BS15 modified by treatment with DCM extract/filter paper

Peak	R.T. (min)	Tentative Identify	Mol. Mass
1	4.75	Unknown	70
2	9.41	1,8-Cineole	154
3	19.15	Unknown	142
4	19.78	Unknown	120
5	21.84	Unknown	126
6	22.35	Phenyl ethyl alcohol	122
7	23.85	2,3-Naphthalenediamine	158
8	24.51	Unknown	182
9	24.75	Unknown	184
10	25.87	Unknown	220
11	26.58	Phenylacetic acid	136
12	27.62	Unknown	298
13	28.72	Diethyl phthalate	222

Peak	R.T. (min)	Tentative Identify	Mol. Mass
14	29.52	Unknown	297
15	30.72	Unknown	213
16	31.47	Unknown	334
17	32.15	Unknown	213
18	32.63	Unknown	192
19	33.27	Unknown	314
20[a]	23.76	3-methyl-2,5-furandione	112
21	26.85	Unknown	216
22[a]	29.82	4,4'-thiobis-benzeneamine	154

[a]Assignment confidence for peaks 20 and 21 are, respectively, 67% and 71

3.3.3 Evaluating the influence of methanol and water extracts on VOC production in BS15

The influence of both the methanol and water extracts from *T. distichum* on BS15 were also evaluated using the process describe above for the DCM extract. The methanol extract/filter paper produced almost no change with the exception that peaks 21 and 22 are again observed. Chromatograms illustrating VOC production before and after addition of methanol extract/filter paper are shown in Figure 3.3. In this case, the control containing only filter paper also induced production of 21 and 22. The production of these compounds also occurred in the DCM extract and their occurrence is thus attributed to the filter paper as a VOC production modifier rather than any compounds extracted by methanol.

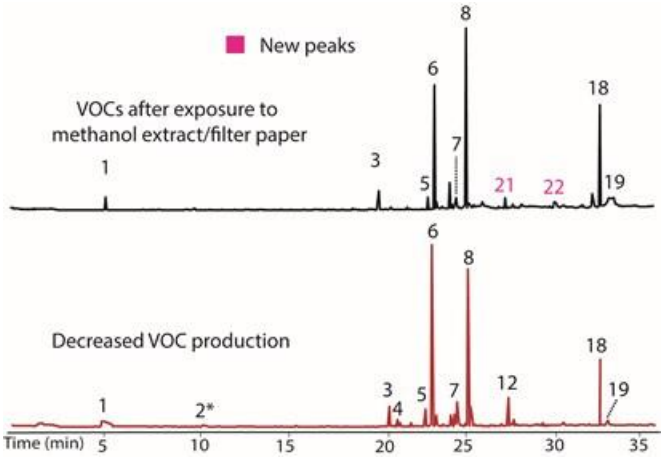

Figure 3.3 Chromatograms showing the negligible influence of the methanol extract/filter paper of VOC production of BS15. The new peaks (21 and 22) observed upon treatment (top plot) also occur in the control containing only filter paper. Their occurrence is therefore attributed to a change from the filter paper rather than the presence of VOC production modifiers extracted by methanol.

The water extract/filter paper was also evaluated (Figure 3.4) using the process described above. This extract increased production of compounds 7, 12 and 18 while decreasing the quantity of 6. The water extract thus likely contains a VOC production modulator. As in the other extracts, peaks 21 and 22 are again observed in both the extract/filter paper and in the control, strengthens the conclusion that filter paper induces their production. All changes from the water extract are inheritable.

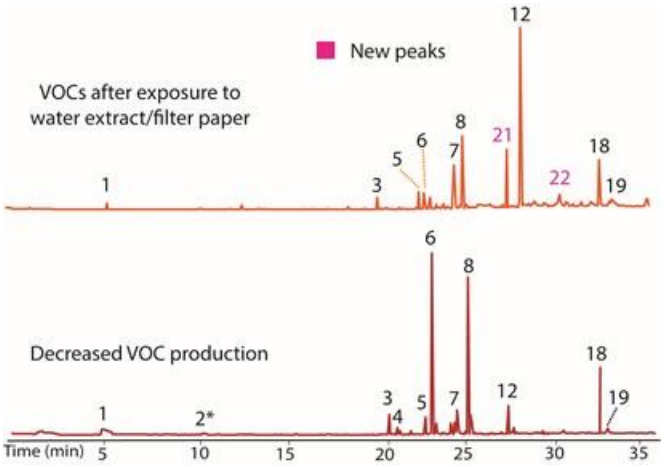

Figure 3.4 Chromatograms showing the influence of the water extract/filter paper of VOC production of BS15. Peaks 7, 12 and 18 increase upon exposure to the water extract while 6 decreased (top plot). The water extract thus appears to contain a modulator of VOC production. Peaks 21 and 22 were again observed to occur in both the water extract and in the control containing only filter paper.

3.3.4 The influence of exhaustively extracted *T. distichum* wood on VOC production in BS15

As a final test of potential modulators of VOC production in BS15, the finely ground extracted woody tissue of *T. distichum* was evaluated. This unusual step was taken because the filter paper was repeatedly found to induce production of peaks 20 and 21 in prior extracts and it was of interest to see if other cellulose contain materials could have the same effect. The extracted wood was observed to decrease production of peaks 7 and 12 while increasing 5 and 18 (Figure 3.5). Of greatest interest was the observation that peaks 21 and 22 appeared as prominent peaks, supporting the contention that these peaks are induced by cellulose containing materials. A comparison of the woody tissue to the filter paper controls from each extract is illustrated in Figure 3.6. The remarkable similarity in the changes induced by filter paper and those induced by exhaustively extracted wood from *T. distichum* indicates that a common production modulator is involved.

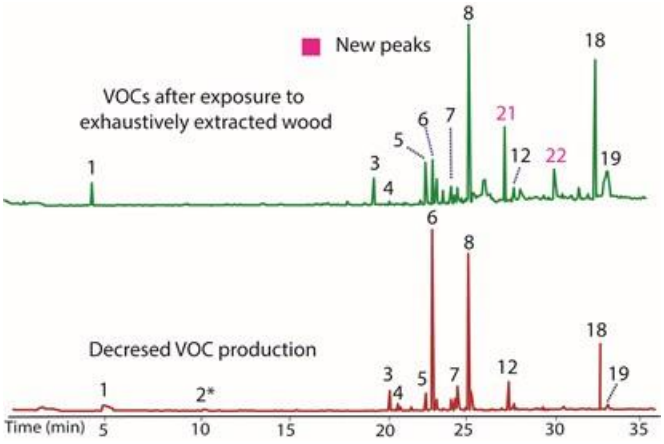

Figure 3.5 Chromatograms showing the influence on VOC production of the addition of exhaustively extracted woody tissue of *T. distichum* to growth media (PDB). Peaks 5, 18 increase while 7 and 12 decrease (top plot). Peaks 21 and 22 appear as prominent components in the top plot, supporting the contention that these peaks are induced by cellulose containing materials.

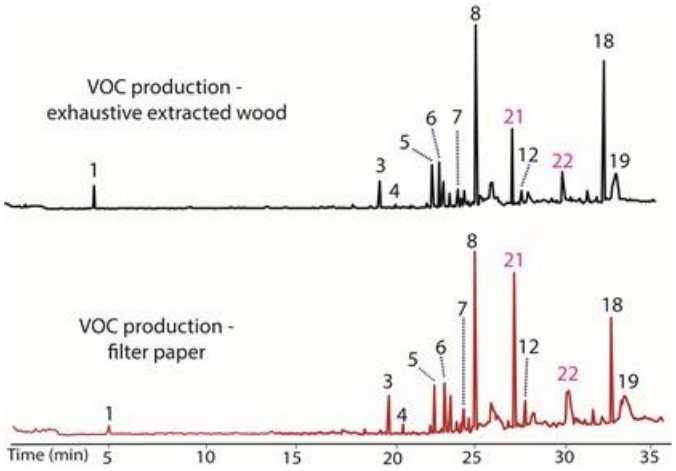

Figure 3.6 Chromatograms illustrating the changes in the production of VOCs by BS15 following exposure to filter paper (bottom) and to exhaustively extracted woody tissue from branches of *T. distichum* (top).

A direct comparison of each extract and its influence on VOC production is summarized in Table 3.4 and shows that the influence of DCM, water and extracted wood (or equivalently, filter paper) clearly differs. This result indicates that multiple modulators are involved in VOC production as opposed to a single modulator.

Table 3.4 A summary of compounds influenced by different extracts of *T. distichum*.[a]

Extract	Increased production	Decreased production	New cmpds. Produced
DCM	18	6	20, 21, 22
Methanol	None	None	21, 22
Water	7, 12, 18	6	21, 22
Wood, extracted	5, 18	7, 12	21, 22
Filter paper	5, 18	7, 12	21, 22

[a]All analyses of VOC production were made on fungi from the 4th generation after initial exposure to the extract or woody tissue/filter paper

An important question regarding the influence of modulators on VOC production is how long the fungus with compromised VOC production should be exposed to media containing ground plant tissue or extractable compounds in order to restore VOC production. In the case of BS15, we observed that continuous exposure to the woody tissue or extracts/filter paper for three generations was sufficient to restore production. For the purposes of this study, a transfer of the fungus was made each week to new media containing modulators (i.e. extracts). Thus, three generation corresponds to three weeks. In all cases, the fungus was removed from the media containing extractables or wood and grown for a week to maturity before transfer to PDB to ensure that all exogenous contaminations were removed. Similarly, it is important to evaluate how long the fungus with restored production can grow in vitro before VOC production begins to decrease. In BS15, a significate decrease in VOC production was observed after 8 generations (8 weeks).

3.4 Conclusions

The research demonstrates that waning production of volatile organic compounds in an endophytic *Hypoxylon* sp. (BS15) can be partially restored by re-exposure to chemical

constituents contained in the DCM and water extracts of the woody tissue of *T. distichum*. Surprisingly, the exhaustively extracted woody tissue also induces changes in VOC production from BS15 by causing production of two compounds never observed in the original growth media and in altering production of 4 other compounds. Filter paper alone can also produce this change, suggesting that the differences are epigenetic changes and that cellulose or its degradation products is the active component in altered VOC production rather than other compounds (e.g. lignans). The cellulose based modification of production may be caused by hydrolysis of the woody tissue or filter paper which could create water soluble carbohydrates and further study of this effect is needed. It is notable that carbohydrates are known to alter gene expression in some bacteria,[38] thus there is precedent for this change in BS15 from cellulose containing materials. An interesting parallel to this observation of activity in cellulose, a material that is essentially insoluble, is a prior study [35]reporting that breakdown products from lignin (i.e. ferulic acid and vanillin) also act as VOC production modulators in a closely related endophytic fungus.

It is notable that the large solubility difference between the water and DCM extracts suggests that the production modulator in DCM differs from that found in water. Thus, it is likely that more than one modulator effectively influences VOC production in BS15. At present, insufficient amounts of the DCM and water extracts are available to allow isolation of individual compounds and future work will focus on identifying compounds in these extracts involve in VOC modulation.

Solid-phase extraction was used in this study rather than the more commonly employed approach of solid-phase micro-extraction in order to more effectively include compounds having lower vapor pressures. One possible limitation to this approach is that compounds having high

vapor pressures may be underrepresented. Further study is underway to directly comparing these two techniques.

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CHAPTER 4: A COMPARISON OF SPE AND SPME FOR THE ANALYSIS OF VOLATILE ORAGNIC COMPOUNDS (VOCS) FROM FUNGI

4.1 Introduction

Recently, several fungi have been discovered that have the ability to produce volatile organic compounds (VOCs).[1,2,3,4,5,6] Among the compounds produced by these organisms are several that have potential value as fuels or fuel additives due to their similarity to compounds in fossil fuels. Specifically, the VOCs reported include branched alkanes, alkyl alcohols, esters, arenes, substituted cycloalkanes and polycyclic aromatic hydrocarbons. In the majority of these prior studies, the most common technique utilized to extract VOCs from fungi was solid phase micro-extraction (SPME).[7] Typically, VOCs are sampled by SPME from the gas phase above a growing fungus and then analyzed by gas chromatography/mass spectroscopy (GC/MS). In this approach, SPME preferentially samples compounds having high vapor pressures. Compared with the more traditional method of liquid-liquid extraction (LLE), SPME is solvent-free, cost-effective, convenient and reliable. Sampling with SPME has been used extensively to quantify volatile and semi-volatile compounds in both liquid and gas phase matrices and is relatively insensitive to matrix effects.

In a recent survey of studies on VOCs from fungi, SPME was found to be the dominant technique employed for extracting VOCs.[8] However, in these studies SPME is invariably used to sample the gas phase, and thus preferentially measures compounds having higher vapor pressures. This means that fungal products having potential use as fuel but with lower vapor pressures could potentially be overlooked. It is important to consider if an alternative sampling procedure could improve the yield of compounds that are volatile enough to boil in the gasoline/diesel range but insufficiently volatile to be sampled by SPME. One convenient and

well established alternative is solid phase extraction (SPE). The aim of the present study is to evaluate the usefulness of SPE in improving the quantity of VOCs harvested from fungi. For the purposes of this study, compounds are defined as VOCs if they eluted on a gas chromatographic analysis in the same temperature range as an external standard having a composition similar to gasoline and diesel fuel. In this initial study, two fungi are evaluated including a recently reported *Hypoxylon* sp. and a commercially available *Streptomycete*.

The SPE phases evaluated herein for their ability to retain VOCs consist of silica particles with a bonded a C-18 stationary phase and are expected to retain moderately polar and non-polar analytes. Highly water soluble compounds from the aqueous growth media (e.g. carbohydrates) are expected to pass through the stationary phase unretained. In recent years, numerous studies have compared SPE against SPME for a wide variety of analytes and sample matrices. For example, Huang *et al.* employed both SPE and SPME methods with GC and high resolution mass spectroscopy (HRMS) to determine monohydroxy PAH in urine samples. In this case, SPE demonstrated major advantages over SPME, including decreased cost of preparation, higher throughput, and better calibration curve linearity.[9] Horak *et al.* combined both SPE and SPME with GC to analyze the free fatty acids in beer samples, SPME was found to be a more suitable technique for medium-chain fatty acid analysis due to its operational simplicity, low cost and repeatability.[10] Konstantinou *et al.* also combined SPME and SPE with gas chromatography to determine antifouling booster biocides in natural waters, while Kin *et al.* compared SPME and SPE techniques for determination of organochlorine and organophosphorus pesticide residues in food matrices.[11,12] However, to the author's knowledge, no previous study has compared SPE and SPME methods for extraction of VOCs from fungi. It is therefore important to compare the effectiveness of these techniques.

The present study compares SPE and SPME and quantifies their ability to extract VOCs from two microbes, namely, *Hypoxylon sp.,* designated as BS15, and *Streptomyces ambofaciens,* designated as SA40053 (Braunschweig, Germany). The VOCs profiles obtained by headspace SPME analysis are compared with the VOCs obtained SPE extraction of liquid growth media. The advantages and limitations for these two techniques are identified and evaluated. Our results showed SPE is more effective than SPME in extracting VOCs having boiling points in the range comparable to the high boiling gasoline fraction and the low boiling diesel fraction. A significantly larger variety of compounds are extracted by SPE in each case.

4.1.1 Materials and regents

Dichloromethane (DCM) and acetonitrile (ACN) were purchased from Fisher Scientific (Salt Lake City, UT, USA). N-Phenethylacetamide, 2-coumaranone, cyclo-gly-pro, and 2,5-dimethyl-4-methoxy-3(2H)-furanone were purchased from Sigma Aldrich (St. Louis, MO, USA). The *Hypoxylon sp*. (BS15C) considered herein was isolated from a bold cypress tree (*Taxodium distichum*) near Orange County, Florida, USA as described elsewhere.[ii] A microbial strain *Streptomyces ambofaciens* (SA40053) was purchased from DSMZ (Braunschweig, Germany). A one mL standard of hydrocarbons representative of compounds found in gasoline and diesel fuel and suitable for use as a qualitative retention time index standard was purchased from Restek (Bellefonte, PA, USA). An SPME manual holder was purchased from Supelco (Bellefonte, PA, USA). Three SPME fibers coated with 50/30 um divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) were also purchased from Supelco. SilicaPrep C-18 columns were purchased from Silicycle (Quebec City, Canada). Potato

dextrose broth (PDB) and agar were purchased from Microtech Scientific (Vista, USA). All reagents were used as received.

4.1.2 Culturing and storing BS15 and SA40053

Culture media for BS15 consisted of Potato dextrose broth (PDB). This was prepared by adding 2.4 g of potato dextrose broth to 100 mL deionized water in a 250 mL Erlenmeyer flask. A nutrient broth growth media was employed for SA40053. This broth was prepared by adding 1.6 g of nutrient broth to 100 mL deionized water in a 250 mL Erlenmeyer flask. Both Erlenmeyer flasks were sealed with aluminum foil and sterilized for 15 min in an autoclave. Growth of BS15 was initiated by transferring small blocks of BS15 on potato dextrose agar (PDA) into the sterile PD broth. This resulting solution was resealed with aluminum foil and left to grow for 30 days at room temperature without stirring. Likewise, growth of SA40053 was initiated by transferring a few mL of SA40053 growing in water/nutrient agar to sterile nutrient broth. This solution was resealed with aluminum foil and left to grow for 14 days at room temperature with stirring using a magnetic stir bar.

4.1.3 Qualitative analysis of VOCs by SPME

The SPME analyses of VOCs produced by BS15C and SA40053 employed the procedures of Strobel *et al*. with slight modification.[14] Specifically, two SPME fibers were prepared by insertion into a GC injection port held at 250° C for 15 min to remove potential contaminants. These fibers were then exposed to the head space of the fungal media for 45 min. Care was taken to not disrupt the headspace by making a very small hole in the aluminum foil covering and exposing the fiber through this opening. The SPME syringes were then inserted

into a splitless injection port of GC with an inlet temperature of 250° C and desorbed for 45 seconds to initiate VOC analysis.

4.1.4 Qualitative analysis of VOCs by SPE

The SPE analyses of VOCs in the aqueous phase produced by both microbes were carried out using C18 cartridges. These cartridges were initially washed with 5mL of methanol, and then 15mL of nanopure water. All particulates were remove from both BS15C and SA40053 growth media by vacuum filtration (Whatman Grade 4 paper). Approximately 100 mL of each broth was then passed through the C18 cartridge under gentle vacuum. Both columns were subsequently washed with 15 mL nanopure water to remove carbohydrates and other unretained components. Columns were then dried by drawing air passing though the column for 45 min. Retained compounds were subsequently eluted by adding 1.5 mL of methanol/acetonitrile (50:50 by volume) through the cartridge. This process produced a colorless solution for SA40053 and a light brown solution for BS15C. This solution was directly injected for GC/MS analysis.

4.1.5 Quantitative analysis of VOCs

In order to identify and quantify several peaks from both cultures, standard solutions of compounds were prepared and then diluted. Specifically, 6.4 mg of N-phenethylacetamide, and 5.4 mg of 2-coumaranone were weighted and dissolved in 1 mL of acetonitrile. A dilution was made by taking 400 uL of a given stock solution and adding 600 uL of acetonitrile. This process was repeated to ultimately make eight increasingly dilute solutions for each standard. Similarly, 2.5 mg of cyclo-gly-pro was dissolved in 1 mL of acetonitrile solvent and eight diluted solutions were made by taking 500 uL of a given solution then diluting with 500 uL of acetonitrile. A

stock solutions of the liquid standard, 2,5-dimethyl-4-methoxy-3(2H)-furanone (furanone) solutions, was made by combining 50 uL of pure compound with 950 uL of acetonitrile. Additional solutions were made by taking 200 uL of a solution and diluting with 800 uL of acetonitrile solvent.

Calibration curves for GC/MS analysis of all external standard solutions were made by injected each three times then plotting average integrated areas versus concentrations. The data was found to be well-fit with a first order polymonial and this relationship provided the mass of a target compound in the sample.

4.1.6 GC/MS conditions

The gas chromatography/mass spectrometry (GC/MS) analysis for the volatile compounds was performed using a method described previously published by our group.[15] An Agilent 6850 was used with a 5975CVC MS detector and a Restek Rxi-5HT capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The carrier gas was ultrahigh purity helium with a one cm^3/min constant flow rate and an initial column head pressure of 77 kPa. The injector mode was set to splitless mode with 1-μL volume per injection. The column oven temperature was programmed with the initial temperature held at 45°C for 1 min then raised 10 °C/min until 100 °C and held for 5 min. The temperature was then raised 5 °C/min until 200 °C and held for 7 min for a total run time of 38.50 min. The detector was held at 280 °C and set to scan a range of 50–650 m/z. The data acquisition and processing were performed on Agilent MSD ChemStation software. The identification of the compounds was made via library comparison using the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) database. Retention indices (R.I.) were calculated by injecting saturated n-alkane standard

solution C_7-C_{33} (100-200 ug/mL in hexane), using the method developed by Vandendool and Kratz for non-isothermal conditions.[16]

4.2 Results and discussions

4.2.1 A qualitative comparison of SPE and SPME in SA40053

The microbial strain SA40053 was selected for study based on the observation that other *Actinomycetes* produces the VOC geosmin,[17] a compound well-known for its earthy odor. It is hypothesized that other volatiles will also be produced by this microbe. The SPE and SPME techniques were employed to qualitatively analyze the VOCs produced. Numerous VOCs was produced, as summarized in Table 1. The nominal masses of all compounds were obtained and thirteen compounds were tentatively identified. Among these compounds, 2-coumaranone and N-phenethylacetamide were confirmed by comparing both the retention time and mass spectra against commercial standards. Figure 1 illustrates the direct comparison between the ability of SPE and SPME to extract SA40053. The differences of VOCs extracted by these two techniques are notable. For example, phenyl ethyl alcohol was the only compound extracted by both techniques. All remaining compounds were extracted exclusively by either SPE or SPME method. Most notably, SPE technique extracts a larger number of compounds than SPME, with most additional compounds in SPE having higher retention times corresponding to greater boiling point and lower vapor pressures. In other words, most of the compounds extracted by SPE do not have significant populations in the headspace of the growth media. Nevertheless,

these compounds have retention times comparable to diesel fuel and thus potentially useful as fuels. Overall, SPE appears to be a more effective method to extract VOCs with fuel potential.

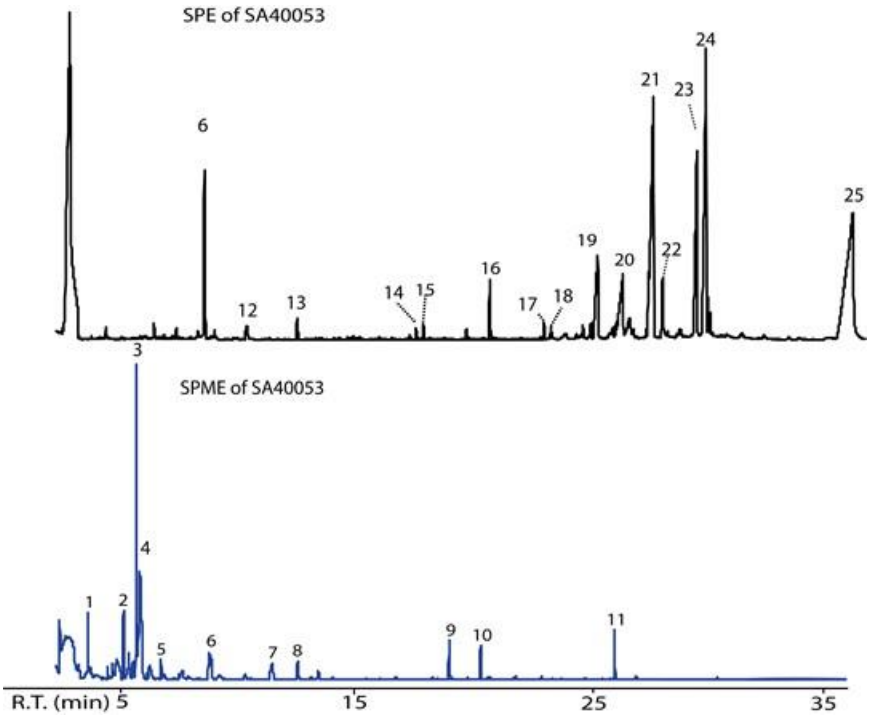

Figure 4.1 A gas chromatogram illustrating VOCs produced by SA40053 using SPME and SPE techniques, respectively. Nominal mass of each peak and tentative identities were listed on table 4.1.

Table 4.1 A GC/MS analysis of the VOCs produced by SA40053.

Peak	R.T. (min)	R. I.	Tentative identify	Mol. mass	Peak area ($\times 10^6$)
1 [a]	4.410	831	S-methylthiobutyrate	118	1.435
2	5.906	929	unknown	118	1.333
3 [a]	6.439	962	Dimethyltrisulfide	126	7.112
4	6.592	972	unknown	126	9.208
5	7.476	1019	unknown	130	0.381
6 [a]	9.476	1106	Phenyl ethyl alcohol	122	2.138
					23.420
7	12.129	1182	unknown	128	1.219
8 [a]	13.198	1212	Dimethyltetrasulfide	158	0.666
9	19.549	1404	Geosmin	182	1.177
10	20.866	1452	unknown	204	0.929
11	26.471	1679	unknown	236	1.307
12 [a]	11.429	1162	2-piperidinone	99	3.491
13 [a]	13.635	1224	2-Coumaranone	134	33.335
14 [a]	18.837	1381	Benzeneacetamide	135	1.780
15	19.161	1391	unknown	196	1.882
16 [a]	22.063	1495	N-phenethylacetamide	163	18.217
17	24.440	1591	unknown	238	2.391
18	24.742	1603	unknown	177	2.203
19	26.788	1693	unknown	210	28.308
20 [a]	27.889	1744	Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	154	30.794
21	29.229	1807	unknown	210	90.783
22	29.629	1824	unknown	210	10.629
23 [a]	31.127	1901	L,L-Cyclo(leucylprolyl) isomer	210	48.613
24 [a]	31.527	1920	L,L-Cyclo(leucylprolyl)	210	80.309
25 [a]	37.959	none	2,5-piperazinedione, 3,6-bis(2-methylpropyl)-	226	133.372

[a]Assignment confidence for peaks 1, 3, 6, 8, 12, 13, 14, 16, 20, 23, 24 and 25 are, respectively, 85.8%, 98.9%, 83.4%, 87.3%, 93.2%, 92.8%, 73.8%, 84.0%, 79.5%, 74.3%, 91.7% and 74.2%. For peak 6, the top peak area and bottom peak area are from SPME and SPE GC chromatogram, respectively. All other peaks did not correspond to compounds in the NIST database.

4.2.2 Qualitative comparison of SPE and SPME in BS15C

BS15C, a new genius belongs to *Hypoxylon sp*., was selected for study based on the observation that compounds having distinctive odors were produced. The phylogenetic characterization of this fungus was published by our group earlier last year.[13] Both BS15C and other *Hypoxylon sp.* have been previously shown to produce a wide variety of volatile organic hydrocarbons.[13,15] In our previous work, liquid-liquid extraction using ethyl acetate/water and SPE methods were both employed to extract VOCs from BS15C. Despite the difference of the amount of some products, the majority of the compounds extracted were the same using either method. However, there were significant differences of both the amount and the profile of the compounds extracted by SPME and SPE methods. The complete list of compounds extracted by both methods are listed in table 4.2. The nominal masses of these compounds were obtained and six of them were tentatively identified. 4-methoxy-2,5-dimethyl-3(2H)-furanone and cyclo-gly-pro were confirmed by comparing both retention time and mass spectra with that of internal standards. Figure 4.2 illustrates a direct comparison of volatile hydrocarbons extracted by both SPME and SPE method. Neither SPME method nor SPE method extracted the same compounds. The number of VOCs extracted by SPE were significantly more than SPME, indicating most of the volatiles hydrocarbons actually remained in the liquid phase. Compounds from peak 40 to peak 52 were considered to have higher boiling point and lower vapor pressure, thus they were eluted later from the column. SPE outperformed SPME in extracting those compounds, which deviates from the common practice that SPME is the most dominantly used technique in extracting VOCs from fungi.

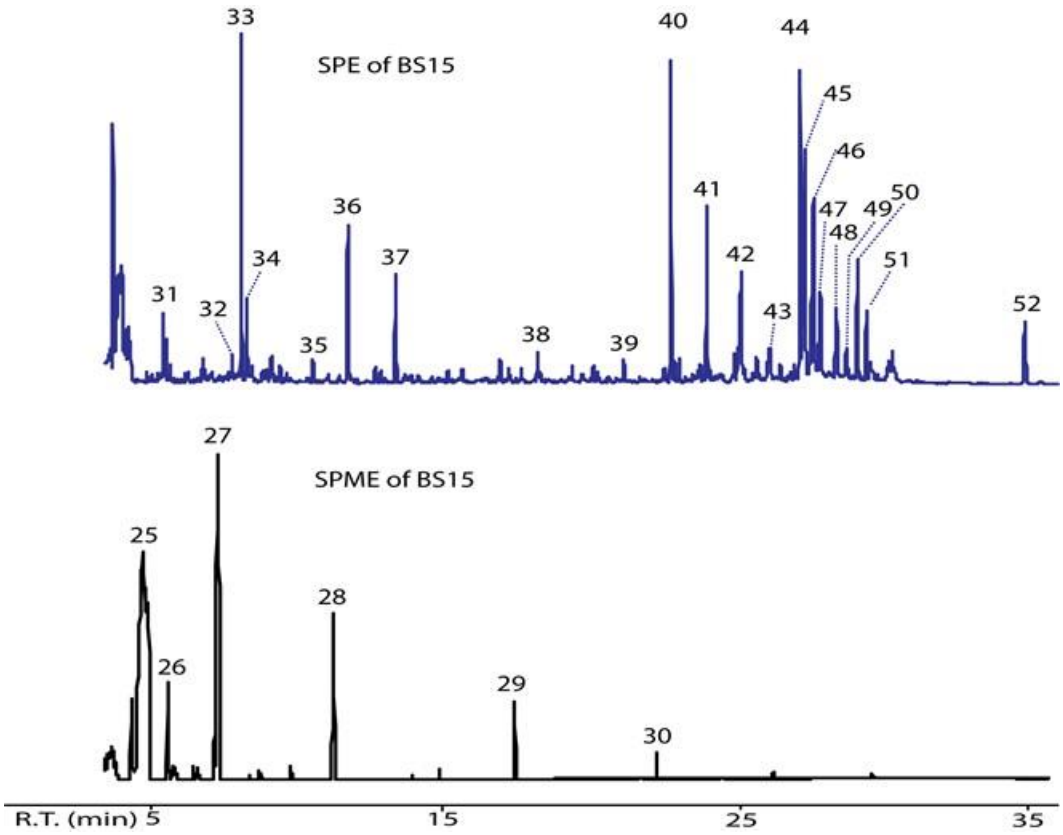

Figure 4.2 A gas chromatogram illustrating VOCs produced by BS15C using SPME and SPE techniques, respectively. Nominal mass of each peak and tentative identities are listed on table 4.2.

Table 4.2 A GC/MS analysis of the VOCs produced by BS15C.

Peak	R.T. (min)	R.I.	Tentative Identify	Mol. mass	Peak area ($\times 10^6$)
25	4.379	829	Unknown	222	1.570
26 [a]	5.250	888	Oxime-, methoxy-phenyl-	151	0.0701
27	6.993	996	unknown	296	0.633
28	11.025	1150	unknown	370	0.231
29	17.316	1332	unknown	444	0.0601
30	22.177	1499	unknown	384	0.0256
31	5.263	888	Unknown	114	2.831
32	7.812	1034	Unknown	170	1.967
33 [a]	8.150	1050	4-methoxy-2,5-dimethyl-3(2H)-furanone	142	17.537
34	8.354	1059	Unknown	120	5.698
35	10.814	1144	Unknown	196	2.071
36	12.121	1181	Unknown	315	12.802
37	13.893	1231	Unknown	156	8.259
38	19.167	1391	Unknown	186	1.737
39	22.375	1507	Unknown	144	1.544
40 [a]	24.117	1578	Dimethyl-1,8-naphthalenedioxysilane	216	18.534
41	25.462	1635	Unknown	194	10.911
42	26.732	1690	Unknown	210	12.981
43 [a]	27.795	1739	Cyclo-gly-pro	154	5.595
44	28.934	1792	Unknown	279	19.633
45	29.100	1800	Unknown	210	28.387
46	29.417	1816	Unknown	222	24.732
47	29.679	1829	Unknown	160	10.888
48	30.277	1859	Unknown	182	7.505
49	30.639	1877	Unknown	208	3.521
50 [a]	31.062	1897	L,L-Cyclo(leucylprolyl) isomer	210	9.866
51 [a]	31.390	1913	L,L-Cyclo(leucylprolyl)	210	4.709
52	37.276	None	Unknown	255	6.757

[a]Assignment confidence for peaks 26, 33, 40, 43, 50 and 51 are, respectively, 90.8%, 96.7%, 93.9%, 71.5%, 72.2% and 96.5%. All other peaks did not correspond to compounds in the NIST database.

Simulated distillation (SimDist) by gas chromatography is one of the analytical methods to determine the properties of crude oils and petroleum products. The system was calibrated by analyzing a standard mixture of n-alkanes from C5 to C40 with boiling temperature range from 36˚C to 525˚C. The calibration curve was built by the boiling point of the n-alkanes with respect to their corresponding retention times, and it was used to determine the boiling point range distribution of the sample.[18] The qualitative retention time index standard that used to calculate the retention indices of the volatile compounds contains 27 n-alkanes, ranging from C7 to C33. Due to the limitation of the highest temperature that the current column can reach, only alkanes within the temperature range (45˚C to 250˚C), which consists of n-alkanes with carbon numbers from C_7 to C_{21}, can be eluted from the column and detected by the mass spectroscopy. Jones *et al.* converted biomass to gasoline and diesel, and ran the SIMDIST test for boiling range distribution of petroleum fractions by gas chromatography, and they found the boiling point range for their products remains both in gasoline range (45˚C to 180 ˚C) and diesel range (180 ˚C to 350 ˚C).[19] Interestingly, both Figure 4.1 and Figure 4.2 show that SPE turned out to be more effective extracting VOCs corresponding to n-C_{16} to n-C_{21} than SPME. The boiling point range for those volatile compounds fall into the higher end of the gasoline range and lower end of the diesel range.

4.2.3 Quantitative analysis of four VOCs from SA40053 and BS15C

Single point external calibration is one of the most common way for standardizing a method. It involves comparison of peak areas from the sample to the peak areas from the external standards in the calibration curve, and the measured peak area is assumed be

proportional to the concentration of the external standard solutions. Under this assumption, equation 1 and equations 2 should be fulfilled by both the analytes and the external standards.

$$[Peak\ area]_{analyte} = \mathrm{k} \times C_{analyte} \quad (1)$$

$$[Peak\ area]_{external\ standard} = k \times C_{external\ standard} \quad (2)$$

k is the response factor for a given external compound and $C_{analyte}$ and $C_{external\ standard}$ are the concentrations of the target analyte and the external standard, respectively. These two equations show that the plot of peak area against concentration should follow a straight line, and the determination of concentrations of the analytes are possible by plugging the linear equations of the straight lines, regardless of potential limitations, such as sample matrix or instrument shifts. Table 4.3 listed of standard solutions with known concentrations for four different external standard compounds and their peak areas in GC chromatogram. In all cases, the average peak area of each solution was calculated and plotted against the concentration of that solution to yield straight trend lines (figure 4.3). The results showed a linear behavior in the range tested with R^2 values ranging from 0.99258 to 0.99882. Which further demonstrated the feasibility of assumptions made earlier that the peak area of the analyte should be proportional to the concentration of that analyte.

In order to quantify the 2,5-dimethyl-4-methoxy-3(2H)-furanone and cyclo-gly-pro produced by BS15C, as well as 2-coumaranone and N- phenethylacetamide produced by SA40053, their average peak areas were plugged in the trend line equations, respectively. Although the limit of detection (LOD) was not validated, and the peak area may not respond to the concentrations of the solutions proportionally beyond the standard solutions prepared here,

this method provides a feasible and simple way to quantify four compounds produced by these two microbial species

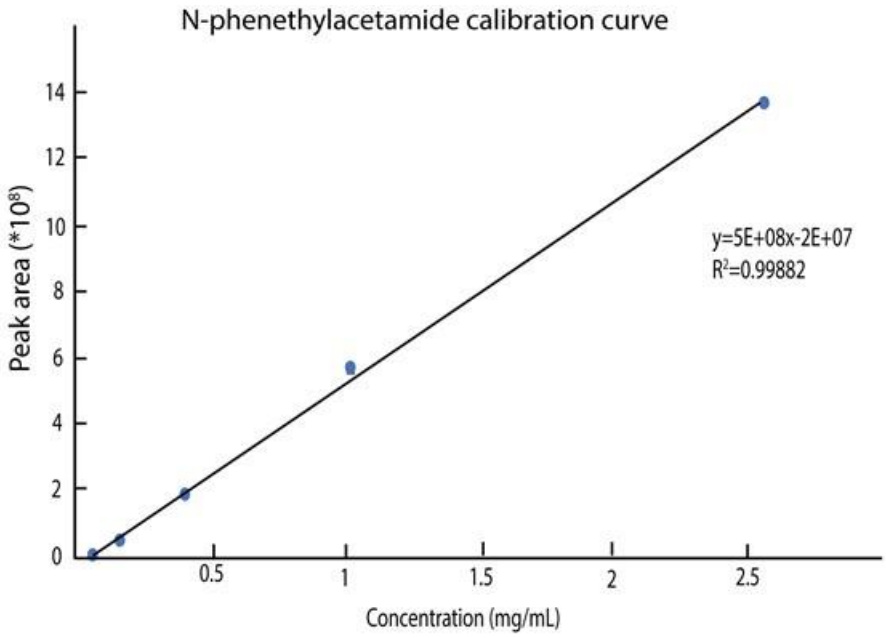

Figure 4.3 Calibration curve of N-phenethylacetamide external standard by plotting peak areas versus concentrations of serial diluted solutions

Table 4.3 Equations of four external standards and their linearity. *****Asterisk denotes the concentrations after taking correctors of evaporation into account. The correctors for evaporation for these four compounds are 100/90.

Compound	Equations	R^2	Concentrations* (mg/mL$\times 10^{-2}$)	Mass (mg) 100mL Broth
N-phenethylacetamide	y=5$\times 10^8 x$-2$\times 10^7$	0.99882	8.493	8.493
2-Coumaranone	y=4$\times 10^8 x$+3$\times 10^7$	0.99283	0.926	0.926
Cyclo-gly-pro	y=4$\times 10^8 x$-3$\times 10^6$	0.99258	2.388	2.388
2,5-dimethyl-4-methoxy-3(2H)-furanone	y=2$\times 10^8 x$+9$\times 10^6$	0.99335	4.743	4.743

4.2.4 Quantitative comparison of SPE and SPME in extracting VOCs from SA40053 and BS15C

Neither qualitative nor quantitative comparison between SPME and SPE in extracting VOCs has been reported yet. It's achievable to accomplish the quantitative comparison if the following assumptions are met: 1), peak areas for all the compounds produced are linear with respect to the concentrations of the analytes in the calibration curves; 2), although calibration curves for every single compound produced by either SA40053 or BS15C cannot be established, their concentrations can still be estimated by plugging peak areas into the known calibration curve, respectively. Thus, the mass of VOCs extracted by both techniques in one liter broth can also be calculated. Given the similarity of slopes between the four calibration curves, especially the magnitudes are the same, it is reasonable to assume calibration curves for the rest of the compounds are similar to N-phenethylacetamide calibration curve. Table 4.4 includes the calculation of concentrations of each compound and their masses in one liter media broth extracted by both SPME and SPE techniques. As expected, the total masses of VOCs extracted by SPE were significantly greater than that of SPME. For BS15C, the mass of VOCs extracted by SPE was five time greater than that of SPME. Even though the hypothesis made earlier might not be perfect, it gave us a clear and obvious way to compare the effectiveness extracting VOCs between these two techniques. SPE is a more efficient technique than SPME extracting VOCs with higher boiling temperature range.

Table 4.4 Qualitative comparison between SPME and SPE techniques extracting VOCs produced by SA40053 and BS15C. * Asterisk denotes the concentrations after taking corrector of evaporation (100/90) into account.

	Peak SA40053	Concentration* (mg/mL× 10^{-2})	Mass (mg/L)		Peak BS15C	Concentration* (mg/mL× 10^{-2})	Mass (mg/L)
SPME	1	4.763	47.63	SPME	25	4.793	47.93
	2	4.740	47.4		26	4.460	44.60
	3	6.027	60.27		27	4.585	45.85
	4	6.491	64.91		28	4.496	44.96
	5	4.529	45.29		29	4.458	44.58
	6	4.920	49.2		30	4.450	44.50
				Total mass (mg)			**271.42**
	7	4.715	47.15	SPE	31	5.074	50.74
	8	4.592	45.92		32	4.882	48.82
	9	4.706	47.06		33	4.743	47.43
	10	4.651	46.51		34	5.711	57.11
	11	4.735	47.35		35	4.905	49.05
Total mass (mg)			**548.69**		36	7.289	72.89
SPE	6	9.649	96.49		37	6.280	62.80
	12	5.220	52.2		38	4.830	48.30
	13	0.926	9.26		39	4.788	47.88
	14	4.840	48.4		40	8.563	85.63
	15	4.863	48.63		41	6.869	68.69
	16	8.493	84.93		42	7.329	73.29
	17	4.976	49.76		43	2.388	23.88
	18	4.934	49.34		44	8.807	88.07
	19	10.735	107.35		45	10.753	107.53
	20	11.288	112.88		46	9.940	99.40
	21	24.618	246.18		47	6.864	68.64
	22	6.806	68.06		48	6.112	61.12
	23	15.247	152.47		49	5.227	52.27
	24	22.291	222.91		50	6.637	66.37
	25	34.083	340.83		51	5.491	54.91
					52	5.946	59.46
	Total Mass (mg)		**1689.69**				**1394.28**

4.3 Conclusions

In the present study, we successfully quantify four peaks produced by BS15C and SA40053 by building calibration curves using multiple external standards, as well as compared the effectiveness between SPME and SPE methods quantitatively. Such comparison between these two methods has never done before. Both SPE and SPME sampling techniques have been widely employed in various matrices. When these two techniques are compared in extracting VOCs with fuel potential for the first time, SPE demonstrates major advantages over SPME, especially in extracting VOCs that fall into the diesel boiling point range. This discovery is significantly different from the fact that SPME is the most dominantly employed techniques in extracting VOCs from fungi. Given both techniques extract different VOCs from the same sample, we should also consider using both technique to get a more complete profile of VOCs from fungi in the future.

4.4 References

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APPENDIX A: SUPPORTING INFORMATION FOR CHAPTER 4

Below are several supplementary tables containing raw data used to build the calibration curves and to calculate the concentration of each compound.

Table 4.5 A series of diluted solutions of 2,5-dimethyl-4-methoxy-3(2H)-furanone and their peak areas in GC chromatogram.

Concentration (mg/mL)	Peak area			Average peak area	Standard deviation
	1st injection	2nd injection	3rd injection		
2.148	373,194,083	367,993,141	361,790,489	367,659,238	5709124.942
0.4296	98,826,810	101,324,848	109,293,664	103,148,441	5466523.193
0.08592	23,081,018	21,334,490	23,840,490	22,751,999	1284989.869
0.017184	2,588,566	3,464,385	3,519,683	3,190,878	522349.764
0.0034368	339,895	364,444	284,385	329,575	41015.154

Table 4.6 A series of diluted solutions of cyclo-gly-pro and their peak areas in GC chromatogram.

Concentrations	Peak area			Average peak area	Standard deviation
	1st injection	2nd injection	3rd injection		
0.15625	51,252,249	62,859,988	52,772,671	55,628,303	6308793.493
0.078125	30,191,040	30,945,846	30,709,751	30,615,546	386120.458
0.0391	12,430,384	11,692,693	11,912,889	12,011,989	378698.509
0.01953	3,530,215	3,737,550	3,448,726	3,572,164	148911.362
0.009766	467,946	622,445	816,026	635,472	174405.290

Table 4.7 A series of diluted solutions of 2-coumaranone and their peak areas in GC chromatogram

Concentrations (mg/mL)	Peak area			Average peak area	Standard deviation
	1st injection	2nd injection	3rd injection		
5.4	2,252473931	2320652199	2313766369	2295630833	37533216.04
2.16	1121968016	1100138757	1075200863	1099102545	23400789.54
0.864	483299253	464696179	458303680	468766370.7	12985357.62
0.3456	168557112	162928034	162262118	164582421.3	3458248.9
0.13824	58642015	56325535	50339337	55102295.67	4284372.923
0.055296	19932299	18654657	19924411	19503789	735380.460
0.0221184	4628406	5936571	5188983	5251320	656306.592

Table 4.8 A series of diluted solutions of N- phenethylacetamide and their peak areas in GC chromatogram

Concentrations (mg/mL)	Peak area			Average peak area	Standard deviation
	1st injection	2nd injection	3rd injection		
2.56	1,375,224,835	1,342,934,601	1,383,075,969	1,367,078,468	21234511.13
1.024	556,642,378	576,304,089	580,542,012	571,162,826	12752355.18
0.4096	189051869	191952061	195216529	192,073,486	3084123.265
0.16384	55850508	53697437	52892406	54,146,784	1529387.518
0.065536	10021426	10523282	10860359	10,468,356	422154.973

Table 4.9 The peak areas of four standard external compounds

Standard sample	Peak area			Average peak area
	1st injection	2nd injection	3rd injection	
Furanone	28,212,821	38,415,452	30,040,343	32,222,872
Cyclo-gly-pro	6,898,257	9,779,296	8,700,356	8,459,303
2-coumaranone	33,335,930	31,051,287	30,143,480	31,510,232
N-phenethylacetamide	18,217,336	19,023,443	16,423,678	17,888,152

Table 4.10 A GC/MS analysis of the VOCs produced by SA40053.

Peak	R.T. (min)	R. I.	Tentative identify	Mol. mass	Peak area
1 [a]	4.410	831	S-methylthiobutyrate	118	1,434,930
2	5.906	929	unknown	118	1,332,928
3 [a]	6.439	962	Dimethyltrisulfide	126	7,122,031
4 [a]	6.592	972	Dimethyltrisulfide	126	9,208,234
5	7.476	1019	unknown	130	380,964
6 [a]	9.476	1106	Phenyl ethyl alcohol	122	2,137,939
					23,419,815
7	12.129	1182	unknown	128	1,218,954
8 [a]	13.198	1212	Dimethyltetrasulfide	158	666,347
9	19.549	1404	Geosmin	182	1,177,137
10	20.866	1452	unknown	204	928,609
11	26.471	1679	unknown	236	1,307,157
12 [a]	11.429	1162	2-piperidinone	99	3,491,081
13 [a]	13.635	1224	2-Coumaranone	134	33,335,930
14 [a]	18.837	1381	Benzeneacetamide	135	1,780,016
15	19.161	1391	unknown	196	1,882,267
16 [a]	22.063	1495	N-phenethylacetamide	163	18,217,336
17	24.440	1591	unknown	238	2,390,862
18	24.742	1603	unknown	177	2,203,478
19	26.788	1693	unknown	210	28,307,884
20 [a]	27.889	1744	Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	154	30,794,094
21	29.229	1807	unknown	210	90,782,610
22	29.629	1824	unknown	210	10,628,868
23 [a]	31.127	1901	L,L-Cyclo(leucylprolyl) isomer	210	48,613,299
24 [a]	31.527	1920	L,L-Cyclo(leucylprolyl)	210	80,309,452
25 [a]	37.959	none	2,5-piperazinedione, 3,6-bis(2-methylpropyl)-	226	133,372,326

Table 4.11 A GC/MS analysis of the VOCs produced by BS15C

Peak	R.T. (min)	R.I.	Tentative Identify	Mol. mass	Peak area
25	4.379	829	Unknown	222	1,570,446
26 [a]	5.250	888	Oxime-, methoxy-phenyl-	151	70,075
27	6.993	996	unknown	296	633,270
28	11.025	1150	unknown	370	231,288
29	17.316	1332	unknown	444	60,125
30	22.177	1499	unknown	384	25,612
31	5.263	888	Unknown	114	2,831,110
32	7.812	1034	Unknown	170	1,967,454
33 [a]	8.150	1050	4-methoxy-2,5-dimethyl-3(2H)-furanone	142	17,536,581
34	8.354	1059	Unknown	120	5,698,438
35	10.814	1144	Unknown	196	2,070,880
36	12.121	1181	Unknown	315	12,802,184
37	13.893	1231	Unknown	156	8,259,160
38	19.167	1391	Unknown	186	1,737,043
39	22.375	1507	Unknown	144	1,543,888
40 [a]	24.117	1578	Dimethyl-1,8-naphthalenedioxysilane	216	18,533,900
41	25.462	1635	Unknown	194	10,910,672
42	26.732	1690	Unknown	210	12,981,294
43 [a]	27.795	1739	Cyclo-gly-pro	154	5,595,189
44	28.934	1792	Unknown	279	19,632,562
45	29.100	1800	Unknown	210	28,387,457
46	29.417	1816	Unknown	222	24,732,239
47	29.679	1829	Unknown	160	10,887,960
48	30.277	1859	Unknown	182	7,505,437
49	30.639	1877	Unknown	208	3,521,321
50 [a]	31.062	1897	L,L-Cyclo(leucylprolyl) isomer	210	9,865,544
51 [a]	31.390	1913	L,L-Cyclo(leucylprolyl)	210	4,708,835
52	37.276	None	Unknown	255	6,757,258

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