FUNGAL PRODUCTION OF BIOFUEL AND FLAVOR COMPOUNDS IN LIQUID AND SOLID STATE

by

Heidi Renee Schoen

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Chemical and Biological Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 2017

©COPYRIGHT

by

Heidi Renee Schoen

2017

All Rights Reserved

ACKNOWLEDGEMENTS

The completion of this dissertation would have not been possible without the support I received from the National Science Foundation EFRI program, the Montana State University Center for Biofilm Engineering, the Chemistry and Biochemistry Department, and the Chemical and Biological Engineering Department. I'd also like to thank the following people, in addition to my family, for support during my graduate studies:

The Peyton and Carlson research groups: Muneeb Rathore, Kristopher Hunt, Ashley Beck, Lee McGill, Todd Pederson, Dr. Robert Gardner, Dr. Brandy Stewart, Dr. Natasha Mallette, Dr. James Folsom, Reed Taffs, Karen Moll, Tizsa Bell, Dr. Hans Bernstein, Dr. Zack Jay, Dr. Lisa Krik, Dr. Chaichi Hwang, Dr. Dana Skorupa, and Lisa Weeks.

The Faculty and Staff in Chemical Engineering: Dr. Brent Peyton, Dr. Ross Carlson, Dr. Stephanie Wettstein, Dr. Stephanie McCalla, Alisha Yoder, Shelley Thomas, Jill Brenden, Dr. Ryan Anderson, and Dr. Jeff Heys.

Many people at the Center for Biofilm Engineering: Katie Davis, Greg Krantz, Matt Jackson, Laura Boegli, Dr. Kelly Kirker, Kylie Bodle, Laura Bickle, Ann Willis, Kristen Brileya, Austin Jacobs, and Coltran Hophan-Nicolas

Others across campus: Dr. Gary Strobel, Dr. Berk Knighton, Dr. Brian Bothner, Jackson Toth, and Jesse Thomas.

TABLE OF CONTENTS

1. INTRODUCTION 1

Significance of Fungi and Fuel 1
Background 2
Endophytic Fungi 2
Consolidated Bioprocessing 4
Solid State Fermentation 6
Fungal Secondary Metabolite Production 7
Analytical Methods 9
Summary 11
Dissertation Summary and Outline 12

2. CARBON CHAIN LENGTH OF BIOFUEL-AND FLAVOR-RELEVANT VOLATLE ORGANIC COMPUNDS PRODUCED BY LEGNOCELLULOLYTIC FUNGAL ENDOPHYTES CHANGES WITH CULTURE TEMPERATURE 17

Contribution of Authors and Co-Authors 17
Manuscript Information Page 18
Abstract 19
Manuscript 20
Substrate Utilization and Optimal Growth Conditions 21
VOC Production 23
Additional Metabolic Byproducts 30
Implications 30
Acknowledgements 32
Supplementary Material 32
Methods 32
Microorganism Isolation, Growth Medium and Storage 32
Fungal Cultivation 33
Biomass Dry Weight Analysis 34
Substrate and Product Analysis 35
Analysis of VOCs 36
Additional Implications 37

3. RAPID TOTAL VOLATILE ORGANIC CARBON QUANTIFICATION FROM MICROBIAL FERMENTATION USING A PLATINUM CATALYST AND PROTON TRANSFER REACTION-MASS SPECTROMETRY 45

TABLE OF CONTENTS CONTINUED

Contribution of Authors and Co-Authors 43
Manuscript Information Page 46
Abstract 47
Introduction 48
Materials and Methods 51
Experimental Setup 51
Solid State Fungal Reactor System 52
Microorganism 53
Growth Conditions 54
CO_2 Detector Calibration and Verification 54
Platinum Catalyst VOC Conversion Verification 55
Bioreactor Measurements 56
Proton Transfer Reaction-Mass Spectrometry (PTR-MS) 56
Ethanol PTR-MS Calibration 58
Results 58
Respiratory CO_2, VOC Production and Reproducibility 64
Discussion 65
Acknowledgements 68
Supplemental Material 69

4. VOLATILE ORGANIC COMPOUND PRODUCTION AT VARYING OXYGEN CONDITIONS IN A SOLID STATE FUNGAL REACTOR 70

Contribution of Authors and Co-Authors 70
Manuscript Information Page 71
Abstract 72
Introduction 73
Materials and Methods 75
Microorganism 75
Growth Conditions 75
Solid State Fungal Reactor System 76
Total Volatile Organic Carbon and Respiratory CO_2 Measurement 77
Selectivity 78
Fungal Biomass Estimates 78
Proton Transfer Reaction-Mass Spectrometry (PTR-MS) 80
Head Space Solid Phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME GC-MS) 81
Conversion to Reported Units 82
Results and Discussion 82

TABLE OF CONTENTS CONTINUED

Total VOC Production 82
Carbon Number Four and Higher VOC Production 84
VOC Production through Culture Growth Phases 88
Selectivity 90
Conclusions 93
Acknowledgements 93
Supplemental Material 94
Supplemental Text 95

5. PRODUCTION OF VOLATILE ORGANIC COMPOUNDS UNDER VARYING NITROGEN CONDITIONS BY *ASCOCORYNE SARCOIDES* 99
Contribution of Authors and Co-Authors 99
Manuscript Information Page 100
Abstract 101
Introduction 102
Materials and Methods 104
Microorganism 104
Growth Conditions 104
Fungal Reactor System 105
Biomass Dry Weight Analysis 106
High Performance Liquid Chromatography (HPLC) 106
Proton Transfer Reaction-Mass Spectrometry (PTR-MS) 107
Head Space Solid Phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME GC-MS) 109
Results 109
Volatile Organic Compound Production 109
Volatile Organic Compound Profiles 112
Liquid Metabolites 116
Discussion 119
Acknowledgements 120
Supplemental Material 121

6. CONCLUSIONS & FUTURE WORK 128
Research Summary 128
Future Work 134

REFERENCES 141

LIST OF TABLES

Table Page

2.1. Growth of three *Nodulisporium* fungal endophyte isolates (EC, CO, TI) on different carbon sources at room temperature 22

2.2 Biofuel- and flavor-relevant VOCs produced by fungal endophyte isolates (EC, CO, TI) growing on glucose medium 25

2.S1 Fungal endophyte isolate (EC, CO, TI) biomass yield on glucose as a function of isolate, culture temperature, and culture pH during batch growth. 38

3.S1 Ethanol, methanol, acetaldehyde and terpene quantification data as measured by PTR-MS and VOC CO_2 as measured by the CO_2 detector. 69

4.1 Ions quantified by PTR-MS. 86

4.S1 Identification of volatile organic compounds produced by the endophytic fungus *Nodulisporium* isolate TI-13 grown in a solid state reactor by head space solid phase microextraction gas chromatography mass-spectrometry 94

5.1 Ions quantified by PTR-MS 112

5.2 Compounds quantified using high performance liquid chromatography from *Ascocoryne sarcoides* grown in liquid culture on five different nitrogen sources. 117

5.S1 Identification of volatile organic compounds produced by the endophytic fungus *Ascocoryne sarcoides* by head space solid phase microextraction gas chromatography mass-spectrometry. 123

LIST OF FIGURES

Figure Page

2.1 Carbon chain length for VOCs produced by three fungal endophyte isolates (EC, CO, TI) as a function of temperature at pH 6 during batch growth on glucose media 29

2.2 Fungal endophyte isolate (EC, CO, TI) VOC carbon chain length as a function of temperature at pH 6 during batch growth on glucose media and as a function of metabolism type 29

2.S1 Fungal endophyte isolate growth on different carbon sources 39

2.S2 Fungal endophyte isolate growth at 30 °C and pH 6 40

2.S3 Fungal endophyte isolate (EC, CO, TI) specific biomass accumulation rate and maximum biomass concentration as a function of pH at 30 °C during batch growth on glucose medium 41

2.S4 Fungal endophyte isolate (EC, CO, TI) specific biomass accumulation rate and maximum biomass concentration as a function of temperature at pH 6 during batch growth on glucose media 42

2.S5 Fungal endophyte isolate (EC, CO, TI) culture volatile organic compounds as a function of pH and temperature during batch growth on glucose media 43

2.S6 Fungal endophyte isolate (EC, CO, TI) culture glycerol concentration as a function of pH and temperature during batch growth on glucose media 43

2.S7 Ethanol production at pH 6 as a function of temperature during batch growth on glucose media 44

3.1 Experimental setup for monitoring VOC production from a fungal solid state reactor 52

LIST OF FIGURES CONTINUED

Figure Page

3.3 VOC production in the fungal reactor based on platinum catalyst VOC conversion to CO_2 61

3.4 PTR-MS spectrum for experiment 3 (119 hours) where background VOCs, reactor off-gas air sampled through the catalyst, have been subtracted from the reactor off-gas spectrum....62

4.1 Total volatile organic carbon (VOC) production rates of the endophytic fungus *Nodulisporium* isolate TI-13 grown in a solid state reactor at four different oxygen conditions as measured by a platinum catalyst and CO_2 detector....84

4.2 Panel a depicts the total specific production of carbon number four (C4) and higher volatile organic compounds (open circles), of which a portion are carbon number eight (C8) and higher (black-filled circles), during an experiment at four different oxygen conditions (average of biological duplicates and standard error) as measured by PTR-MS. Panel b depicts average specific production rates of C4 and C8 VOCs during an entire experiment. Panel c depicts the maximum specific production rates identified during each experiment....87

4.3 Specific production rates of ethanol (panel a), phenylacetaldehyde (panel b), ethyl acetate (panel c), and an ester at m/z 103 (panel d) at four different oxygen conditions as measured by PTR-MS 89

4.4 Selectivity, the amount of volatile organic carbon divided by respiratory CO_2 carbon, at all four oxygen concentrations 91

LIST OF FIGURES CONTINUED

Figure Page

5.1 *Ascocoryne sarcoides* VOC total production as the average and standard error of three biological triplicates on each of the five nitrogen sources tested, as well as a control with no nitrogen source. 111

5.2 Carbon number four and higher VOCs (average and standard error of biological triplicates) as measured by PTR-MS from *Ascocoryne sarcoides* grown on ammonium chloride (closed circles), phenylalanine (open diamonds), and leucine (x) as nitrogen sources. 114

5.3 3-methylbutanol as measured by PTR-MS from *Ascocoryne sarcoides* cultures grown on ammonium chloride (panel a), leucine (panel b), phenylalanine (panel c), and asparagine (panel d).. 115

5.S1 Total VOC production (closed circles) as measured by the platinum catalyst and CO_2 detector and cell dry weight (open squares) with ammonium chloride as the nitrogen source as a representative experiment....125

5.S2 *Ascocoryne sarcoides* specific growth rate and standard error of three biological replicates grown on five different nitrogen sources and a control with no nitrogen source provided. 126

5.S3 *Ascocoryne sarcoides* maximum biomass by dry weight and standard error of three biological replicates grown on five different nitrogen sources and a control with no nitrogen source provided....127

6.1 A conceptual packed bed reactor the conversion of agricultural byproduct, here beet pulp, to VOCs including ethanol and smaller amounts of longer chain hydrocarbons by a cellulosic fungus....139

ABSTRACT

Oil reserves are limited, so new sources of fuels and petroleum byproducts must be found. Some endophytic, filamentous fungi produce fuel and flavor relevant compounds from minimally pretreated cellulosic materials. Additionally, fungal volatile organic compounds can act synergistically as mycofumigants to inhibit bacteria, insects, and fungi. This dissertation identifies and quantifies fungal volatile organic compounds.

A new method was created to quantify the total volatile organic carbon in the gas phase. A platinum catalyst was used to completely oxidize organic compounds to carbon dioxide, which was then measured with a carbon dioxide detector. This method agreed to within 94% of volatile organic carbon measurements taken with proton transfer reaction-mass spectrometry. Additionally, fungal production of fuel and flavor relevant volatile organic compounds was measured with varying pH and temperature in liquid cultures from *Nodulisporium* isolates EC, CO and TI. Production was also measured for TI when grown in solid state on the agricultural byproduct beet pulp at varying oxygen conditions. Finally, the model volatile organic compound producing organism *Ascocoryne sarcoides* was grown in liquid state with varying nitrogen sources, including amino acids.

The three *Nodulisporium* isolates produced longer carbon number compounds at lower temperatures, which are better biofuels and are more likely to be bioactive. This trend was especially strong among volatile organic compounds associated with fatty acid metabolism. The fungi produced fewer compounds at lower pH. In solid state, TI had the highest total production of ethanol and carbon number four and higher compounds under anoxic conditions, but the highest production rates under microaerophilic conditions. Additionally, ethanol appeared to be the only major anoxic fermentation byproduct. Finally, *A. sarcoides* produced the most ethanol and carbon number four and higher compounds in the gas phase with ammonium chloride as the nitrogen source. Nitrogen sources that are reactants for volatile organic compounds, like leucine and phenylalanine, had lower gas phase concentrations of volatile organic compounds.

CHAPTER ONE

INTRODUCTION

Significance of Fungi and Fuel

Fossil fuel is a key contributor to the world economy, and economic and social stability depends on its supply (Liao et al. 2016). However, oil reserves are predicted to be completely spent by 2066 (BP, 2015), so alternative sources of fuels and petroleum byproducts are required. Lignocellulosic products, like agricultural residues, yard waste, wood, and paper are renewable, do not complete with food resources, and are one of the most abundant resources on earth (Grigoriev et al. 2011; Saini et al. 2015). These properties make lignocellulosic material an ideal source to replace fossil fuels (Grigoriev et al. 2011a). Some strains of filamentous fungi turn cellulosic materials directly into compounds similar to those in gasoline and diesel fuel (Strobel et al. 2008).

Fungi produce volatile organic compounds (VOCs) that have the same carbon chain lengths as the compounds currently found in gasoline and diesel fuel (Mallette et al. 2014; Strobel et al. 2008; Zhi-Lin et al. 2012). VOCs can be classified as biofuel-relevant if the carbon backbone falls within the range of molecules used in gasoline ($C_4 – C_{12}$) or diesel fuel ($C_8 – C_{25}$) ((U.S. Department of Energy 2013). The endophytic fungus *Ascocoryne sarcoides* produced VOCs of these carbon chain lengths directly from cellulosic feedstocks, showing it has potential as a fuel producing microorganism (Mallette et al. 2014; Strobel et al. 2008). However, initial yields of VOCs produced by *A. sarcoides* are too low to be industrially relevant, and the fungus had lower titer and

slower growth rates than most currently employed fungal industrial strains (Mallette 2013).

Three new fungal isolates were examined for faster growth rates and higher VOC yields and titers. A solid state reactor system was employed in hopes of discovering higher production yields from a cellulosic agricultural byproduct. With this, an additional method to quickly and accurately identify and quantify VOCs was developed so that VOC modulation could be determined more reliably. Temperature, pH, oxygen, and nitrogen sources were varied in attempts to learn more about the endophytic fungi and conditions under which they produce different types and amounts of VOCs.

Background

Endophytic Fungi

Much remains to learn about fungi, with only an estimated 100,000 of 1.5 to 5 million species described so far (Hibbett et al. 2011). Filamentous fungi have many potential industrial applications as they grow well on inexpensive substrates, express and secrete proteins effectively, and make numerous secondary metabolites (Meyer, 2008). Additional metabolic and genetic understanding of filamentous fungi will likely yield environmentally friendly production of many industrial products such as antimicrobials, biofuels, flavor compounds, and pigments (Grigoriev et al. 2011). One poorly studied class with great potential industrial application is endophytic fungi, or fungi that live within plants without causing apparent harm (Abrahão et al. 2013).

Endophytes are a significant part of the ecosystem of plants, (Mukherjee et al. 2013; Porras-Alfaro & Bayman, 2011), with endophytes having been isolated from every sampled plant (Suryanarayanan, 2013). Different endophytes may function as mutualists, pathogens, saprotrophs, or temporary residents with little impact, and their relationship may vary based on environmental conditions and throughout the fungal lifecycle (Saikkonen et al. 1998; Suryanarayanan, 2013). When acting as mutualists, endophytic secondary metabolites may help plants defend against microbial pathogens, insects, nematodes, and animal herbivores (Mukherjee et al. 2013; Porras-Alfaro & Bayman, 2011; Rodriguez et al. 2009; Saikkonen et al. 1998). Endophytes may also provide drought resistance, heat resistance, and improve seed dispersal among other benefits for the plant (Saikkonen et al. 1998). Alkaloid compounds specifically are attributed to herbivore resistance in grasses, although *in situ* data linking the production of specific endophyte metabolites to plant benefits is limited, especially in non-grass plants (Saikkonen et al. 1998; Suryanarayanan, 2013).

Ex situ, hundreds of fungal endophytic bioactive compounds have been identified, including alkaloids, terpenoids, flavonoids, and steroids, which can act as anticancer, antitumor, antibiotic, and mycofumigation compounds (Guo et al. 2008; Zhi-Lin et al. 2012). This research focuses on bioactive VOCs, such as alcohols, esters, ketones, acids, and lipids, that can inhibit bacterial and fungal growth (Strobel et al. 2001). Fungal endophytes have also produced VOCs, like naphthalene and naphthalene derivatives, which repel insects (Daisy et al. 2002; Strobel et al. 2008). The combinations of VOCs produced by endophytic fungi work synergistically to further inhibit or kill fungi and

bacteria (Strobel et al. 2001). These VOC suites may act by damaging DNA and disrupting cell membrane integrity (Campos et al. 2015). Endophytic VOCs have potential as industrial biofumigants that can be used to treat human wastes, fresh produce, and agricultural soils (Guo et al. 2008; Strobel et al. 2008c; Zhi-Lin et al. 2012).

Additionally, endophytic fungi produce numerous industrially relevant flavor compounds, such as eucalyptol; limonene; benzaldehyde; 1-butanol, 3-methyl-; and phenylethyl alcohol (Berger, 2009; Jager et al. 1996; Mallette et al. 2012; Rodriguez-Bustamante & Sanchez, 2007; Tomsheck et al. 2010). Flavor compounds make up a quarter of all food additives and globally are a multi-billion dollar business annually (Ben Akacha & Gargouri, 2015). Many flavor compounds are currently produced from petroleum byproducts, so new sources will need to be found as fossil fuels are depleted (Schwab et al. 2008). Fungal flavor compounds are considered all natural and can be sold for several times more than the same fossil fuel derived product (Ben Akacha & Gargouri, 2015; Krings & Berger, 1998). These higher value specialty chemicals may become industrially viable fungal products before low value fungal biofuels are economically competitive.

In this research, the VOC products of four endophytic fungi were identified. Previously, *Ascocoryne sarcoides* (NRRL 50072) was isolated from *Eucryphia cordifolia* in a temperate Patagonian rainforest. Additionally, three *Nodulisporium* isolates from tropical locations were investigated in hopes of finding higher titers and faster growth rates than those determined for *A. sarcoides*. *Nodulisporium* isolate EC38-12 (NRRL 50503) was obtained from *Neea floribunda* sampled from the Ecuadorian Amazon.

Nodulisporium isolate CO27-5 (NRRL 50500) was obtained from *Rhizophora mangle* (red mangrove) sampled from the Western coast of the mid-region of Columbia. *Nodulisporium* isolate TI-13 (NRRL 50502) was obtained from *Cassia fistula* sampled from the highlands of Thailand.

Consolidated Bioprocessing

Several VOC producing fungi are candidates for consolidated bioprocessing, the conversion of lignocellulose into product in one reactor, since they can utilize a variety of waste biomass sources and produce VOCs with biofuel potential (Lynd et al. 2005; Strobel et al. 2008). Consolidated bioprocessing has the potential to reduce bioprocessing costs up to 75% compared to traditional multi-reactor approaches (Lynd et al. 2005). Consolidated bioprocessing generally focuses on the genetic engineering of a high fuel yielding organism to additionally degrade cellulose and utilize numerous types of sugars (Lynd et al. 2005; Xu et al. 2009). Alternatively, a cellulose degrading organism may be genetically engineered to have higher yields of biofuel (Lynd et al. 2005; Xu et al. 2009). Difficulties have been encountered in engineering a high fuel yielding organism to produce cellulases; low activities of heterologous cellulytic enzymes have been reported as well as problems expressing and secreting the multiple enzymes required in appropriate ratios (Hasunuma et al. 2013; Lynd et al. 2005; Xu et al. 2009). Additionally, many consolidated bioprocessing schemes focus on bioethanol production, and the VOCs identified here are more energy dense and could replace a larger portion of fuel (Atsumi et al. 2008; Lynd et al. 2005). This makes cellulolytic organisms producing VOCs similar to gasoline and diesel fuel desirable as consolidated bioprocessing organisms.

Solid State Fermentation

Solid state fungal fermentation is an excellent resource for conversion of lignocellulosic biomass to biofuels and flavor compounds (Holker et al. 2004). Solid state reactors require less energy, space, and water than submerged fermentations. Additionally solid state systems typically produce less waste water and have easier separation processes (Ali & Zulkali, 2011; Holker et al. 2004). Often higher yields of desired compounds are obtained in fungal solid state fermentations compared to liquid phase (Holker et al. 2004). Solid state conditions mimic the natural environment of most filamentous fungi; in fact, sometimes product will only be expressed in solid state (Ali & Zulkali, 2011; Ben Akacha & Gargouri, 2015; Holker et al. 2004; Singhania et al. 2009). Fermentations on solid cellulosic carbon sources may be more cost effective and environmentally friendly one-step processes compared to schemes that include a separate cellulase production step (Lynd et al. 2005). Additionally, the complex carbon source cellulose was shown to yield higher VOC production in the endophyte *Ascocoryne sarcoides* than glucose in previous experiments (Mallette et al. 2014).

Despite these advantages, solid state fermentation has been a neglected technology. Penicillin production made submerged fermentation the default biotechnology commercial production scheme for industrialized nations; the ease of monitoring biomass and controlling oxygen, temperature and nutrient levels was very appealing (Pandey, 2003). The last couple of decades have seen a solid state renaissance including the creation of novel mathematical modeling, reactor designs, and monitoring technology necessary for solid state fermentation to become commercially viable

(Thomas et al. 2013). Solid state fermentation production of enzymes is currently a multibillion dollar business globally (Thomas et al. 2013). Attempting to expand the industrial applicability of solid state fermentations, a model reactor has been constructed in China for economically competitive bioethanol production from sweet sorghum stems (Li et al. 2013). A tray solid state reactor design with the agricultural residue beet pulp as the solid substrate was used in this research for the production of biofuel and flavor VOCs.

Fungal Secondary Metabolite Production

Many endophytic VOCs of interest are secondary metabolites. Secondary metabolites are compounds that are not directly required for cell growth and are often produced once growth has slowed in association with stationary phase (Brakhage, 2013; Demain, 1986). Many secondary metabolites are bioactive and are produced for cell-to-cell communication, inhibition of other organisms, to control morphological changes, and sometimes for yet unknown reasons (Brakhage, 2013; Keller et al. 2005). Fungi do not produce secondary metabolites under all culture conditions, and expression of genetically predicted secondary metabolites can be difficult or currently impossible to induce (Brakhage, 2013; Keller et al. 2005). The active expression of metabolic pathways is required for techniques like transcriptomics to identify the RNA responsible for production of products. Since fungi may not express secondary metabolites under many culture conditions, identification of pathways utilized for hydrcarbon production can be difficult. For example, the pathway for octane, the potental biofuel product of *Ascocoryne sarcoides*, was not identified in an initial transcriptomic study and is still

unknown (Gianoulis et al. 2012). This research demonstrates conditions which change fungal VOC expression and analytical techniques to readily quantify changes in production, which can aid in future transcriptomic studies.

There are several known types of pathways which contribute to VOC production in fungi. The Ehrlich pathway assimilates amino acids for nitrogen and produces VOCs as byproducts. This pathway deaminates seven amino acids, resulting in an α-keto acid, which is then decarboxylated into a fusel aldehyde. The aldehyde is oxidized to a fusel acid, such as isovaleric acid or reduced to a fusel alcohol, such a 3-methyl, 1-butanol, depending on the redox state of the cell (Hazelwood et al. 2008). Amino acid conversion to the cellular nitrogen pool and fuel alcohols and acids is performed predominately during exponential growth phase, and is therefore associated with primary metabolism, but induction may also occur in association with secondary metabolism (Gianoulis et al. 2012; Roze et al. 2010a). A second source is the modification of linoleic acid by lipoxygenases resulting in the formation of C8 alcohols and ketones, such as 1-octen-3-ol and 3-octanone (Gianoulis et al. 2012; Wurzenberger & Grosch, 1984). Many VOCs are produced in association with fatty acid pathways, such as the mevalonate pathway producing precursors for terpenes like myrtenol, d-limonene, α-humulene, and pinocarveol (Mallette et al. 2014; Quin et al. 2014; Strobel et al. 2008b). Finally some smaller VOCs are produced from fermentation pathways such as ethanol or 2,3-butanediol (Xiao et al. 2012).

Non-corresponding VOC production (e.g. changing nitrogen source to an Ehrlich pathway associated amino acid will not only increase Ehrlich metabolites, but fatty acid

pathway related products as well) was also quantified in this research. Induction or changes in yield of secondary metabolites by altering culture conditions has been well documented, including different strains, temperatures, media, oxygen conditions, and co-culturing (Ahamed & Ahring, 2011; Bok & Keller, 2004; Bunge et al. 2008; Kai et al. 2009; Mallette et al. 2014; Roze et al. 2010). Differences in secondary metabolite production were identified in this dissertation by altering temperature, pH, oxygen and nitrogen sources in experiments and utilizing different fungal strains.

Analytical Methods

A lack of analytical methods to quickly and reliably identify and quantify microbial VOCs has held back their study (Morath et al. 2012). A combination of analytical methods are utilized in this dissertation to confidently quantify fungal secondary metbolite production through growth phases and under different culturing conditions. The methods are focused on the headspace, since the products of interest are typically very volatile.

Headspace Solid Phase Microextration Gas Chromotography-Mass Spectroscopy (HS SPME GC-MS) has been utilized widely to identify fungal VOCs (Griffin et al. 2010; Mallette et al. 2012; Stoppacher et al. 2010). This method is attractive because it can confidently identify metabolites without extensive laboratory preparation. In this method a fiber is placed in the headspace of a microbial sample, and VOCs present in the gas phase adsorb onto the fiber. Unfortunately this means the method is inherently biased as some metabolites adhere more readily to the fiber resulting in competitive adsorption effects that can reduce reliability of concentration measurements (Mallette et al. 2012).

A complimentary method which can confidently quantify headspace volatiles is Proton Transfer Reaction-Mass Spectrometry (PTR-MS). PTR-MS is a gentle chemical ionization method, where a proton is transferred to volatiles with proton affinities greater than water from hydronium ions, often leaving the protonated molecular ion intact (Lindinger et al. 1998). PTR-MS can directly sample the off-gas of liquid or solid state fungal cultures and many VOCs can be quantified down to the 10 – 100 parts per trillion range with reliable results (Biasioli et al. 2011; de Gouw et al. 2003; Lindinger et al. 1998; Luchner et al. 2012). These real-time gas phase measurements readily correlate VOC concentration variations to experimental conditions and temporal changes (Luchner et al. 2012). While PTR-MS provides information on identity with mass to charge ratios, prior knowledge of likely compound identification is required for firm identification. Methods like SPME GC-MS, liquid chromatography-mass spectrometry (LC-MS), or nuclear magnetic resonance may be used in conjunction with PTR-MS to aid in ion identification (Mallette et al. 2012).

While PTR-MS is inherently quantitative, total VOC quantification can be inhibited by a number of issues, like compounds that are not detected by PTR-MS (e.g. alkanes) or complex mixtures where multiple compounds contribute to the same mass to charge ratio (Lindinger et al. 1998). An additional method was employed to accurately quantify the total VOC content of reactor off-gas and to aid in identifying any issues in PTR-MS measurements. In this method, a platinum catalyst was utilized to oxidize VOCs completely to CO_2. A sensitive CO_2 detector then collected two streams of data: respiratory CO_2 directly from the reactor off-gas and a combination of respiratory CO_2

and the CO_2 that was produced from the platinum catalyst oxidation of VOCs. The difference between these two data streams is the total carbon content of the VOCs in the off-gas. By continuously recording these data, a record of respiratory CO_2 and total VOC production can be created.

Many metabolites, including glucose, ethanol, ethyl acetate, and Ehrlich pathway products, can be measured using high performance liquid chromatography (HPLC). HPLC provides quantification of the compounds in the liquid phase, which is helpful because some metabolites, like Ehrlich pathway acids, are not particularly volatile. For compounds that may be present in the liquid and vapor phase, Henry's law (Mallette et al. 2012) and PTR-MS branching fractions (Ezra et al. 2004) can assist in identification and quantification of vapor phase compounds and resolving multiple compounds contributing to the same mass to charge ratio in a PTR-MS spectrum.

Summary

Endophytic fungi are an untapped source of bioactive VOCs with biofuel, flavor, and biofumigant potential. These fungi can utilize cellulosic feedstocks to produce VOCs in one-step solid state fermentation processes, which are often more environmentally friendly and economical than traditional multiple liquid phase reactor schemes. Varying culture conditions can significantly change the types and amounts of VOCs produced. A greater understanding of these fungi is necessary to produce industrially relevant yields of valuable VOCs. The methods described here can also be applied to the study of headspace metabolites in other microorganisms to gain a deeper understanding of microbiology.

Dissertation Summary and Outline

The chapters in this dissertation identify the industrially relevant VOCs produced by four endophytic fungi at varying culture conditions, quantify products at varying culture conditions for TI-13 and *A. sarcoides*, and introduce a new method to readily quantify total VOC production. A brief summary of each chapter's highlights is provided below:

Chapter 2: Carbon chain length of biofuel- and flavor-relevant volatile organic compounds produced by lignocellulolytic fungal endophytes vary with culturing temperature. In this chapter, three VOC producing *Nodulisporium* isolates from tropical environments were studied in an attempt to identify fungi that grew at industrially relevant rates and temperatures. Additionally, the VOCs produced were categorized as being associated with one of the following four pathway types: Ehrlich, fatty acid, degradation of linoleic acid, and fermentation. The fungal VOCs produced were identified under varying pH and temperature conditions to determine culture conditions for production of industrially desirable compounds. Longer carbon chain products, which are more desirable biofuels, were identified at lower temperatures. This trend was strongest for VOCs associated with fatty acid metabolism, such as 2,6-dimethyl octane and eucalyptol. Interestingly, more fermentation products, like acetoin and 2,3-butanediol, were observed at higher temperatures. This chapter demonstrates that higher value VOCs were produced at lower temperatures by the three *Nodulisporium* isolates. Also in Chapter 2, optimal pH was 6 and temperature 30 to 33 °C for titer and growth rate for all three strains with increased fungal biomass concentrations of up to 19 +/- 1.1

g/L for isolate CO compared to 5 g/L for *A. sarcoides*. Growth rates were not improved with a maximum of 1.6 +/- 0.084 day $^{-1}$ for TI compared to 2.1 day $^{-1}$ for *A. sarcoides*.

Chapter 3: Rapid total volatile organic carbon quantification from microbial fermentation using an oxidizing platinum catalyst and proton transfer reaction-mass spectrometry. In this chapter, a new total VOC quantification method consisting of a heated platinum catalyst and sensitive CO_2 detector was introduced to study the complex mixtures of VOCs produced by fungi. The platinum catalyst completely and quantitatively oxidized VOCs to CO_2 to readily provide total VOC production in nearly real-time. This new method is more accurate at measuring total VOC production than other available measurement systems. In this chapter, the platinum catalyst and sensitive CO_2 detector were employed to quantify total VOC production of a complex mixture produced in a non-homogenous solid state reactor system by *Nodulisporium* isolate TI-13. This new method can be used to quantify total VOC production, monitor processes, help understand new processes as well as to compare total quantification with other measurement systems. Chapter 3 employed proton transfer reaction-mass spectrometry (PTR-MS) to quantify individual compounds over time. In this model system, the total VOC production measured by the PTR-MS agreed with the platinum catalyst total quantification to within 94 % on average. The comparison of these two systems can help identify issues like compounds that cannot be measured by PTR-MS (i.e. alkanes) or issue where multiple compounds with different calibration factors are contributing to the same mass to charge ratio. This method quickly and accurately measured VOCs in

complex mixtures from a heterogeneous fungal solid state reactor over several biological replicate experiments.

Chapter 4: Fungal volatile organic carbon production in a solid state reactor at varying oxygen conditions. In this chapter, production of VOCs by *Nodulisporium* isolate TI-13 was quantified in a solid state reactor at four oxygen concentrations because varying oxygen condition has been shown to change secondary metabolite expression in fungi. In Chapter 4, production of carbon chain length four and longer (C4) compounds, increased six fold at anoxic conditions compared to aerobic conditions, including compounds like 3-methylbutanol, benzaldehyde, and ethyl acetate as quantified by PTR-MS and identified by SPME GC-MS. Yields of C4 and higher VOCs were highest under microaerophilic conditions. Additionally, total volatile organic compound (of any length) production increased by 24 times at anoxic conditions compared to aerobic conditions. Selectivity, defined here as the carbon from VOCs measured by the platinum catalyst, divided by carbon in respiratory CO_2, achieved the theoretical maximum of 2 briefly under anoxic conditions, predominantly due to ethanol. *Nodulisporium* isolate TI-13 produces concentrations of ethanol near the theoretical maximum in headspace selectivity, which is a desirable byproduct in C4 and higher VOC production schemes, since it is currently a widely used biofuel. This is interesting because the cellulosic fungus TI-13 produces ethanol as a fermentations product, as opposed to the mixed acid fermentation of the previously studied VOC producing fungus *Ascocoryne sarcoides.* While overall yields of C4 and higher compounds were several orders of magnitude

below industrially viability, Chapter 4 demonstrated confident quantification and modulation of these secondary metabolites.

Chapter 5: Production of volatile organic compounds with varying nitrogen sources by *Ascocoryne sarcoides*. In this chapter, the model VOC producing organism, *Ascocoryne sarcoides*, was studied, since it has an annotated genome and metabolic model. Gas phase VOC and liquid metabolite production data were collected. These data can be used in conjunction with the genome and metabolic model to better understand fungal physiology and potentially aid in future genetic engineering work. Nitrogen variation was chosen because some amino acids, which were used as nitrogen sources, feed directly into the Ehrlich pathway to produce biofuel and flavor relevant VOCs. Additionally, nitrogen source can activate secondary metabolites promoters in fungi. This chapter identified that ammonium chloride resulted production of the highest concentrations of C4 and higher compounds, including compounds such as phenyl ethyl alcohol; propanoic acid, 2-methyl; and ethyl acetate. VOC concentrations per biomass are listed in order from highest to lowest for the following nitrogen sources: ammonium chloride, leucine, phenylalanine, methionine, and asparagine. Maximum biomass titer and growth rate were achieved with asparagine, although it produced the lowest VOC concentrations. This chapter demonstrated high VOC yields are likely caused by genetic regulation and not directly by pathway reactants.

Each chapter of this dissertation provides a unique understanding of ways to modulate or measure volatile organic compound production by cellulosic fungi. The next four research chapters are composed of an introduction, methods, research results and

discussion section. Afterwards a conclusion chapter summarizes the results of this research and presents ideas for future work that follows as a result of these chapters.

CHAPTER TWO

CARBON CHAIN LENGTH OF BIOFUEL- AND FLAVOR-RELEVANT VOLATILE ORGANIC COMPOUNDS PRODUCED BY LIGNOCELLULOLYTIC FUNGAL ENDOPHYTES CHANGES WITH CULTURE TEMPERATURE

Contribution of Authors and Co-Authors

Author: Heidi R. Schoen

Contributions: Assisted in designing the study, collected data, analyzed data, and wrote the paper.

Co-Author: Kristopher A. Hunt

Contributions: Assisted in designing the study, analyzed data, discussed results and implications, and edited the manuscript.

Co-Author: Gary A. Strobel

Contributions: Assisted in designing the study, discussed results and implications, and edited the manuscript.

Co-Author: Brent M. Peyton

Contributions: Assisted in designing the study, discussed results and implications, and edited the manuscript.

Co-Author: Ross P. Carlson

Contributions: Assisted in designing the study, discussed results and implications, and edited the manuscript.

Manuscript Information Page

Heidi R. Schoen, Kristopher A. Hunt, Gary A. Strobel, Brent M. Peyton, Ross A. Carlson
Mycoscience
Status of Manuscript:
_____Prepared for submission to a peer-reviewed journal
__X__Officially submitted to a peer-reviewed journal
_____Accepted by a peer-reviewed journal
_____Published in a peer-reviewed journal

Submitted 08/18/2016

CARBON CHAIN LENGTH OF BIOFUEL- AND FLAVOR-RELEVANT VOLATILE ORGANIC COMPOUNDS PRODUCED BY LIGNOCELLULOLYTIC FUNGAL ENDOPHYTES CHANGES WITH CULTURE TEMPERATURE

Heidi R. Schoen[1,2], Kristopher A. Hunt[1,2], Gary A. Strobel[3], Brent M. Peyton[1,2], Ross P. Carlson[1,2,*]

1. Department of Chemical & Biological Engineering, 306 Cobleigh Hall, Montana State University, Bozeman MT 59717
2. Center for Biofilm Engineering, 366 Barnard Hall, Montana State University, Bozeman MT 59717
3. Department of Plant Sciences, 119 Plant Bioscience Building, Montana State University, Bozeman MT 59717

Abstract

Three fungal endophyte isolates from the genus *Nodulisporium* were studied for volatile organic compound (VOC) production. All three fungi grew on a wide range of carbon substrates ranging from simple sugars to waste biomass sources, including wood chips, corn stover, and sugar beet pulp. The fungi produced more concentrated biomass, up to 19 g cell dry weight per liter, than previously reported VOC-producing fungal endophytes. The fungi synthesized a number of long and short-chain VOCs, including eucalyptol; 1-butanol, 3-methyl; 1-octen-3-ol; and benzaldehyde, all with potential applications as biofuel or flavor compounds. As culture temperature decreased, average VOC carbon chain length increased especially for VOCs associated with fatty acid metabolism. To the best of our knowledge, this is the first report of temperature impacting the carbon chain length of VOCs secreted from microbes. The results provide a

template for controlling synthesis of desired VOCs through selection of species and culturing conditions.

Manuscript

Endophytes live within plant tissues without causing apparent symptoms and have been found in all plant species examined to date (Rodriguez et al. 2009; Porras-Alfaro and Bayman 2011). Fungal endophytes produce a large range of compounds with antibacterial, antifungal or antitumor activity like the anticancer agent Taxol as well as industrially relevant volatile organic compounds (VOCs) (Strobel et al. 1996; Keller et al. 2005). VOC synthesis by fungal endophytes has been known for years (Stinson et al. 2003), but the examination of biofuel and flavor compounds produced by endophytes is relatively new (Tomsheck et al. 2010; Ahamed and Ahring 2011; Mallette et al. 2012). Production of biofuels from waste lignocellulosic biomass is a major global research goal due to finite petroleum reserves and atmospheric greenhouse gas increases (Sánchez and Cardona 2008). Flavor compounds are a large industry with 2013 global sales estimated at more than 23 billion U.S. dollars (Leffingwell and Associates 2014). Endophyte-produced flavor molecules can be marketed as ‘all natural’ making them as much as three orders of magnitude more valuable than compounds produced via synthetic chemistry (Krings and Berger 1998).

Filamentous fungi utilize at least four major pathways to produce VOCs. The fatty acid and mevalonate pathways, referred to here collectively as fatty acid synthesis,

can produce alkanes, fatty alcohols, terpenes and terpenoids (Strobel et al. 2008; Grigoriev et al. 2011; Mallette et al. 2014). The Ehrlich pathway is another VOC production pathway which is used to catabolize amino acids as a source of nitrogen and can be associated with either primary or secondary metabolism (Hazelwood et al. 2008). Beta-oxidation of linoleic acid by lipoxygenases is a third VOC producing pathway resulting in the formation of C_8 alcohols and ketones, such as 1-octen-3-ol and 3-octanone (Wurzenberger and Grosch 1984; Gianoulis et al. 2012). Finally, fermentative metabolism can produce VOCs, such as glycerol and 2,3-butanediol (Huang et al. 2007).

The current study analyzes three new endophytic *Nodulisporium* isolates in detail. The *Nodulisporium* isolates are from distinct, tropical locations (Ecuador, Thailand, Colombia) and distinct plant hosts (Supplementary Material). The isolates were selected to study similarities and differences in growth properties and VOC production profiles across geographical location and to expand the number of physiologically-characterized fungal endophytes in the literature (Ahmed and Ahring, 2011; Mallette et al 2014).

Substrate Utilization and Optimal Growth Conditions

The three *Nodulisporium* fungi isolates (EC, CO, TI) grew on a range of simple and complex carbon sources including xylose, glucose, sucrose and cellobiose as well as the polymers cellulose and xylan (see Supplementary Material for culturing details). The three fungi also demonstrated robust growth on complex agricultural wastes including sugar beet pulp and corn stover (Table 2-1; Supplementary Figure 2-S1). The three fungi

did not grow under anoxic conditions on potato dextrose agar (Sigma-Aldrich, St. Louis, MO, USA).

Table 2-1. Growth of three *Nodulisporium* fungal endophyte isolates (EC, CO, TI) on different carbon sources at room temperature.

Carbon Source	EC	CO	TI
Glucose	+++	+++	+++
Xylose	+++	+++	+++
Glycerol	+++	+++	+++
Sucrose	+++	+++	+++
Cellobiose	+++	+++	+++
Xylan	+++	+++	+++
Cellulose	+++	+++	+++
Lignin	-	-	-
Sugar Beet Pulp	+++	+++	+++
Corn Stover	+++	+++	+++
Grass	+++	+++	+
Paper	+	+	-
Woodchips	+	+	+

+++: Significant growth. More than two centimeter radius of fungal growth in ten days.
+: Growth. Visible growth in ten days.
-: No significant growth. No growth in ten days.

Cultures grew as hyphal suspensions in shake flasks and demonstrated exponential biomass accumulation rates followed by a brief linear biomass accumulation phase (Supplementary Figure 2-S2). Reported specific biomass accumulation rates were taken from the initial growth phase, which formed a straight line on a cell dry weight vs. time semi-log plot. The effect of pH on specific biomass accumulation rate and final biomass titer was measured over a range of initial pH values (4, 5, 6, or 7) at 30 ℃ (Supplementary Figure 2-S3). The fastest specific biomass accumulation rates were observed at pH 6 with specific biomass accumulation rates of 1.1–1.2 d^{-1}; all three fungi had their highest final biomass titer at pH 6. The effect of temperature (room temperature, 27, 30, 33, 37 ℃) on fungal growth was tested using glucose medium with an initial pH

value of 6 (Supplementary Figure 2-S4). The optimal temperature range, based on specific biomass accumulation rate, was 30 to 33 °C. The liquid cultures did not show consistent growth at room temperature (data not shown). Maximum specific biomass accumulation rates and final biomass titers ranged from 1.1–1.6 d^{-1} and 12.9–19.0 g cell dry weight L^{-1}, respectively, for the three isolates. Biomass yields on glucose were calculated for each isolate after exponential biomass accumulation phase (Supplementary Table 2-S2). Typical biomass yields on glucose ranged from 0.15 to 0.3 g cell dry weight per g glucose consumed.

VOC Production

VOC production was measured as a function of culture pH and temperatures because fungal secondary metabolites are well known to vary with culturing conditions (Keller at al. 2005; Mallette et al. 2014). Thirty-six biofuel- and flavor-relevant compounds were identified using SPME GC-MS. Table 2-2 lists the identified VOCs with quality match, relative concentration, as well as the associated fungal isolate and culturing condition. It should be noted that SPME GC-MS-based quantification of metabolite concentration can be complicated by competitive adsorption of compounds (Mallette et al. 2012). VOCs were classified as biofuel-relevant if the carbon backbone fell within the range of molecules used in gasoline (C_4 – C_{12}) or diesel fuel (C_8 – C_{25}) (U.S. Department of Energy 2013). Classification of a chemical as a flavor compound was based on the compound being described in a published report as a flavor compound (Jager et al. 1996; Rodriguez-Bustamante and Sanchez 2007; Berger 2009; Zhi-Lin et al.

2012). All three fungal isolates produced flavor compounds including benzaldehyde; 1-octen-3-ol; and 1-butanol, 3 methyl. Some additional VOCs of interest include 2,3-butanediol secreted by isolate CO (Ng et al. 2012), eucalyptol, a cyclic ether terpenoid, secreted by isolate TI (Jager et al. 1996; Nigg et al. 2014) and limonene also secreted by isolate TI (U.S. EPA 1994; Mallette et al. 2014). The total concentration of secreted VOCs increased substantially with increasing pH for all three isolates, while the highest total VOC concentration measured for all strains was at 27 °C (Supplementary Figure 2-S5). Isolate CO secreted the highest amount of total VOC, up to 277 mg L^{-1}, of the studied isolates with the majority of the VOC being 1-octen-3-ol.

Table 2-2. Biofuel- and flavor-relevant VOCs produced by fungal endophyte isolates (EC, CO, TI) growing on glucose medium. Temperature and pH indicate growth conditions where the compound was detected. Compounds were sorted by production associated with the following metabolic pathway types: Fatty acid synthesis, Ehrlich pathway, linoleic acid degradation, Fermentative metabolism, or Undetermined.

Compound		Isolate and Conditions								
		EC			CO			TI		
	Carbon Number	pH	T (°C)	Concentration (mg L^{-1}) / Quality Match	pH	T (°C)	Concentration (mg L^{-1}) / Quality Match	pH	T (°C)	Concentration (mg L^{-1}) / Quality Match
Fatty Acid Synthesis										
1-Propanol*	3				7	30	3.89/90	6	37	1.91/90
Pentane	5				6	30	20.8/91	6	30	7.77/78
Hexanal*	6				6	30	2.29/87			
Hexanoic acid*	6				6	37	2.31/83			
2-Heptanone*	7	7	30	1.15/80						
Nonanoic acid	9				4	30	0.80/93			
Nonanal*	9	6	27	1.76/91						
Eucalyptol*	10							6	27	32.5/90
Limoene*	10							6	30	1.45/70
3-Cyclohexene-1-methanol, .alpha., .alpha. 4-trimethyl-	10				7	30	6.83/87	6	27	23.3/91
Octane, 2,6-dimethyl-	10				6	27	3.39/72			
Octadecanoic acid	18	6	27	41.6/99						

Ehrlich Pathway										
1-Propanol, 2-methyl-	4							6	37	2.47/72
Butanal, 3-methyl	5							7	30	4.29/90
1-Butanol, 3-methyl-*	5	7	30	2.04/90	6	27	5.07/78	6	30	2.38/78
3-Buten-1-ol, 3-methyl-	5	6 6	27 30	2.96/90 1.44/94	6 6	27 30	3.57/90 1.48/70			
Benzaldehyde*	7	6 7	30 30	1.24/97 1.79/97	6 7	30 30	1.31/97 0.49/97	6 7 6	27 30 37	21.6/97 7.93/97 5.65/97
Methylbenzaldehyde*	8	4 6	30 30	3.00/97 1.79/96	6	30	1.44/94	6	30	1.53/97
Phenylethyl alcohol*	8				7 6	30 37	6.16/94 2.29/93	6	27 30 37	17.1/97 7.29/95 19.6/94
Dimethylbenzaldehyde*	9	6	30	1.01/90	6 6	27 30	2.29/87 0.97/70			
Linoleic Acid Degradation										
1-Octen-3-ol*	8	6 7	27 30	233/78 5.95/90	6 6	27 30	18.0/86 2.44/90	7	30	27.7/83
3-Octanone*	8	6	27	5.71/94						
Fermentative Metabolism										
2-Butanone, 3-hydroxy-	4				7 6	30 37	3.29/78 18.1/83	6	37	14.4/78
2,3-Butanediol	4				6	37	3.83/90			
Butyrolacetone*	4	6	37	1.00/72						
2,4-Pentanedione	5	6	37	1.29/87						
Undetermined										

2-Furancarboxaldehyde, 5-methyl-*	6	6	37	1.16/94			
2-Furancarboxaldehyde, 5-hydroxymethyl	6	6	30	1.15/70			
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	12	6	27	11.7/80			
1,3-Propanedione, 1,3-diphenyl-	15	6	27	15.3/93			
1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	16	6	27	30.4/87			
Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	16				6	30	1.99/87

* VOCs that have documented roles as flavor compounds. Classification of a chemical as a flavor compound was based on the compound being described in a published report as a flavor compound (Jager et al. 1996; Rodriguez-Bustamante and Sanchez 2007; Berger 2009).

All three isolates demonstrated a strong relationship between the number of carbon atoms in the secreted VOCs and the culturing temperature (Table 2-2; Figure 2-1). VOC molecules from cultures grown at 27 °C had an average length of 9 carbon atoms, while VOC molecules from cultures grown at 37 °C had an average length of 5 carbon atoms. The data was further analyzed as a function of four VOC synthesis pathways (production associated with i. fatty acid synthesis, ii. Ehrlich pathway, iii. linoleic acid degradation and iv. fermentative metabolism) and fungal isolate (Table 2-2; Figure 2-2). The fatty acid synthesis-associated VOCs showed an increase in carbon length with decreasing culture temperature; the relationship was observed for all three isolates. Ehrlich pathway-associated VOCs demonstrated a similar increase in carbon chain length with decreasing temperature in TI cultures, while CO and EC had an opposite trend. VOCs associated with beta-oxidation of linoleic acid (8 carbon atoms) were detected more often at lower culturing temperatures. Fermentative metabolism VOCs were shorter in carbon chain length and detected more often at higher temperatures. The VOC carbon chain length analysis was also performed as a function of starting culture pH. Generally, a smaller number of VOC compounds were detected at lower pH values (Table 2-2).

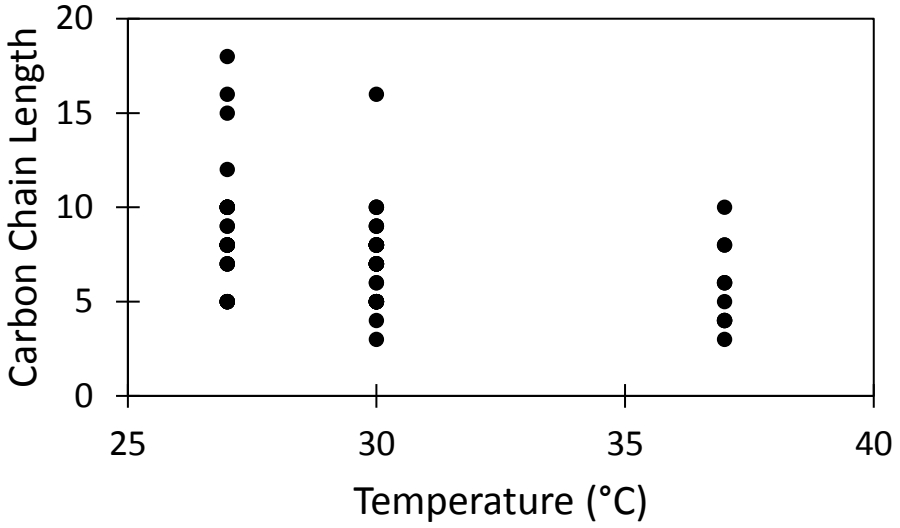


Figure 2-1. Carbon chain length for VOCs produced by three fungal endophyte isolates (EC, CO, TI) as a function of temperature at pH 6 during batch growth on glucose media.

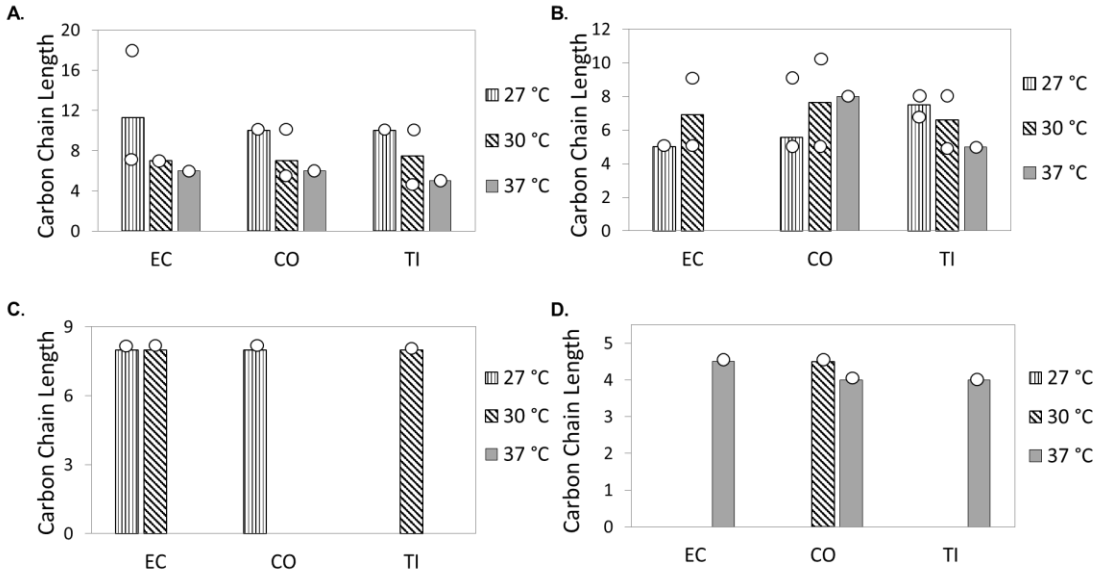


Figure 2-2. Fungal endophyte isolate (EC, CO, TI) VOC carbon chain length as a function of temperature at pH 6 during batch growth on glucose media and as a function of metabolism type (**A**: Fatty acid synthesis, **B**: Ehrlich, **C**: Linoleic acid degradation, **D**: Fermentative metabolism). The height of the bars represents the average carbon chain length of the VOCs and the dots the minimum and maximum carbon chain length identified at each condition.

Additional Metabolic Byproducts

Culture supernatants were analyzed for soluble organic byproducts using HPLC. All three *Nodulisporium* isolates produced substantial amounts of glycerol, up to 4 g L^{-1} (Supplementary Figure 2-S6). Glycerol production was highest near optimal temperatures and did not demonstrate a statistically significant trend with pH. Ethanol production was detected for isolates CO and TI under a variety of conditions. Ethanol concentrations increased as the specific biomass accumulation rate decreased suggesting a correlation to culturing stress like oxygen depletion (Supplementary Figure 2-S7).

Implications

Filamentous fungi produce a wide variety of bioactive and industrially relevant metabolites, including numerous VOCs (Strobel et al. 1996; Keller et al. 2005). Longer carbon chain lengths are desirable for biofuels, since they are more energy dense (Strobel et al. 2008). The presented data showed longer chain VOCs were favored at lower culturing temperatures; this is the first report of such behavior. This is opposite of the trend observed in fungal membrane fatty acid carbon chain length; typically membrane fatty acids decrease in length and degree of saturation as temperatures decrease to maintain membrane properties (Suutari et al. 1997). The increase in secreted VOC chain length may be due to temperature dependent kinetics of fatty acid synthesis reactions (Kates and Baxter 1962) or temperature dependent differences in cell membrane properties that increase excretion of longer carbon chain length VOCs (Liu et al. 2011; Kawahara et al. 2016). Alternatively, many of the VOCs detected are bioactive and their

production may have evolved to support endophyte ecological functions. Plant transpiration, the transport and evaporation of water, can maintain plant tissues at cooler than ambient temperatures which could be a key ecological parameter for these tropical isolates (Edwards and Hanson 1996; Stockfors 2000). Several large bioactive compounds, including terpenes, were detected at lower culturing temperatures and may play a role protecting the living plant host against pathogens and herbivores (Rodriguez et al. 2009; Porras-Alfaro and Bayman 2011). Endophytes live part of their lifecycle within plants but can switch to saprophytic phenotypes upon host death (Porras-Alfaro and Bayman 2011). Warmer culturing temperatures may replicate conditions associated with fungal growth within a dead plant, where a switch toward fermentative metabolism would be ecologically competitive.

In summary, three *Nodulisporium* isolates produced biofuel- and flavor-relevant VOCs. The carbon chain length of the VOCs increased as culturing temperatures decreased. The fungi grew on a wide variety of low cost feedstocks, including sugar beet pulp, corn stover, and grass clippings. The *Nodulisporium* isolates grew quickly with specific biomass accumulation rates up to 1.6 d^{-1} and produced relatively high biomass titers with maximum concentrations ranging from 13-19 g cell dry weight L^{-1}. The presented work also increases the number of physiologically-characterized fungal endophyte isolates from four to seven.

Acknowledgements

This research was supported by the National Science Foundation (NSF) EFRI Program (Grant No. 0937613). Kristopher Hunt was supported by NSF IGERT (DGE 0654336). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors(s) and do not necessarily reflect the views of the NSF. Analytical work was supported by National Institutes of Health award P20RR024237. The authors would also like to thank Laura Bickle and Elle Pankratz for performing initial experiments, Eunsung Kan for technical assistance, and Natasha Mallette for technical advice.

Supplementary Material

Methods

Microorganism Isolation, Growth Medium and Storage

Three endophytic, filamentous fungi isolates were isolated from distinct geographical locations and plant hosts using established collection and isolation methods (Ezra et al. 2004). *Nodulisporium* isolate EC38-12 was isolated from *Neea floribunda* sampled from the Ecuadorian Amazon. The EC38-12 genome is available at genome.jgi.doe.gov under the name *Hypoxylon* sp. EC38 and is here after referred to as isolate EC. *Nodulisporium* isolate CO27-5 was isolated from *Rizophora mangle* (red mangrove) sampled from the Western coast of the mid region of Columbia. The CO27-5 genome is available at genome.jgi.doe.gov under the name *Hypoxylon* sp. CO27-5 and is here after referred to as isolate CO. *Nodulisporium* isolate TI-13 was isolated from

Cassia fistula sampled from the highlands of Thailand. The TI-13 ITS1-5.8 S-ITS2 ribosomal gene sequence is available in GenBank as KJ558391 (Nigg et al. 2014), and the isolate is here after referred to as isolate TI. The three fungi were characterized as having the perfect stage of *Hypoxylon* sp. and as *Nodulisporium* sp. based on ITS1-5.8 S-ITS2 ribosomal gene sequence information (Nigg et al. 2014). The *Nodulisporium* isolates EC, CO and TI are deposited as NRRL 50503, NRRL 50500 and NRRL 50502 respectively in the Agriculture Research Service Culture Collection at the U.S. Department of Agriculture.

Purified cultures were grown on either complex potato dextrose agar (PDA) medium or defined salts medium with a noted carbon source. Basal defined salts growth medium with glucose had the following composition (g L^{-1}): 60 glucose, 0.05 yeast extract, 15 NH_4Cl, 8.25 $NaH_2PO_4 \cdot 2H_2O$, 13.6 KH_2PO_4, 0.86 $MgSO_4 \cdot 7H_2O$, 0.28 $Ca(NO_3)_2 \cdot 4H_2O$, 0.006 $FeCl_3 \cdot 6H_2O$, 0.0075 $ZnSO_4 \cdot 7H_2O$, 0.18 KCl, 0.24 KNO_3, 0.0042 H_3BO_3, and 0.024 $MnCl_2 \cdot 4H_2O$. To avoid component precipitation, the medium was filter sterilized (0.2 μm), and the pH was adjusted using 1 N NaOH or 1 N HCl to a final desired pH. No variance in fungal growth linearity was seen between media concentrations, and so filtering is not believed to impact media component availability or growth (Mallette 2013).

Fungal Cultivation

Inoculation cultures were generated by adding two Microbank microbeads containing frozen fungal culture to 100 mL of fresh medium in 250 mL sterile baffled

flasks. Cultures were grown for six days at the desired temperature (room temperature, ~22 ˚C, to 37 ˚C) and agitated on an orbital shaker at 150 rpm. All experimental planktonic cultures were started at an initial biomass concentration of 0.2 g cell dry weight L^{-1} as estimated using a modified Bradford method (Bradford 1976), grown in 250 mL baffled flasks containing 140 mL of medium and agitated at 150 rpm at the desired temperature. Timely inoculum cell dry weights were estimated using a modified Bradford protein method and calibration curve. Shake flasks were covered with Kimguard to permit gas exchange and to prevent contamination. All fungal cultures were grown in triplicate, and an abiotic control flask was also run at each culture condition. Dry weight measurements were taken every 12 hours for the first four days and then once per day on the fifth and sixth days. Specific biomass accumulation rates were determined from the exponential growth phase identified as the linear region on a semi-log plot of biomass concentration versus time. The filamentous fungi demonstrated growth that was well described by first order kinetics while flocs were small. Abiotic control shake flasks were used to estimate culture evaporative water loss. Prior to culture sampling, the abiotic control flask medium volume was measured. An appropriate volume of autoclaved, deionized water was then added to all flasks to replace the evaporative losses.

For agar plate growth experiments, one microbead was placed in the center of each agar plate containing a water soluble carbon source. Growth on insoluble carbon sources (e.g. wood chips) was tested by first autoclaving the substrate dry at 121°C for 20 min, then the substrate was aseptically placed in a petri dish, and inoculated by placing a

1 cm by 1 cm section of fungus inoculum grown on a PDA plate in the center of the new petri dish.

For anoxic testing, fungal cultures were grown on PDA plates until they covered >60 % of the surface. Fungi and agar plugs, approximately 1 cm x 1 cm, were placed on new PDA plates in an anaerobic jar (Gas Pak System; Becton, Dickinson and Company) at room temperature. Control PDA plates inoculated with the same procedure were allowed to grow under aerobic conditions.

Biomass Dry Weight Analysis

Glass microfiber filters were dried in an oven at 80 ˚C for approximately 24 h. The filters were placed in a desiccator for one hour before being weighed. Fungal biomass from 5 mL culture samples was collected via vacuum filtration. The biomass containing filters were washed with approximately 20 mL of 30 ˚C deionized water, placed in an 80 ˚C oven for 24 h, transferred to a desiccator for one hour and then re-weighed to determine the mass of collected fungal culture.

Substrate and Product Analysis

Glucose, glycerol and ethanol concentrations were measured using an Agilent 1200 HPLC. Supernatant samples were collected within 24 h of the culture reaching stationary phase. Samples were filtered (0.2 µm) to remove biomass and debris. Sample filtrate (20 µL) was injected on an HPX-87H column (Bio-Rad) at 40°C, with a 0.005M H_2SO_4 mobile phase (0.6 mL min^{-1}) for analysis of glycerol and ethanol. Sample filtrate

(20 µL) was diluted 1:50 prior to injection for glucose analysis. All concentrations were determined using a Refractive Index Detector (RID) and appropriate calibration curves.

Analysis of VOCs

VOC identification was performed by Solid Phase MicroExtraction (SPME) as previously described (Griffin et al. 2010; Mallette et al. 2012). Experimental samples were collected within 24 hours of the culture reaching stationary phase. An abiotic medium sample was analyzed for each condition to determine background signal. Samples were filtered with 750 µL centrifuge filters (nylon, 0.2 µm, National Scientific) to remove biomass and debris and stored at 4 ˚C until analysis. Samples were analyzed by RJ Lee Group (Pasco, WA). Briefly, a SPME fiber (divinylbenzene/carboxen on polydimethylsiloxane (Supelco Bellefonte)) was exposed to the sample headspace while the liquid phase was stirred for 45 min. The exposed fiber was inserted into the modified GC injection port operated at 240 °C. The GC column temperature was held at 40 °C for 2 min and then ramped to 230 °C at 5 °C min^{-1}. The mass spectrometer was tuned to meet EPA method 8260 BFB tuning criteria. Compounds were identified via library comparison using the National Institute of Standards and Technology (NIST) database. All compounds reported had quality matches of at least 70 (Griffin et al. 2010; Mallette et al. 2012; Mends et al. 2013); direct manual comparisons were made to spectra of known compounds when there were multiple high quality matches. SPME GC-MS compound quantification is unreliable with complex VOC mixtures because of competitive

adsorption between VOCs including smaller compounds like ethanol (Mallette et al. 2012), and reported values are given tentatively.

Additional Implications

Many industrial flavor compounds are either synthesized chemically from petroleum or extracted with variable efficiency from plants (Schwab et al. 2008). Endophytes have been found to produce many secondary metabolites previously isolated from plants including flavor compounds (Abrahão et al. 2013). Endophyte cultures could help fill the increasing demand for sustainable, all natural flavor compounds from renewable feedstocks and can likely produce the compounds more consistently than plants without the need for dedicated land usage (Krings and Berger 1998; Schwab et al. 2008; Abrahão et al. 2013). Additionally, naturally produced flavor compounds can be marketed at prices many times higher than synthetically made compounds (Kriggs & Berger, 1998).

Table 2-S1. Fungal endophyte isolate (EC, CO, TI) biomass yield on glucose as a function of isolate, culture temperature and culture pH (gray cells) during batch growth. Yields calculated as dried biomass produced per glucose consumed. Standard error based on biological triplicates.

Isolate		**EC**		**CO**		**TI**	
Temperature (°C)	pH	biomass (g) /glucose (g)	standard error	biomass (g) /glucose (g)	standard error	biomass (g) /glucose (g)	standard error
27	6	0.148	0.011	0.179	0.006	0.086	0.003
30	6	0.185	0.053	0.172	0.029	0.635	0.136
33	6	0.140	0.009	0.159	0.014	0.364	0.039
37	6	0.171	0.009	0.249	0.014	0.170	0.049
30	4	0.281	0.024	0.213	0.067	0.160	0.033
30	5	0.212	0.039	0.153	0.026	0.398	0.070
30	6	0.185	0.053	0.172	0.029	0.635	0.136
30	7	0.244	0.017	0.168	0.059	0.014	0.007

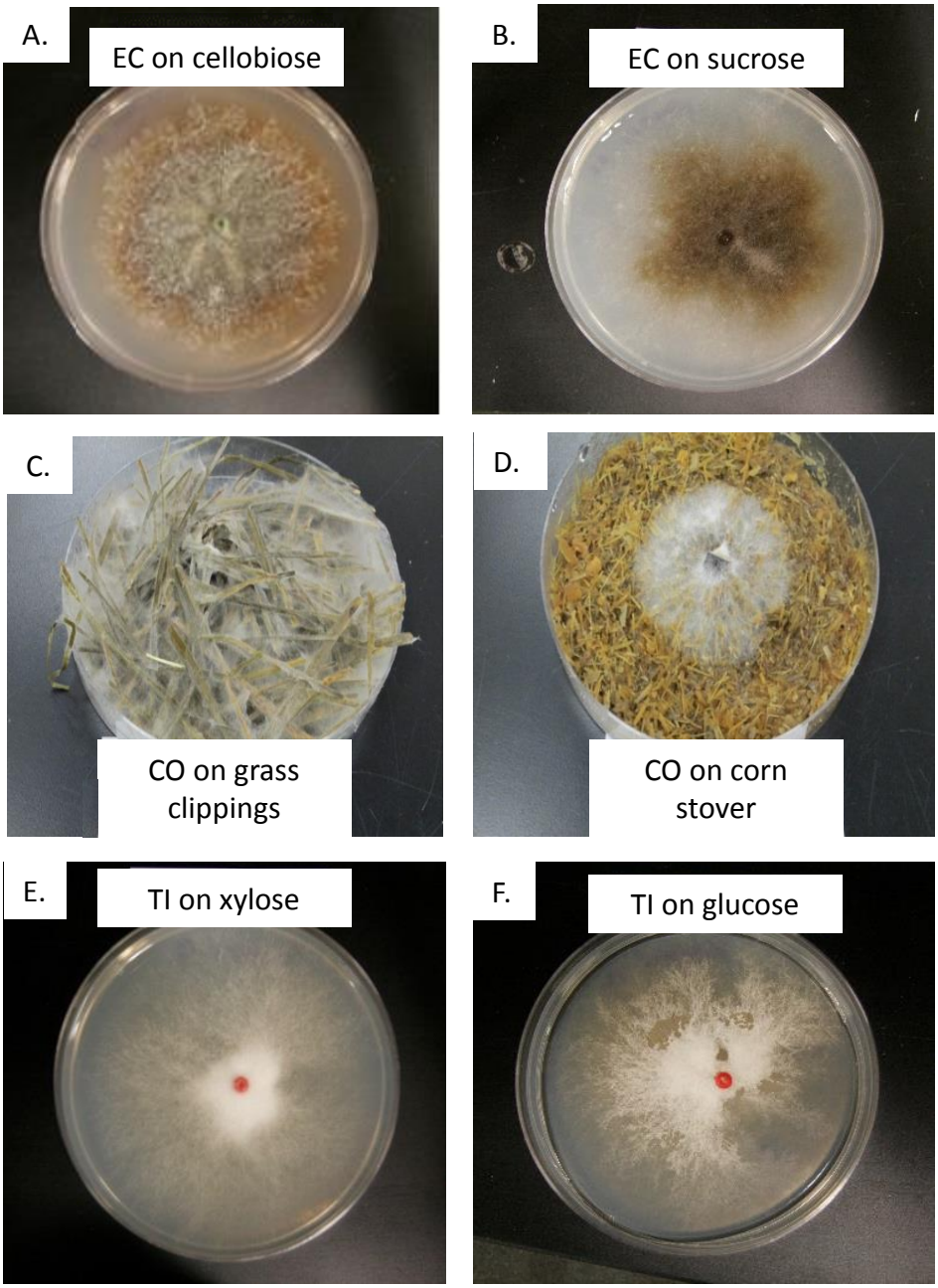


Figure 2-S1. Fungal endophyte isolate growth on different carbon sources. **a)** Isolate EC grown on cellobiose. **b)** Isolate EC grown on sucrose. **c)** Isolate CO grown on grass clippings. **d)** Isolate CO grown on corn stover. **e)** Isolate TI grown on xylose. **f)** Isolate TI grown on glucose.

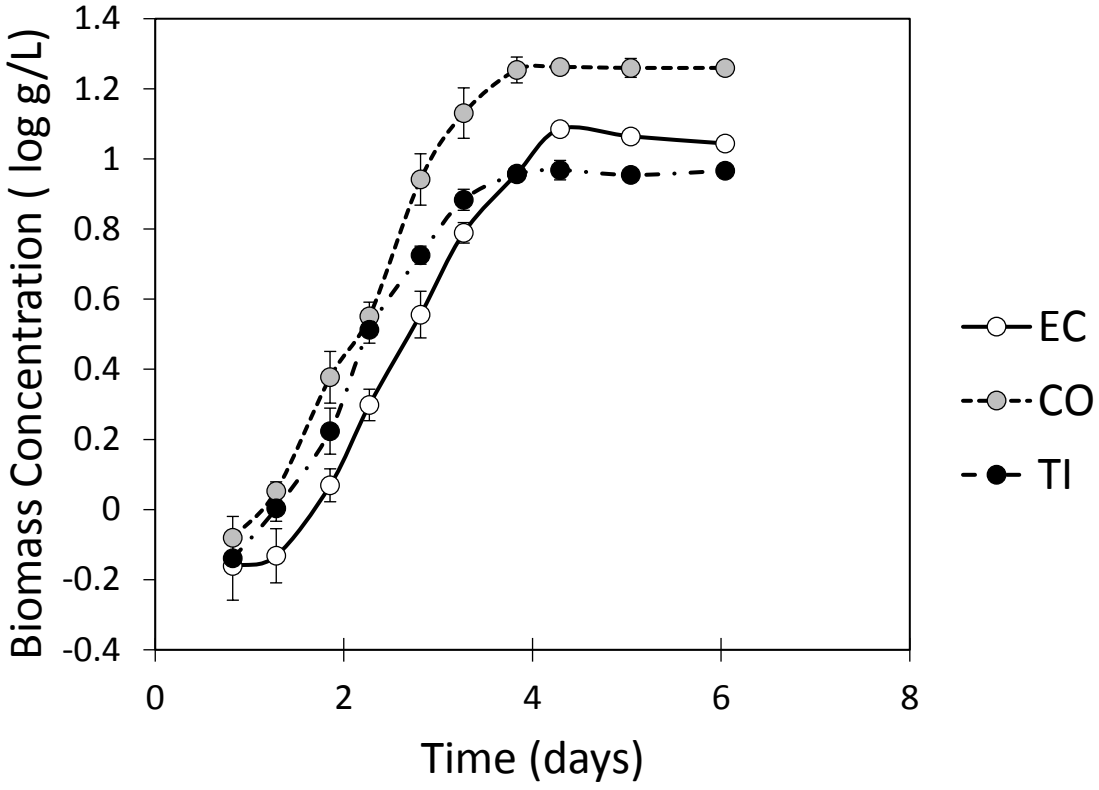


Figure 2-S2. Fungal endophyte isolate growth at 30 °C and pH 6. Values represent the average fungal dry weight of three biological replicates. Error bars show standard error of three biological replicates.

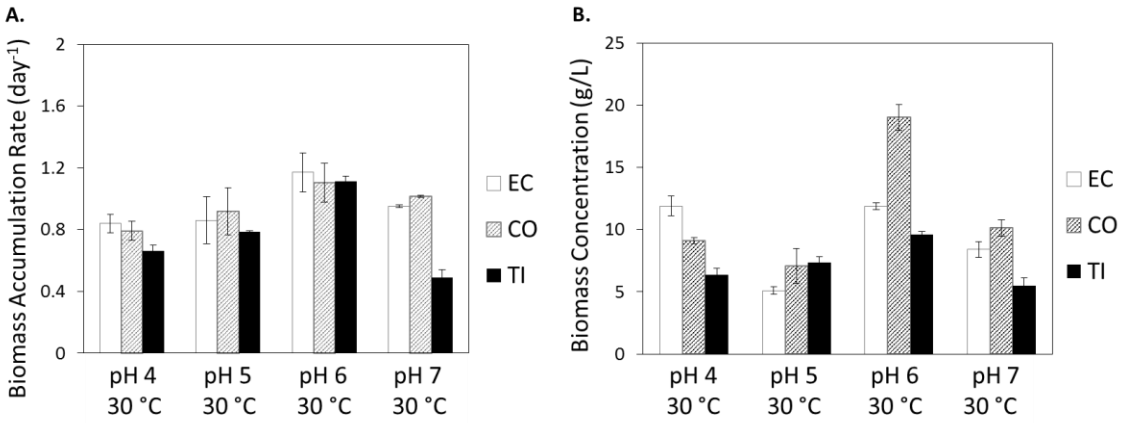


Figure 2-S3. Fungal endophyte isolate (EC, CO, TI) specific biomass accumulation rate and maximum biomass concentration as a function of pH at 30 °C during batch growth on glucose medium. **A:** Maximum specific biomass accumulation rate. **B:** Maximum biomass concentration at stationary phase. Error bars show standard error of three biological replicates.

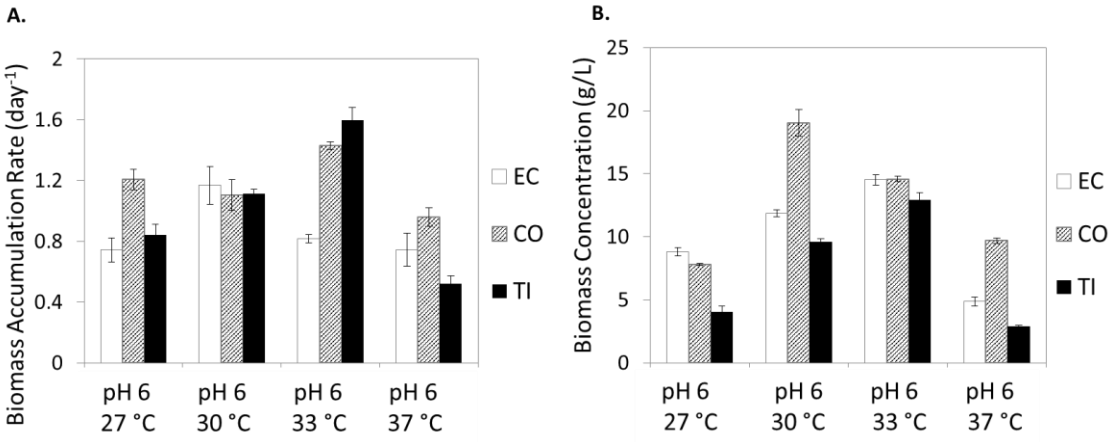


Figure 2-S4 – Fungal endophyte isolate (EC, CO, TI) specific biomass accumulation rate and maximum biomass concentration as a function of temperature at pH 6 during batch growth on glucose media. **A:** Maximum specific biomass accumulation rate. **B:** Maximum biomass concentration at stationary phase. Error bars show standard error of three biological replicates.

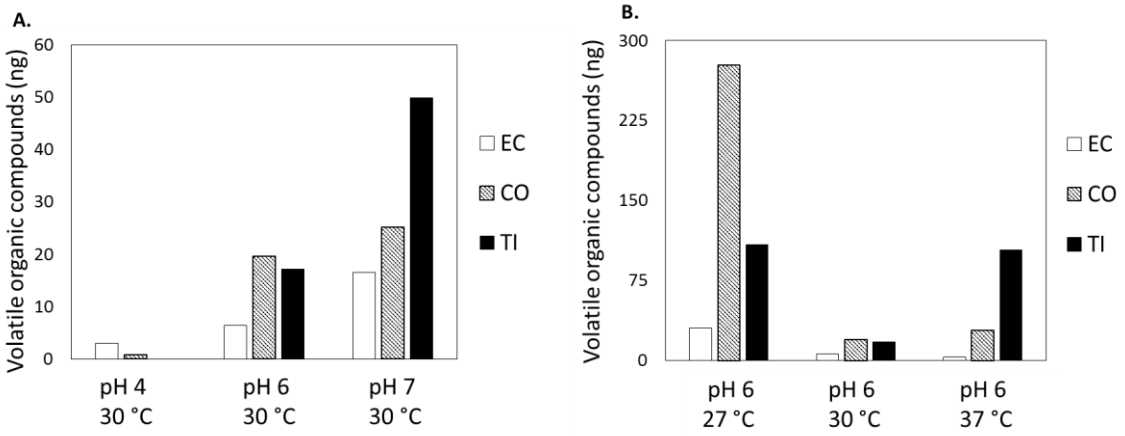


Figure 2-S5. Fungal endophyte isolate (EC, CO, TI) culture volatile organic compounds as a function of pH and temperature during batch growth on glucose media. Quantification is estimated based on SPME GC-MS values.

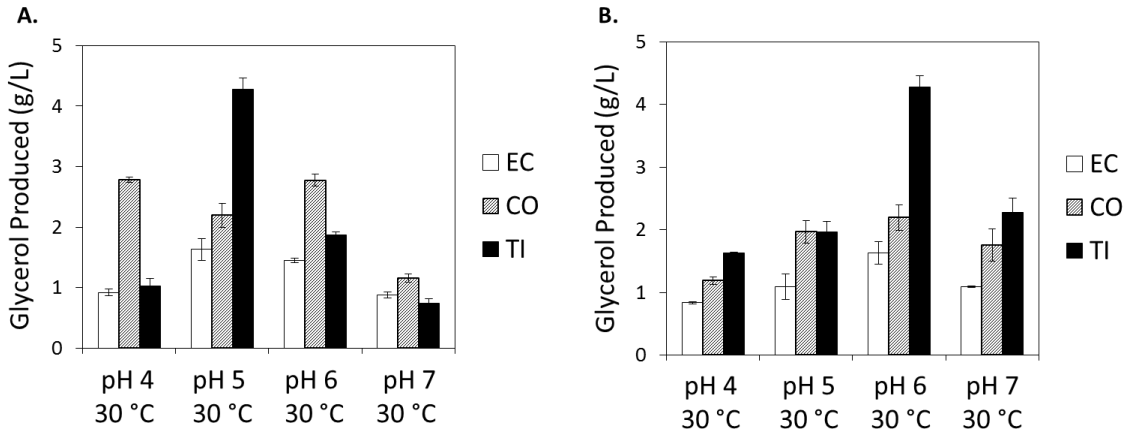


Figure 2-S6. Fungal endophyte isolate (EC, CO, TI) culture glycerol concentration as a function of pH and temperature during batch growth on glucose media. Error bars show standard error of three biological replicates.

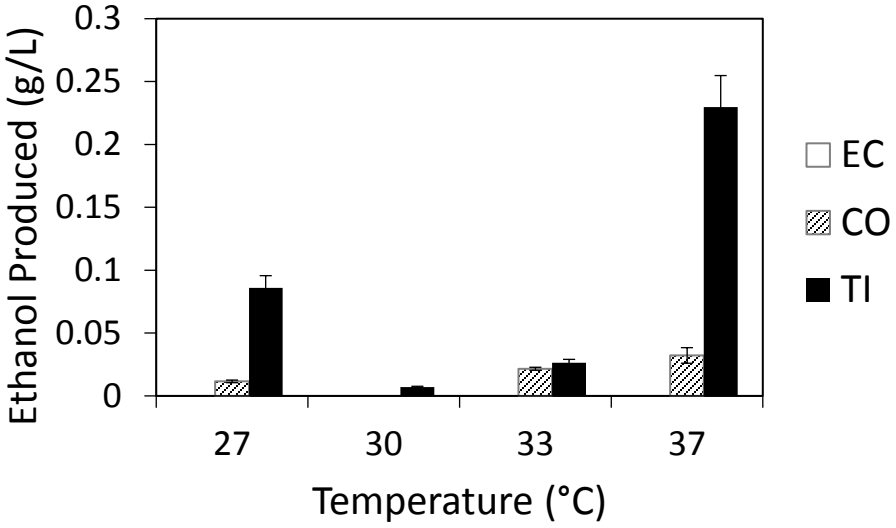


Figure 2-S7. Ethanol production at pH 6 as a function of temperature during batch growth on glucose media. Error bars show standard error of three biological replicates. Additionally, TI produced ethanol at 30 ˚C and pH 5 (0.24 +/- 0.020 g L^{-1}), 6 (0.0070 +/- 0.00062 g L^{-1}), and 7 (0.15 +/- 0.011 g L^{-1}).

CHAPTER THREE

RAPID TOTAL VOLATILE ORGANIC CARBON QUANTIFICATION FROM MICROBIAL FERMENTATION USING A PLATINUM CATALYST AND PROTON TRANSFER REACTION-MASS SPECTROMETRY

Contribution of Authors and Co-Authors

Author: Heidi R. Schoen

Contributions: Assisted in designing the study, collected data, analyzed data, and wrote the paper.

Co-Author: Brent M. Peyton

Contributions: Discussed results and implications and edited the manuscript.

Co-Author: W. Berk Knighton

Contributions: Designed the study, analyzed data, discussed results and implications, and edited the manuscript.

Manuscript Information Page

Heidi R. Schoen, Brent M. Peyton, W. Berk Knighton
AMB Express
Status of Manuscript:
_____Prepared for submission to a peer-reviewed journal
_____Officially submitted to a peer-reviewed journal
_____Accepted by a peer-reviewed journal
__X__Published in a peer-reviewed journal

Published Online: 5 October 2016

RAPID TOTAL VOLATILE ORGANIC CARBON QUANTIFICATION FROM MICROBIAL FERMENTATION USING A PLATINUM CATALYST AND PROTON TRANSFER REACTION-MASS SPECTROMETRY

Heidi R. Schoen[1,2], Brent M. Peyton[1,2], W. Berk Knighton[3*]

1. Department of Chemical & Biological Engineering, 305 Cobleigh Hall, PO Box 173920, Montana State University, Bozeman MT 59717
2. Center for Biofilm Engineering, 366 Barnard Hall, P.O. Box 173980, Montana State University, Bozeman MT 59717
3. Department of Chemistry and Biochemistry, 103 Chemistry and Biochemistry Building, PO Box 173400, Montana State University, Bozeman MT 59717

Abstract

A novel analytical system was developed to rapidly and accurately quantify total volatile organic compound (VOC) production from microbial reactor systems using a platinum catalyst and a sensitive CO_2 detector. This system allows nearly instantaneous determination of total VOC production by utilizing a platinum catalyst to completely and quantitatively oxidize headspace VOCs to CO_2 in coordination with a CO_2 detector. Measurement of respiratory CO_2 by bypassing the catalyst allowed the total VOC content to be determined from the difference in the two signals. To the best of our knowledge, this is the first instance of a platinum catalyst and CO_2 detector being used to quantify the total VOCs produced by a complex bioreactor system. Continuous recording of these CO_2 data provided a record of respiration and total VOC production throughout the experiments. Proton transfer reaction-mass spectrometry (PTR-MS) was used to identify and quantify major VOCs. The sum of the individual compounds measured by PTR-MS

can be compared to the total VOCs quantified by the platinum catalyst to identify potential differences in detection, identification and calibration. PTR-MS measurements accounted on average for 94% of the total VOC carbon detected by the platinum catalyst and CO_2 detector. In a model system, a VOC producing endophytic fungus *Nodulisporium* isolate TI-13 was grown in a solid state reactor utilizing the agricultural byproduct beet pulp as a substrate. Temporal changes in production of major volatile compounds (ethanol, methanol, acetaldehyde, terpenes, and terpenoids) were quantified by PTR-MS and compared to the total VOC measurements taken with the platinum catalyst and CO_2 detector. This analytical system provided fast, consistent data for evaluating VOC production in the nonhomogeneous solid state reactor system.

Introduction

Fungi and bacteria produce hundreds of volatile organic compounds (VOCs) with industrial applications including biofuels, insecticides, quorum sensing and biocontrol, flavor and aroma compounds, antibacterials and antifungals (Hung et al. 2015; Kai et al. 2009; Morath et al. 2012; Strobel 2014). Bioprospecting has identified many new microorganisms that produce valuable VOCs, and the types and amounts of these compounds often change with substrate, culturing conditions and growth phase (Bunge et al. 2008; Kai et al. 2009; Morath et al. 2012; Strobel 2014). Higher yields of most microbial VOCs must be achieved to make these bioprocesses industrially viable, but fast and accurate analytical methods to determine the type and amount of VOCs produced are lacking (Morath et al. 2012). Efficient ways to screen new and genetically modified

strains, and changes in culture conditions, are required to rapidly identify improvement to VOC yields (Morath et al. 2012).

Solid Phase MicroExtraction (SPME) coupled with Gas Chromatography – Mass Spectrometry (GC-MS) has been used to identify compounds produced by microbial fermentation (Mallette et al. 2012; Morath et al. 2012). With little sample preparation, SPME GC-MS readily identifies VOCs using compound libraries, but does a poor job of quantifying the amounts of compounds produced (Luchner et al. 2012; Mallette et al. 2012; Morath et al. 2012) and is not well suited to screen for increased yields of VOCs. Traditional extraction techniques to concentrate VOCs can be time intensive, and large organic solvent volumes may lead to poor resolution of volatile compounds (Kai et al. 2009; Morath et al. 2012). Further, extraction techniques may identify fewer VOCs than SPME GC-MS (Kai et al. 2009; Morath et al. 2012). Another analytical method, proton nuclear magnetic resonance can quantify VOCs quickly, but has poor sensitivity, cannot determine carbon length and some oxygenated VOCs cannot be measured because signals are confounded with sugar peaks (Mallette et al. 2014).

Proton Transfer Reaction-Mass Spectrometry (PTR-MS) is an effective analytical technique for measuring VOCs in the headspace of both liquid and nonhomogeneous solid state microbial reactor systems (Bunge et al. 2008; Ezra et al. 2004b; Luchner et al. 2012; Mallette et al. 2012). With PTR-MS, many VOCs can be quantified down to the 10 – 100 parts per trillion range with reliable results (Biasioli et al. 2011; de Gouw et al. 2003; Lindinger et al. 1998). PTR-MS provides data quickly including a likely compound identification, without the extensive preparatory laboratory work required for extractions

(Ammann et al. 2004; Luchner et al. 2012). With real-time gas phase measurement capability, the PTR-MS readily ties VOC data to experimental conditions and temporal changes, and can non-invasively utilize reactor off-gas for measurements (Luchner et al. 2012; Romano et al. 2015).

Previously, PTR-MS has been used to explore microbial systems, but estimates of error in VOC quantification can be between 15 and 40% depending on system conditions (Bunge et al. 2008; Ezra et al. 2004b; Luchner et al. 2012; Mallette et al. 2012; Romano et al. 2015; Schmidberger et al. 2014; Singer et al. 2009). Quantification of total VOC production with PTR-MS can be improved with calibration, but significant uncertainties may remain due to compounds that are not readily detected by PTR-MS (e.g. alkanes) and complex mixtures where multiple compounds with different sensitivity (calibration) factors contribute to the same mass to charge ratio.

Alternatively, the platinum catalyst and sensitive CO_2 detector provide a robust VOC quantification system (Baasandorj et al. 2015; Veres et al. 2010) that confidently provides the total amount of gas phase organic carbon in complex mixtures of VOCs produced by microbial systems. The platinum catalyst oxidizes VOCs completely to CO_2, which allows a sensitive CO_2 detector to accurately quantify the total VOC production in real-time, as well as record respiratory CO_2. This accurate VOC total can be used to quantify headspace carbon for carbon balances and as a real-time process monitoring tool. Additionally, the total VOC measurements can be compared to less certain total quantifications performed with other instruments, such as PTR-MS, to identify discrepancies in the system. To the best of our knowledge, this is the first time the

combination of a platinum catalyst and CO_2 detector has been used to quantify total headspace VOC production from a complex bioreactor system.

This paper demonstrates total VOC quantification in a non-homogenous solid state reactor system, where both growth phase and VOC data can be cumbersome to collect (Desgranges et al. 1991; Krishna 2005). The system described here provides a virtually instantaneous method for quantifying total VOC production.

Materials and Methods

Experimental Setup

Figure 3-1 provides a schematic of the experimental setup. The platinum catalyst, CO_2 detector and PTR-MS sampled approximately 200 mL/min of the diluted reactor off-gas stream. The flow path was alternated every three hours using an automated three-way valve to flow either directly to the CO_2 detector to measure respiratory CO_2 only or through the VOC oxidizing platinum catalyst and then to the CO_2 detector, measuring CO_2 from both VOCs and fungal respiration. The PTR-MS sampled both the gas steams that entered and bypassed the platinum catalyst once per day. Gas flow that passed through the catalyst was measured to verify complete removal of VOCs from the gas steam and as a background with which to compare the bypass flow stream PTR-MS measurements. Three biological replicate experiments were performed to assess the reproducibility of the system.

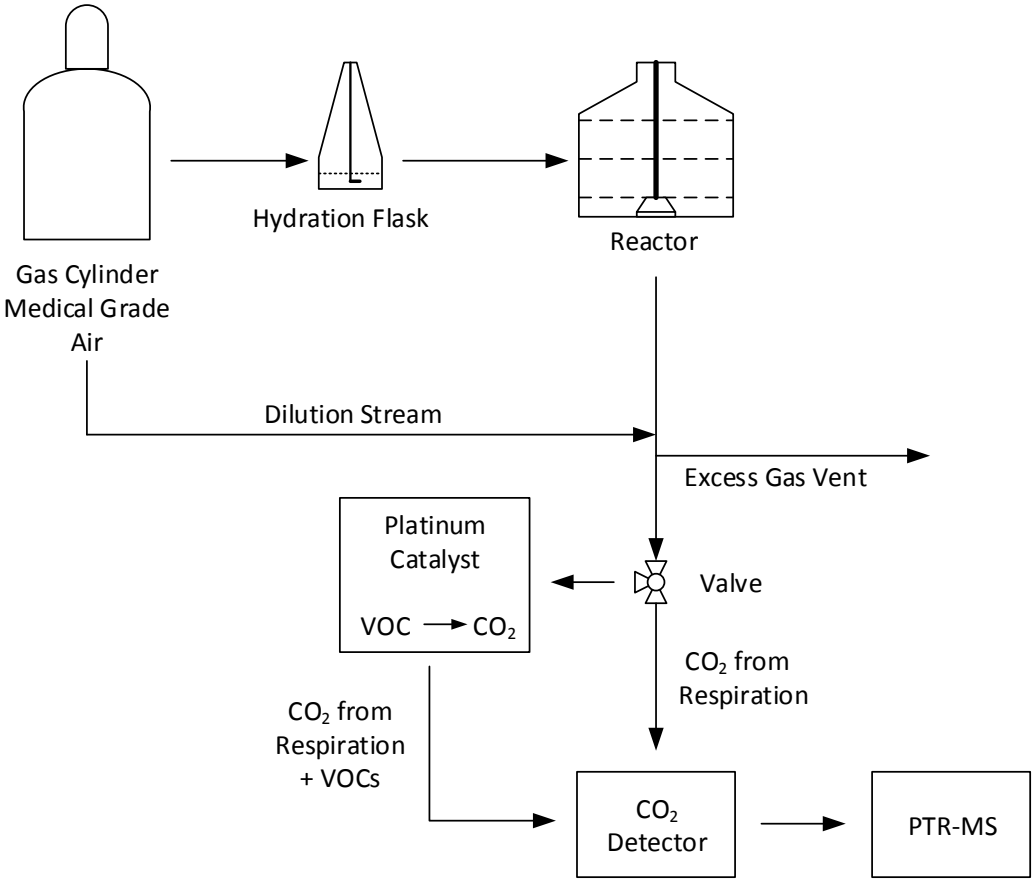


Figure 3-1. Experimental setup for monitoring VOC production from a fungal solid state reactor. Hydrated air was sparged through the bottom of the reactor. Reactor off-gas was sampled from the top of the reactor, diluted and sent to a CO_2 detector to measure respiratory CO_2 and then to the PTR-MS for quantification of major VOC species. Alternatively the off-gas was sent to a platinum catalyst where VOCs were oxidized to CO_2 and measured by the CO_2 detector as the sum of the VOCs produced and respiratory CO_2.

Solid State Fungal Reactor System

Solid state reactors constructed from a 2-liter, air tight, borosilicate glass container with three stainless steel mesh shelves were inoculated with 250 mg of biomass as determined by a correlation with protein concentration using a modified Bradford method (Bradford 1976). As a substrate, 50 g of beet pulp were autoclaved for 20 min with 250 mL of water and allowed to cool. The reactor, hydration flask and all tubing

were autoclaved at 121 °C for 20 min before use. Fungal biomass was added to the beet pulp, mixed by inversion and evenly distributed to the three shelves of the reactor. Biological triplicate experiments were run for approximately five days each until total VOC production approached zero.

The solid state reactor (See Figure 3-1) was continuously sparged with 100 mL/min of hydrated medical grade compressed air. The air was delivered via a stainless steel tube (0.5 cm I.D.) running to the bottom center of the reactor. Inlet air pressure forced reactor gas out of the top of the reactor. Reactor off-gas was then diluted with 900 mL/min of dry medical grade air to keep water from condensing in the system and to keep concentrations within the linear range of the CO_2 detector and PTR-MS. A portion of the diluted flow stream, ~200 mL/min, was then pulled through the CO_2 detector and PTR-MS with the PTR-MS diaphragm pump.

Microorganism

Nodulisporium isolate TI-13 was discovered as an endophyte of *Cassia fistula* in the highlands of Thailand using established collection and isolation methods as described previously (Ezra et al. 2004a). The TI-13 ITS1-5.8 S-ITS2 ribosomal gene sequence is available in GenBank as KJ558391, and the filamentous fungus is stored as sample NRRL 50502 in the Agriculture Research Service Culture Collection at the U.S. Department of Agriculture (Nigg et al. 2014). The fungus was characterized as having the perfect stage of *Hypoxylon* sp. and as *Nodulisporium* sp. based on ITS1-5.8 S-ITS2 ribosomal gene sequence information (Nigg et al. 2014).

Growth Conditions

Inoculum cultures were generated by growing the fungus on potato dextrose agar until hyphae covered ≥60 % of the plate surface. A 20 % glycerol solution (10 mL) was added to each plate, scraped with a sterile glass rod and the suspended fungal biomass solution was collected. The solution was mixed thoroughly, added to Microbank microbead vials, and stored at -80 ˚C until use.

The composition of the inoculum medium was 60 g/L glucose and 0.5 g/L yeast extract. 250 mL of sterile filtered (0.22 µm) medium was added to 500 mL sterile baffled flasks. Three microbeads were added to each inoculum culture and grown for nine days at 160 rpm and 30°C. All inoculum flasks were covered with sterile Kimguard (Kimberly-Clark; Roswell, GA, USA) to allow gas exchange, but prevent microbial contamination.

CO_2 Detector Calibration and Verification

An LI-840 CO_2/H_2O non-dispersive infrared gas analyzer (Li-cor Biosciences; Lincoln, NE, USA), quantified CO_2 in the reactor off-gas continuously without impacting it. This optical technique is non-destructive and allowed the PTR-MS to be placed in series after the CO_2 detector. The CO_2 detector was calibrated using a certified CO_2 free gas standard to set the zero point (1% methane, balance nitrogen Scotty Analyzed Gases, Plumsteadville, PA, USA) and another gas standard for the span (a gas standard of 1010 ppm CO_2, balance nitrogen, Scotty Analyzed Gases, Plumsteadville, PA, USA). Linearity of the CO_2 detector was assessed by diluting the gas standard (1010 ppm CO_2, balance

nitrogen) with medical grade compressed air using mass flow controllers to produce final CO_2 concentrations of 50.5, 101, 505 and 1010 ppm. The CO_2 detector response agreed with the prepared gas standard to within 3.3% for the four values measured, with values creating a line with a slope of 1.0006 and an R^2 value of 0.9998 when plotting measured concentration versus expected concentration (data not shown).

Platinum Catalyst VOC Conversion Verification

Complete oxidation of VOCs to CO_2 by a heated platinum catalyst (Shimadzu, Kyoto, Japan, High Sensitivity Catalyst 630-00996 maintained at 400 ^oC) was confirmed by diluting a gas standard (0.1 % propane, balance air, Cal Gas Direct, Huntington Beach, CA, USA) and medical grade compressed air with mass flow controllers to produce final propane concentrations in the range of 10 – 100 ppm, which spanned the total VOC concentration presented to the catalyst in the diluted sample flow. Based on the 10-fold dilution of the bioreactor effluent and accounting for the 3 carbons in propane, this experiment produced a test matrix that was equivalent to total bioreactor VOC concentration of 300 – 3000 ppm C. Overall, the platinum catalyst and CO_2 detector measurement agreed with the gas standard for conversion of propane to CO_2 by the platinum catalyst to within 5 % for the four measured values, and created a line with a slope of 0.95 and an R^2 value of 0.9991 when measured concentration was plotted against expected concentration. The catalyst exhibited near complete conversion efficiency for propane concentrations up to 50 ppm of propane, which decreased to 95% at the highest, 100 ppm test point. This experiment established the effective maximum working

concentration of the employed catalyst system at 1500 ppm C after adjusting for dilution. Catalyst systems employing higher temperatures and greater amounts of catalyst could be used to achieve higher maximum working concentrations. Similar catalyst systems have been used previously to confirm concentrations of calibration gases with high accuracy (Baasandorj et al. 2015; Veres et al. 2010).

Bioreactor Measurements

The reactor off-gas flow path was alternated with a valve on a three hour timer to run either directly to the CO_2 detector or through the platinum catalyst to oxidize VOCs to CO_2 before the CO_2 detector. By alternating the air stream either around or through the platinum catalyst every three hours, a near-continuous record of respiratory CO_2 and total gas phase carbon (CO_2 and VOC) production was created for each experiment. The CO_2 detector measurement was recorded once per second during the experiments. Background CO_2 and VOCs measurements for each tank of medical grade compressed air were subtracted from these values.

Proton Transfer Reaction-Mass Spectrometry (PTR-MS)

PTR-MS was used to provide compositional information about the VOCs in the reactor off-gas. PTR-MS uses H_3O^+ ions to protonate molecules (e.g. VOCs) with proton affinities greater than water (Lindinger et al. 1998). The singly charged ions are then typically detected as protonated molecules (ions with a mass-to-charge ratio, m/z, equal to the molecular weight plus 1 for the proton) by a quadrupole mass spectrometer

(Lindinger et al. 1998). Identity is assigned based on the mass of the ion and prior knowledge of the likely products of a specific reactor system (Luchner et al. 2012). Constituents of air like O_2, N_2 and CO_2 have proton affinities lower than water so are not protonated and do not interfere with measurements. Alkanes also have proton affinities lower than water, so PTR-MS does not efficiently detect alkanes (Lindinger et al. 1998).

Initial experiments with this solid state system showed the major VOC products were ethanol, methanol, acetaldehyde and monoterpenes, so care was taken to calibrate the PTR-MS for these compounds. Quantification was performed using sensitivity factors derived from calibration experiments by dynamically diluting a multicomponent standard containing methanol, acetaldehyde and terpenes (Apel-Riemer Environmental Inc., Broomfield, CO, USA). Ethanol calibration was performed separately using a permeation tube at a variety of humidities as described below. Sensitivity factors of 4.1, 17.4, 18.2 and 2.2 ncps (normalized counts per second)/ppbv for ethanol (m47, m65, m93), methanol (m33, m51), acetaldehyde (m45) and terpenes and terpenoids (m137) were used, respectively. The off-gas of the abiotic control and fungal cultures were both analyzed by the PTR-MS. The total VOCs measured in the abiotic control were subtracted from the fungal VOC production measurements.

Ethanol PTR-MS Calibration

Ethanol calibrations were performed using a calibrated permeation tube (KIN-TEK; La Marque, TX, USA) at a variety of humidity values approaching dry air as the experiments were performed with 90% dry air. Compressed air (19.5 mL/min) was

sparged through a temperature-controlled oven (50°C) holding a calibrated ethanol permeation tube (emission rate 209 ng/min at 50°C). Mass flow controllers were used to control medical grade compressed air flowing through the permeation tube and a second dilution stream, introduced after the permeation tube, to produce concentrations of 101, 199, 484 and 924 ppb of ethanol based on the calibrated permeation tube emission rate. The concentration of ethanol produced with the permeation tube was checked using the platinum catalyst system as described previously (Baasandorj et al. 2015) resulting in a line with a slope of 1.1 and an R^2 value of 0.997 (data not shown). The temperature of the permeation tube was then increased to 80 °C to produce ethanol concentrations comparable to those observed in the fungal experiment (1.2 – 6.2 ppm). The humidity of the air was varied by utilizing a hydration flask before the dilution. Humidity was monitored via the ratio of the $H_3O^+(H_2O)$ to H_3O^+ ion intensities with calibration experiments performed under conditions that closely matched the humidity in the fungal experiments. At low humidity, the sensitivity factor had a relatively constant value of approximately 4.0. Calibrations were repeated after the experiments and yielded a sensitivity factor of approximately 3.9.

Results

The record of respiratory CO_2 and VOCs plus respiratory CO_2 is shown as Figure 3-2. The platinum catalyst oxidized these VOCs nearly completely to CO_2 as demonstrated by the +/- 5% conversion of propane to CO_2 verification performed as

described above. Additionally, no breakthrough was detected by the PTR-MS when the reactor off-gas was passed through the platinum catalyst, beyond decreasing system memory effects. The stair step shape of the CO_2 data reflects the change of flow paths between going straight from the reactor to the CO_2 detector, quantifying respiratory CO_2 as the lower dashed line, and oxidation of VOCs to CO_2 by the platinum catalyst before the CO_2 detector, quantifying the sum of respiratory and VOC CO_2 as the upper dotted line. The LOESS function of IGOR 6 (Wavemetrics; Portland, OR), a nonparametric regression method that smooths using locally weighted regression, was utilized to interpolate the lower line yielding a continuous respiratory CO_2 record. The shape of the respiration lower line follows a typical growth curve and was used to estimate lag, exponential, stationary and death phases as shown in Figure 3-2. Also, the lower line was integrated to determine the total amount of respiratory CO_2 produced in parts per million carbon (ppm C) during each experiment and was then converted to the mass of carbon using the flow rate, temperature and pressure of the system. Similarly, the upper line, representing the sum of VOC production and respiratory CO_2 in ppm C, was interpolated with the LOESS function to yield a continuous upper line. The lower line was subtracted from the upper line at each point in time to determine the VOC production in ppm C (Figure 3-3). The area under the VOC production curve can be integrated to find the total amount of VOCs produced during the experiment in ppm C, which can then be converted to the mass of carbon using the flow rate, temperature and pressure of the system.

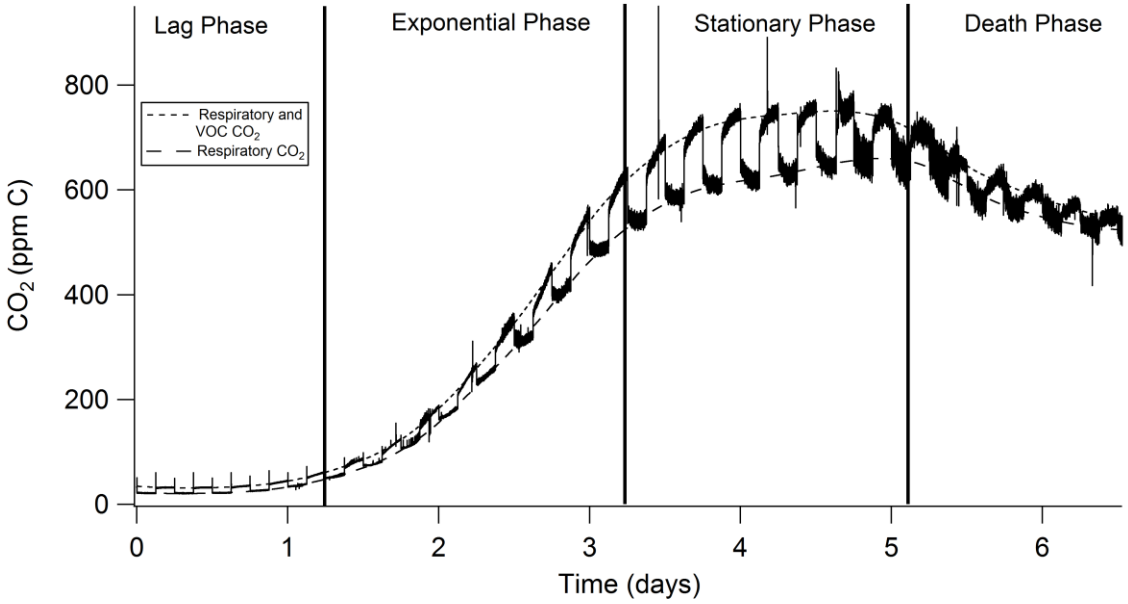


Figure 3-2. A representative CO_2 profile of a fungal solid state reactor experiment after correcting for dilution. The lower line represents respiratory CO_2 as indicated by the interpolated dashed line and the upper line is the sum of respiratory and oxidized VOC CO_2 as represented by the interpolated dotted line. Values switch between respiratory CO_2 and CO_2 from both fungal metabolism and VOCs every three hours. The graph is split into growth phases based on the shape of the respiratory CO_2 curve.

Figure 3-3 shows the total VOCs quantified by the platinum catalyst and CO_2 detector, which were extracted from Figure 3-2 as described above. The sections of the bars displayed in Figure 3-3 represent the relative carbon fractions of the major products as quantified by PTR-MS (as determined from spectra such as Figure 3-4): ethanol, methanol, acetaldehyde, monoterpenes, and terpenoids (See Supplemental Table 3-S1 for raw data). The predominant compound produced was ethanol in this and most of the samples. Total VOC production, as well as individual compound measurements, varied with growth phase of the fungus.

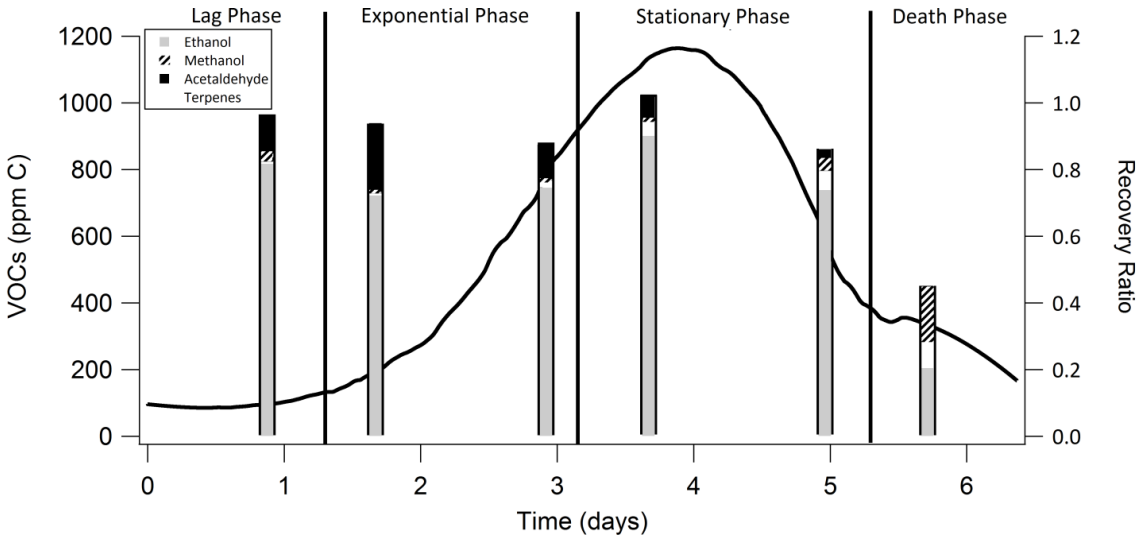


Figure 3-3. VOC production in the fungal reactor based on platinum catalyst VOC conversion to CO_2 during experiment 3 corrected for dilution. Phase lines match the phases in Figure 3-2. The bars represent PTR-MS measurements of ethanol (grey), terpenes (white), acetaldehyde (black) and methanol (slash pattern). The product areas are proportional to the relative percentage of carbon detected of each compound. The height of the bars represents the recovery ratio (shown on the right axis), which is the ratio of carbon detected by PTR-MS to carbon detected by the CO_2 detector. A recovery ratio of one represents an equal amount of carbon detected.

Additionally for these data, we can examine the VOC content quantified with PTR-MS for comparison to the total VOC production measured by the platinum catalyst and CO_2 detector. Figure 3-4 shows a difference PTR-MS spectrum, where a background spectrum (obtained by measuring reactor off-gas passed through the catalyst) was subtracted from the off-gas measurements, from late stationary phase (119 hours into Experiment 3). Additional reactor off-gas VOC ions are present in the spectra, but they represented less than three percent of the ion intensity before death phase and were not quantified. Results show counts for ethanol (m/z 47, 65, 93) were highest for most spectra. Large amounts of methanol (m/z 33, 51) and acetaldehyde (m/z 45) ions are present in this spectrum. The terpene and terpenoid (m/z 81, 137) peaks and

concentrations are smaller, despite accounting for more carbon per mole since they have ten carbons per molecule, compared to one or two carbon compounds like methanol and ethanol. Higher counts of two ions (m/z 57, 71), each of up to three percent of total ion intensity, and small but increased amounts of several other ions (less than one percent of total ion intensity each) were detected in death phase spectra (data not shown).

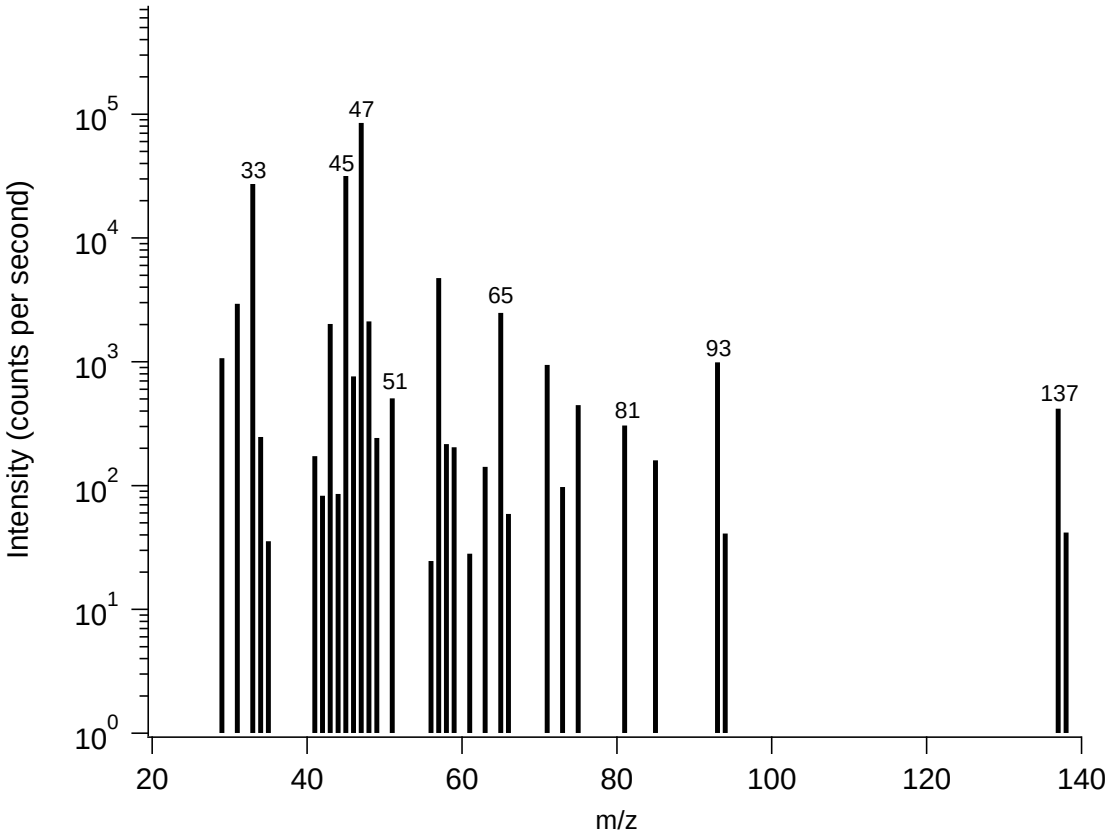


Figure 3-4. PTR-MS spectrum for experiment 3 (119 hours) where background VOCs, reactor off-gas air sampled through the catalyst, have been subtracted from the reactor off-gas spectrum. The figure uses a logarithmic scale of intensity to aid in illustrating both major ions and ions of interest. Ions used for quantificaction ethanol (47, 65, 93), methanol (33, 51), acetaldehyde (45) and terpenes and terpenoids (81, 137) are marked accordingly.

The compounds quantified by the PTR-MS (acetaldehyde, ethanol, methanol and terpenes) were multiplied by their carbon number and summed to determine the total concentration of VOCs in ppm C. This concentration was then divided by the total VOC production in ppm C determined by the platinum catalyst and CO_2 detector to calculate recovery as shown by the height of the bars compared to 1 on the right axis in Figure 3-3. Ideally, recovery ratios would be 1.0 if the PTR-MS detected all carbon containing compounds in the headspace. The average recovery ratio observed here was 0.945 +/- 0.047 (average and standard error; see Supplemental Table 3-S1) indicating that any unidentified VOC products constitute only a small fraction of the total VOC production. Recovery ratios were furthest from 1.0 at lag and death phases. Total VOC production was also lowest during lag and death phases, so small inaccuracies in CO_2 concentration differences between bypass and catalyst measurements likely contributed to the recovery ratio error. Additionally, multiple small intensity ion signals that were not quantified appeared in death phase, which is expected due to cellular degradation and changing metabolism (Hazelwood et al. 2008). Fungal production of small concentrations of alkanes, which are undetectable by the PTR-MS, and small errors in calibration of the CO_2 detector and PTR-MS may also contribute to errors in total quantification.

Two types of deviations from the anticipated response appeared in the CO_2 detector signals later in the experiments. “Rolling” deviations in the CO_2 signal were observed when switching between modes in which the signal exhibited some hysteresis and was slow to achieve a steady measurement immediately following the change in flow paths. This is most readily apparent starting in late-exponential phase (about 60 hours) as

observed in Figure 3-2. The rolling characteristic was observed only in this experimental set and is likely due to pressure variations that occurred when switching between lines. Yegneswaran et al. (1990) observed similar patterns of CO_2 concentrations and determined they were caused by small pressure changes in the bioreactor, but quickly returned to steady state. This type of deviation could be avoided by using two CO_2 detectors. Additionally, some noise was observed in the infrared based CO_2 measurements starting in late stationary phase and increased until the end of the experiments, but no cause was determined for this deviation. While this deviation persisted in later experiments, a definitive cause has not been determined. The combined momentary inaccuracies are estimated at less than 5% of the signal, and do not significantly influence total VOC calculations for the majority of each experiment. During death phase, these perturbations were larger compared to the total signal and may contribute to less accurate death phase VOC recovery results.

Respiratory CO_2, VOC Production and Reproducibility

Three biological replicate experiments were performed to assess the reproducibility of the system. Total respiratory CO_2, total organic carbon in the headspace (from VOCs) and substrate percentage of carbon converted to VOCs and CO_2 were very reproducible for the three experiments. The total amount of respiratory CO_2 produced was 0.73 +/- 0.040 carbon g with three biological replicates (average and standard error) calculated by integrating the lower respiratory CO_2 line in Figure 3-2 as described above. Total VOC production was calculated in an analogous fashion by

integrating data such as that shown in Figure 3-3. Overall, 0.087 +/- 0.0055 carbon g of total VOC were produced in each experiment. The average amount of beet pulp carbon (43% carbon) (Stoppok and Buchholz 1985) converted to respiratory CO_2 and VOCs was 3.8 +/- 0.19 %. A biomass measurement assay was not utilized in these experiments. Whole experiment headspace selectivity was calculated as 0.12 +/- 0.0080 by dividing the VOCs by the respiratory CO_2 as measured by the CO_2 detector throughout the experiment as described above.

Discussion

The combination of PTR-MS, platinum catalyst and sensitive CO_2 detector allowed for real-time VOC sampling with a quantitative determination of the compounds produced. The system described provides a robust measurement of the total VOC production as well as composition of major species produced by the non-homogenous solid state reactor system. To the best of our knowledge, this is the first time the total gas phase VOC content of a solid state reactor has been reported.

The platinum catalyst provides an accurate measurement of total headspace VOC production in complex microbial systems. Catalyst systems are effective at oxidizing nearly all the VOCs present in a sample, and are routinely used to purify air of VOCs for background measurements in PTR-MS (de Gouw and Warneke 2007) and to measure total organic carbon (Sugimura and Suzuki 1988). The platinum catalyst and CO_2 detector agreed with the propane gas standard to within 5% in our verification, and similar systems used to verify gas calibration standards agree to within 4 to 5% of

standards (Baasandorj et al. 2015; Veres et al. 2010). Catalyst systems are more accurate at measuring VOCs under many conditions than other detection systems currently available, such as PTR-MS and GC-MS (Ammann et al. 2004; Baasandorj et al. 2015; de Gouw et al. 2003; Kajos et al. 2015; Veres et al. 2010). An accurate total VOC measurement allows confident determination of the fraction one compound represents in a VOC mixture. Also, the platinum catalyst and CO_2 detector are an affordable system to accurately quantify total headspace VOC carbon and respiratory CO_2 for carbon balances. However, the platinum catalyst and CO_2 detector do not provide any information on the identity of the VOCs present in a sample.

The platinum catalyst can also be used as an assessment tool to determine if the majority of VOCs produced are being detected, accurately identified and accurately quantified by the PTR-MS. A difference in the total VOC content determined by the platinum catalyst and CO_2 detector from that provided by the PTR-MS can alert the user to check for issues such as the following: additional ions with significant carbon content may need to be quantified in the PTR-MS spectrum, some compounds (e.g. alkanes) are not being detected, sensitivity factors may be inaccurate, or identification of the VOC precursor responsible for part or all of an ion's intensity may be incorrect. Ideally, all but one of these issues would be ruled out using prior system knowledge before using this assessment tool to identify discrepancies in PTR-MS VOC quantification. In this system, the PTR-MS total VOC content agreed on average to within 5.5 +/- 4.7 % of that determined with the platinum catalyst and CO_2 detector, which is considered to be excellent agreement. This level of agreement suggests that the PTR-MS quantification is

providing an accurate measurement of VOC composition and that there are no significant errors in the calibration of the four major products, especially the detailed calibration of the major product, ethanol. The system described here is rapid and efficient at measuring the composition of VOCs with high confidence compared to other available measurement methods.

Identifying and quantifying headspace metabolites with PTR-MS throughout growth phases may aid in understanding metabolic processes (Bunge et al. 2008; Crespo et al. 2011; Luchner et al. 2012) and improve monitoring in production systems (Schmidberger et al. 2014). For example, acetaldehyde production appears to be an early marker of impending robust growth in some bacterial and fungal species and can be detected before increases in cell dry weight (Bunge et al. 2008) or respiratory CO_2. Similarly, total VOC measurements can help verify robust growth, product formation and aid in understanding novel processes. This system can also determine headspace selectivity and productivity for total VOC production or individual compounds. For facilities without a PTR-MS, utilizing a platinum catalyst and CO_2 detector in conjunction with SPME GC-MS could be employed. This would provide real-time respiratory CO_2 and total VOC production rates, with SPME GC-MS used to identify specific compounds in complex VOC mixtures. While SPME GC-MS does not provide reliable quantification, it can be used in coordination with other assays to measure products (Mallette et al. 2012).

This total VOC quantification system can be applied in many different types of bioreactors. Bioremediation projects could utilize the detection system to quantify VOC

removal in soil, water, or air samples. This system has applications in screening VOC producing organisms, VOC yield changes from varying process conditions, identifying appropriate target genes for genetic engineering and verifying increased VOC production in genetically engineered strains. The VOC measurement system could also be used for online process monitoring of bioreactors (Schmidberger et al. 2014), especially in solid state reactor systems where accurate and immediate online measurements are extremely difficult (Lui 2013). Similarly, many novel biological systems under study present challenges to traditional rapid monitoring methods, where complications with standard methods arise from a number of conditions such as complex carbon sources, fungi growing with pellet morphology and biofilms. These systems can benefit from utilizing the method described here to provide additional metabolic data in real-time.

Acknowledgements

This research was supported in part by the National Science Foundation (NSF) EFRI Program under Grant No. 0937613. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF. Thank you to the Montana State University Center for Biofilm Engineering, the Chemistry and Biochemistry Department and the Chemical and Biological Engineering Department for support. The authors would also like to thank Drs. Gary Strobel and Natasha Mallette for sound technical advice.

Supplemental Material

Table 3-S1. Ethanol, methanol, acetaldehyde and terpene quantification data as measured by PTR-MS and VOC CO_2 as measured by the CO_2 detector. The recovery ratio is calculated as carbon in ppm detected by the PTR-MS divided by the carbon in ppm detected by the CO_2 detector after oxidation by a platinum catalyst. The control in the bottom row is a sterile, uninoculated beet pulp reactor.

Experiment	Time (hours)	Phase	Ethanol (ppm C)	Methanol (ppm C)	Acetaldehyde (ppm C)	Terpenes (ppm C)	Total Carbon PTR-MS (ppm C)	VOC CO_2 detector (ppm C)	Recovery Ratio
1	18	Lag	9.73	0.308	1.59	0.00789	11.6	12.9	0.90
1	42	Exponential	33.4	0.206	5.24	0.209	39.1	39.1	1.00
1	66	Exponential	88.1	1.4	4.88	6.19	101	88.4	1.14
1	90	Stationary	49	3.7	1.4	7.23	61.3	54.8	1.12
1	114	Death	0.198	5.25	0	2.12	7.56	6.61	1.14
2	23	Lag	4.69	0.767	3.45	0.00399	5.8	8.08	0.72
2	49	Exponential	8.29	0.425	2.24	0.032	11	11.9	0.92
2	73	Exponential	32.1	0.33	4	0.731	37.1	39.2	0.95
2	97	Stationary	75.2	1.21	3.65	4.54	84.6	81.7	1.04
2	118	Stationary	64.4	2.34	1.82	5.21	73.8	68.4	1.08
3	21	Lag	4.18	0.153	0.582	0.00392	4.92	5.12	0.96
3	40	Exponential	6.57	0	1.88	0.0159	8.47	9.1	0.93
3	70	Exponential	31	0	4.5	0.825	36.3	41.6	0.87
3	88	Stationary	61.3	0.557	4.79	2.84	69.5	68.1	1.02
3	119	Stationary	49.7	2.52	1.98	3.77	57.9	67.3	0.86
3	137	Death	4.94	4	0.128	1.87	10.9	24.2	0.45
4	65.3	Stationary	83.6	1.9	3.91	7.59	97	89.6	1.08
4	98	Death	1.27	5.25	0	2.74	9.27	18.7	0.50
4	113	Death	0.13	5.28	0	1.06	6.36	5	1.27
Control	23	Control	0.0197	1.73	0.107	0	1.86	1.62	1.14

CHAPTER FOUR

VOLATILE ORGANIC COMPOUND PRODUCTION AT VARYING OXYGEN CONDITIONS IN A SOLID STATE FUNGAL REACTOR

Contribution of Authors and Co-Authors

Author: Heidi R. Schoen

Contributions: Assisted in designing the study, collected data, analyzed data, and wrote the paper.

Co-Author: W. Berk Knighton

Contributions: Assisted in designing the study, analyzed data, discussed results and implications, and edited the manuscript.

Co-Author: Brent M. Peyton

Contributions: Assisted in designing the study, analyzed data, discussed results and implications, and edited the manuscript.

Manuscript Information Page

Heidi R. Schoen, W. Berk Knighton, Brent M. Peyton
Bioresource Technology
Status of Manuscript:
_____Prepared for submission to a peer-reviewed journal
__X__Officially submitted to a peer-reviewed journal
_____Accepted by a peer-reviewed journal
_____Published in a peer-reviewed journal

Submitted 12/06/16

VOLATILE ORGANIC COMPOUND PRODUCTION AT VARYING OXYGEN CONDITIONS IN A SOLID STATE FUNGAL REACTOR

Heidi R. Schoen[1,2], W. Berk Knighton[3], Brent M. Peyton[1,2]*

1. Department of Chemical & Biological Engineering, Montana State University, 305 Cobleigh Hall, PO Box 173920 Bozeman, MT 59717
2. Center for Biofilm Engineering, Montana State University, 366 Barnard Hall, PO Box 173980, Bozeman MT 59717
3. Department of Chemistry and Biochemistry, Montana State University, 103 Chemistry and Biochemistry Building, PO Box 173400, Bozeman MT 59717

Abstract

Production of volatile organic compounds (VOCs) was quantified in a solid state fungal reactor system at four oxygen concentrations (0, 1, 13, and 21 %). The endophytic, filamentous fungus *Nodulisporium* isolate TI-13 was grown on the agricultural byproduct beet pulp as a substrate. Total VOC production, including small molecules like ethanol and acetaldehyde, increased by 23 times between aerobic and anoxic conditions, predominately due to increased production of ethanol. Production of carbon number four (C4) and higher VOCs increased by 4.6 times between aerobic and anoxic conditions. The highest specific production rates of C4 and higher VOCs were obtained under microaerophilic conditions. These compounds include the biofuel and flavor target molecules benzaldehyde, 2-methylpropan-1-ol, and phenylacetaldehyde. Anoxically, the fungal culture produced VOCs substantially longer, compared to aerobic or microaerophilic conditions, in a resting state with ethanol as the only major anoxic fermentation byproduct.

Introduction

Filamentous fungi are known to produce over 300 volatile organic compounds (VOCs) with applications as biofuels, flavor compounds, insecticides, food preservatives, and antimicrobials (Hung et al. 2015; Strobel, 2014). Fungal endophytes, fungi that live in plants without causing apparent harm, produce a large number of bioactive VOCs, which are hypothesized to protect the plant host from harm due to other organisms (Hyde & Soytong, 2008; Porras-Alfaro & Bayman, 2011). Many of these bioactive VOCs are also compounds of the same carbon numbers as those found in gasoline and diesel fuel (Strobel et al. 2008). Endophytes also produce ethanol, which is a major source of bioenergy, with more than 23 billion gallons produced in 2013 (Guo et al. 2015). However, the higher carbon number fungal biofuels explored here are more energy dense and could replace a higher percent of gasoline than ethanol (Atsumi et al. 2008; Strobel et al. 2008).

The endophytic fungus *Nodulisporium* isolate TI-13 investigated here can use abundant cellulosic waste (Schoen et al. 2016) directly to produce ethanol and higher value VOCs. Lignocellulosic biomass has the greatest potential to replace oil, as it is renewable, cheaply produced, and available in vast quantities (Grigoriev et al. 2011). Solid state fungal fermentation may be a promising technology for conversion of lignocellulosic biomass to biofuel and flavor compounds, as it utilizes less energy, space, water, and produces less waste water than submerged fermentation processes (Ali & Zulkali, 2011; Hölker et al. 2004). Solid state reactors offer easier separations than liquid cultures, and higher fungal product yields are often obtained, as the conditions mimic the natural environment of most filamentous fungi (Ali & Zulkali, 2011; Ben Akacha &

Gargouri, 2015; Hölker et al. 2004). Solid state fermentation on waste cellulosic feedstocks could provide a very environmentally friendly fuel source.

Many of the C4 and higher VOCs of interest are secondary metabolites (Zhi-Lin et al. 2012). The types and quantities of secondary metabolites produced may vary based on the fungal strain, substrate, temperature, oxygen, and growth phase of the culture (Bunge et al. 2008; Kai et al. 2009; Strobel, 2014). Triggers for the production of secondary metabolites are not yet completely understood, especially in less studied fungi such as endophytes (Gianoulis et al. 2012; Keller et al. 2005). Varying oxygen concentrations has been known to promote secondary metabolite production in fungi (Barker et al. 2012; Brakhage, 2013), and it has been suggested that lowering oxygen concentration increases yields of C4 and higher VOCs in endophytes (Mallette, 2013; Strobel et al. 2008). Few studies have been performed that use strong quantification methods, such as Proton Transfer Reaction-Mass Spectrometry (PTR-MS), to study fungal VOC production throughout growth phases or between culture conditions (Mallette et al. 2012; Nigg et al. 2014).

The current study quantifies total VOC production and examines gasoline and diesel range compounds produced throughout the lifecycle of a solid state fungal culture under different oxygen conditions. A VOC producing fungal endophyte, *Nodulisporium* isolate TI-13, was grown on the agricultural byproduct beet pulp with minimal pretreatment and directly produced potential biofuel and flavor compounds. Quantification of compounds was performed using a combination of a platinum catalyst and CO_2 detector for total gas phase VOC carbon, and PTR-MS for individual

components as previously described by Schoen et al. (2016) with identifications confirmed using Head Space Solid Phase MicroExtraction Gas Chromatography-Mass Spectrometry (HS-SPME GC-MS).

Materials and Methods

Microorganism

Nodulisporium isolate TI-13 was discovered as an endophyte of *Cassia fistula* in the highlands of Thailand using established collection and isolation methods as described previously (Ezra et al. 2004a). The TI-13 ITS1-5.8 S-ITS2 ribosomal gene sequence is available in GenBank as KJ558391, and the filamentous fungus is stored as sample NRRL 50502 in the Agriculture Research Service Culture Collection at the U.S. Department of Agriculture (Nigg et al. 2014). The fungus was characterized as having the perfect stage of *Hypoxylon* sp. and as *Nodulisporium* sp. based on ITS1-5.8 S-ITS2 ribosomal gene sequence information (Nigg et al. 2014).

Growth Conditions

Inoculum cultures were generated by growing the fungus on potato dextrose agar until hyphae covered ≥60 % of the plate surface. A 20 % glycerol solution (10 mL) was added to each plate, scraped with a sterile glass rod, and the suspended fungal biomass solution was collected. The solution was mixed thoroughly and added to Microbank microbead vials and stored at -80 ˚C.

The composition of the inoculum medium was 60 g * L^{-1} glucose and 0.5 g * L^{-1} yeast extract. 250 mL of sterile filtered (0.22 µm) medium was added to 500 mL sterile baffled flasks. Three microbeads were added to each inoculum culture and grown for nine days at 160 rpm and 30 °C. All inoculum flasks were covered with sterile Kimguard (Kimberly-Clark; Roswell, GA, USA) to allow gas exchange but prevent microbial contamination.

In a separate experiment for anoxic growth testing, fungal cultures were grown on PDA plates until they covered ≥60 % of the surface. Fungi and agar plugs, approximately 1 cm x 1 cm, were placed on beet pulp plates in an anoxic jar (Gas Pak System; Becton, Dickinson and Company; Franklin Lakes, NJ, USA) at room temperature. Control beet pulp plates inoculated with the same procedure were allowed to grow under aerobic conditions.

Solid State Fungal Reactor System

Solid state reactors constructed from a 2-liter, air tight, borosilicate glass container with three stainless steel mesh shelves were inoculated with 250 mg of biomass as determined by a correlation with protein concentration using a modified Bradford method (Bradford, 1976). As a substrate, 50 g of beet pulp were autoclaved for 20 min with 250 mL of water and allowed to cool. The reactor, hydration flask, and all tubing were autoclaved at 121 °C for 20 min before use. Fungal biomass was added to the beet pulp, mixed by inversion, and evenly distributed to the three shelves of the reactor. The reactor was placed in an incubator at 27 °C during experiments. Biological duplicate

experiments were run for each of the four oxygen conditions until total VOC production approached zero.

The solid state reactor was continuously sparged with 100 mL * min^{-1} of hydrated gas mixed with two mass flow controllers to have concentrations of 21, 13, 1, or 0 % oxygen. Oxygen concentrations were switched from 21 % to 0, 1, or 13 % in mid-exponential phase to allow for accumulation of biomass. The air was delivered via a stainless steel tube (0.5 cm I.D.) running to the bottom center of the reactor. Reactor off-gas was then diluted with 900 mL * min^{-1} of medical grade air and pulled through the CO_2 detector and PTR-MS with the PTR-MS diaphragm pump as previously described (Schoen et al. 2016). The reactor off-gas flow path was alternated every three hours using an automated three-way valve to direct the gas either straight to the CO_2 detector to measure respiratory CO_2 only or through the VOC oxidizing platinum catalyst and then to the CO_2 detector, measuring CO_2 from both VOCs and fungal respiration. The PTR-MS sampled both gas steams that entered and bypassed the platinum catalyst once per day. Two biological replicate experiments were performed at each oxygen condition. Background CO_2 and VOCs measured for each tank of medical grade compressed air and nitrogen were subtracted from these values.

Total Volatile Organic Carbon and Respiratory CO_2 Measurement

An LI-840 CO_2/H_2O non-dispersive infrared gas analyzer (Li-Cor Biosciences; Lincoln, NE) was used to measure respiratory CO_2. To measure total gas phase organic carbon, a heated platinum catalyst (Shimadzu High Sensitivity Catalyst 630-00996

maintained at 400 ^{o}C) completely oxidized VOCs to CO_2 before the CO_2 detector so that total volatile organic carbon and respiratory CO_2 were measured. Data from the CO_2 detector were analyzed using IGOR 6 (Wavemetrics; Portland, OR) as described previously (Schoen et al. 2016) to determine respiratory CO_2 and total VOC production.

Selectivity

Selectivity was determined by dividing the total amount of VOC product in ppm C by respiratory CO_2 in ppm C (numerator and denominator were determined as previously described in Schoen et al. 2016). Selectivity is an instantaneous ratio of the carbon channeled into VOCs instead of respiratory CO_2. This measurement does not examine carbon utilized to produce biomass or non-VOC metabolites, but selectivity is a relative measure of desired VOC products to carbon wasted in respiratory CO_2 as measured in the gas phase.

Fungal Biomass Estimates

Measuring fungal biomass on solid substrates can be extremely difficult, so a correlation was determined based on *Nodulisporium* isolate TI-13 CO_2 evolution when grown on filters over beet pulp plates where biomass can be readily measured. Fungal biomass was then estimated using respiratory CO_2 measurements of reactor off-gas in the primary experiments as described by Couriol et al. (2001). Estimates were made by determining growth-associated CO_2 production as well as maintenance CO_2 evolution with using Equation 1.

$$\frac{dCO_2}{dt} = A\frac{dx}{dt} + BX \qquad (1)$$

Where CO_2 is gas phase respiratory CO_2 (concentration) and t is time (time), such that the term on the left-hand side represents the total respiratory CO_2 leaving the reactor per time. X is the total biomass in the system (mass). A is a constant (unitless) associated with CO_2 evolved due to growth, and B is a constant ($time^{-1}$) associated with maintenance CO_2 evolution (Couriol et al. 2001). The constants A and B were estimated from two biological replicate experiments relating respiratory CO_2 evolution to growth of *Nodulisporium* isolate TI-13 grown on agar beet pulp plates made with 15 g * L^{-1} noble agar (Becton, Dickinson and Company; Sparks, MD, USA) and 20 g * L^{-1} ground beet pulp. Nitrate cellulose filters (0.2 µm; GE Healthcare; Buckinghamshire, UK) were dried at 80 °C overnight, weighed, and placed on the beet pulp plates. Abiotic beet pulp plates with filters were placed in the reactor to measure CO_2 evolution as a control, and this CO_2 was subtracted from respiratory CO_2 in the experiments. To determine the constants A and B, inoculated plates were placed in the reactor, sparged with medical grade air, and duplicate plates were removed twice per day to measure biomass. Fungus and membrane filters were dried again at 80 °C overnight, and weighed to determine dry weights. Constants were identified using linear regression in Excel (Microsoft Corporation; Redmond, WA, USA) which gave an R^2 of 0.82 (data not shown). The constants A and B were then applied to respiratory CO_2 measurements to estimate biomass dry weights throughout the solid state reactor experiments.

Proton Transfer Reaction-Mass Spectrometry (PTR-MS)

PTR-MS was used to provide compositional information about the VOCs in the reactor off-gas. PTR-MS uses H_3O^+ ions to protonate molecules (e.g. VOCs) with proton affinities greater than water (Lindinger et al. 1998). The singly charged ions are then typically detected as protonated molecules (ions with a mass-to-charge ratio, m/z, equal to the molecular weight plus 1 for the proton) by a quadrupole mass spectrometer (Lindinger et al. 1998). Carbon number was assumed for each mass to charge ratio based on HS-SPME GC-MS data, known fungal metabolites, and identification of endophytic fungal VOCs in previous studies (Luchner et al. 2012).

A multicomponent standard containing methanol, acetaldehyde, and alpha-pinene (Apel-Riemer Environmental Inc., Broomfield, CO, USA) was dynamically diluted to determine PTR-MS sensitivity factors for those compounds. Ethanol calibration was performed separately using a permeation tube at a variety of humidities as described previously (Schoen et al. 2016). Sensitivity factors of 4.1, 17.4, 18.2, and 2.2 ncps (normalized counts per second) * $ppbv^{-1}$ for ethanol (m47, m65, m93), methanol (m33, m51), acetaldehyde (m45), and terpenes and terpenoids (m137) were used, respectively. The off-gas of the abiotic control and fungal cultures were both analyzed by PTR-MS, and VOCs measured in the abiotic control were subtracted from the fungal VOC production measurements. Concentrations of all other reported ions were estimated using Equation 2:

$$[R] = \frac{I_{RH^+}}{\mathrm{ktz}\left(I_{\mathrm{m/z21}} * 500 + I_{\mathrm{m/z39}} * 250\right)} \quad (2)$$

where R is the concentration of the compound of interest, k is the reaction rate constant (here assumed to be $k = 2 \times 10^{-9}$ cm^3 $molecule^{-1}$ s^{-1}), t is the reaction time (Lindinger et al. 1998), and z is a mass dependent correction factor for transmission bias (Knighton et al. 2009; Steinbacher et al. 2004) determined for this PTR-MS. Ions representing two percent or greater of the total ion signal were quantified, with the exception of isotopes.

Head Space Solid Phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME GC-MS)

HS-SPME GC-MS was performed (Montana State University Mass Spectrometry Facility) as previously described (Griffin et al. 2010; Mallette et al. 2012) to assist in assignment of identities to PTR-MS ions. Briefly, a SPME fiber (divinylbenzene/carboxen on polydimethylsiloxane (Supelco; Bellefonte, PA, USA) was conditioned at 240 °C for 15 min before being exposed to the headspace of the solid sample for 35 min. The exposed fiber was inserted into a modified GC injection port operated at 240 °C. The Zebron ZB-WAX (30 m × 0.25 mm ID × 0.50 μm) GC column (Phenomenex; Torrance, CA, USA) temperature was held at 30 °C for 2 min and then ramped to 230 °C at 7 °C min^{-1}. Compounds were identified via library comparison using the National Institute of Standards and Technology (NIST) database. All compounds reported had quality matches of at least 70; direct manual comparisons were made to spectra of known compounds when there were multiple high quality matches.

Conversion to Reported Units

The platinum catalyst and CO_2 detector measured CO_2 from VOCs in ppm which is effectively measuring the ppm carbon from VOCs (By measuring the difference between respiratory CO_2 and respiratory CO_2 plus oxidized VOC CO_2 as previously described in Schoen et al. 2016). The ppm of carbon from VOCs was then converted to millimoles of carbon produced per hour based on the temperature, pressure, and flow rate through the reactor (production rate [mol carbon * hr^{-1}]).

The PTR-MS measured counts of ions that were then converted to ppb of VOC carbon produced per hour in a similar fashion (production rate [mol carbon * hr^{-1}). To examine the productivity per biomass this number was divided by the grams of biomass (specific production rate [mol carbon * hr^{-1} * g $biomass^{-1}$]). To find the production of VOCs throughout an experiment, the daily PTR-MS measurements that had been converted to production rates were integrated over time using the trapezoid rule and divided by the biomass estimate (total specific production [mol carbon * g $biomass^{-1}$]). To determine the experiment which produced the most VOCs per time on average, the total specific production of VOCs per experiment was divided by the length of the experiment in hours (average specific production rate [mol carbon * hr^{-1} * g $biomass^{-1}$]).

Results and Discussion

Total VOC Production

The VOCs produced by *Nodulisporium* isolate TI-13 in a beet pulp solid state reactor were investigated at varying oxygen conditions. Total VOCs were accurately

quantified by oxidation with a platinum catalyst and CO_2 detector, including small VOCs like ethanol and acetaldehyde, as well as larger tentatively identified compounds like 2-methylpropan-1-ol and benzaldehyde. As shown in Figure 4-1, the total VOC production of the reactor increased from 0.052 +/- 0.017 g carbon of VOCs (average and standard error) under fully aerobic conditions (21 % O_2) to 1.2 +/- 0.060 g carbon under anoxic conditions (0 % O_2). This represents a factor of 23 increase in total VOC production between oxic and anoxic conditions. Production rates were also highest anoxically at 4.0 +/- 0.15 mg carbon * hr^{-1}. This increase was predominately due to increases in ethanol production and is in part due to the fungus entering an extended VOC production state without growing under anoxic conditions. All experiments were sparged with 21% oxygen through mid-exponential phase to allow for accumulation of biomass. *Nodulisporium* isolate TI-13 did not show growth on beet pulp after one month in an anoxic chamber. Likely no growth occurred in the reactor once anoxic conditions were initiated, but the cells entered a metabolically active, VOC producing, resting state that lasted approximately 12 days. VOC production approached zero approximately three days after mid-exponential growth phase for other oxygen concentrations.

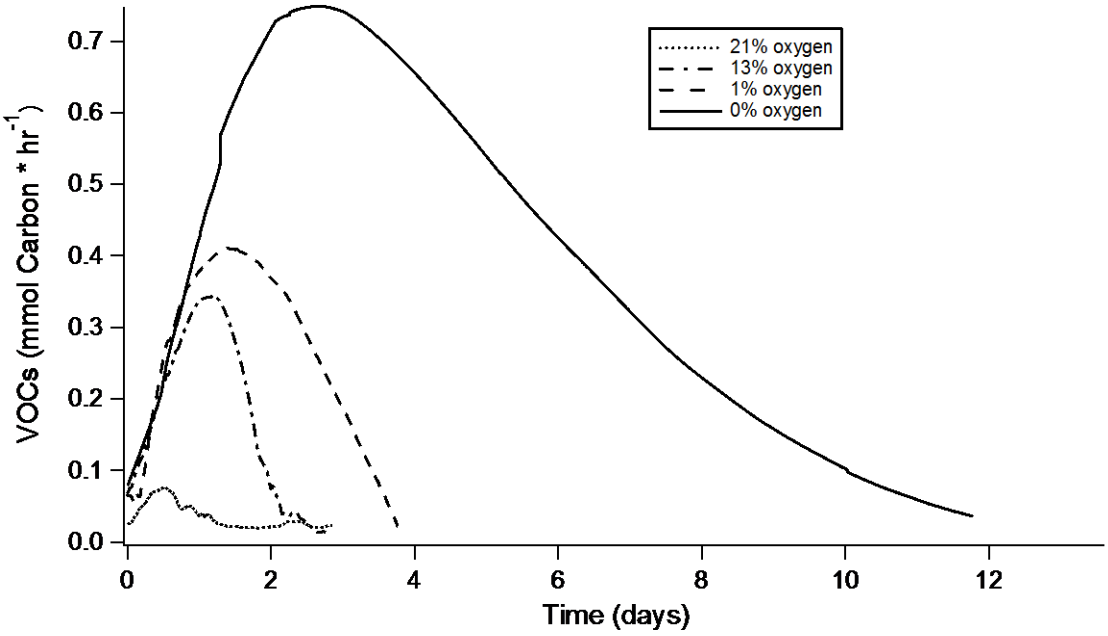


Figure 4-1. Total volatile organic carbon (VOC) production rates of the endophytic fungus *Nodulisporium* isolate TI-13 grown in a solid state reactor at four different oxygen conditions as measured by a platinum catalyst and CO_2 detector. This figure shows a representative run for each of the four oxygen conditions starting at the point the oxygen conditions were changed at approximately mid-exponential phase.

Carbon Number Four and Higher VOC Production

While ethanol is a widely used biofuel, production rates here are low, and the higher value products made by the fungus are of more novel interest. These products were investigated using PTR-MS to quantify individual compounds (See Table 4-1 for tentative identifications of quantified ions). Carbon number four (C4) and higher VOCs (gasoline range) and carbon number eight (C8) and higher VOCs (diesel range) groupings specifically were investigated (U.S. Department of Energy, 2013). Tentative identification of PTR-MS ions was assisted using HS-SPME GC-MS. No compounds with a carbon number greater than ten were identified. Lowering oxygen conditions

significantly increased production of higher carbon number compounds as well. Figure 4-2 shows C4 and higher VOC total specific production (mol carbon produced throughout experiments) increased by 4.6 times from aerobic (110 +/- 32 µmol carbon * g biomass^{-1}) to anoxic conditions (510 +/- 19 µmol carbon * g biomass^{-1}). This increase in production was due to tentatively identified compounds such as 2-methylpropan-1-ol, benzaldehyde; and ethyl butanoate, which are each flavor compounds as well as potential biofuels based on their carbon number (U.S. Department of Energy, 2013; Gillies et al. 1987; Pino et al. 2004; Schwab et al. 2008). These values were lower than the estimated 60 mg * g biomass^{-1} total specific production from *Ascocoryne sarcoides*, although this estimate used less accurate instrumentation and may include large fractions of C3 and smaller volatiles (Mallette, 2013). Total specific production of diesel range compounds, C8 and higher VOCs, increased by 2.7 times from aerobic (52 +/- 37 µmol carbon * g biomass^{-1}) to anoxic conditions (140 +/- 11 µmol carbon * g biomass^{-1}) due to tentatively identified compounds such as 2-phenylethan-1-ol and phenylacetaldehyde.

Table 4-1. Ions quantified by PTR-MS. Ions representing two percent or greater of the total ion signal were quantified. The table provides tentative ion and compound identities based on HS-SPME GC-MS data, known fungal metabolites, and identification of endophytic fungal VOCs in previous studies. No compounds were clearly identified for m/z 59, 99, 103, and 135.

Ion	Tentative Ion Identification	Tentative Compound Identification	Formula	MW
33	CH_4OH^+	Methanol	CH_4O	32.04
41	$C_3H_5^+$	Propan-1-ol	C_3H_8O	60.10
43	$C_3H_7^+$	Propan-1-ol	C_3H_8O	60.10
45	$C_2H_4OH^+$	Acetaldehyde	C_2H_4O	44.05
47	$C_2H_6OH^+$	Ethanol	C_2H_6O	46.07
51	$CH_4OH^+(H_2O)$	Methanol	CH_4O	32.04
57	$C_4H_9^+$	2-Methylpropan-1-ol	$C_4H_{10}O$	74.12
59	$C_3H_6OH^+$		C_3H_6O	58.08
65	$C_2H_6OH^+(H_2O)$	Ethanol	C_2H_6O	46.07
61	$C_2H_5O_2^+$	Ethyl Acetate	$C_4H_8O_2$	88.11
71	$C_5H_{11}^+$	3-Methylbutan-1-ol	$C_5H_{12}O$	88.15
75	$C_3H_6O_2H^+$	Methyl acetate	$C_3H_6O_2$	74.08
79	$C_2H_5O_2^+(H_2O)$	Ethyl Acetate	$C_4H_8O_2$	88.11
81	$C_6H_9^+$	1,8-Cineole	$C_{10}H_{18}O$	154.25
85	$C_6H_{13}^+$	Hexan-1-ol	$C_6H_{14}O$	102.18
89	$C_4H_8O_2H^+$	Ethyl Acetate	$C_4H_8O_2$	88.11
93	$H^+(C_2H_6O)_2$	Ethanol	C_2H_6O	46.07
99	$C_6H_{10}OH^+$		$C_6H_{10}O$	98.16
103	$C_5H_{10}O_2H^+$		$C_5H_{10}O_2$	116.16
105	$C_8H_8^+$	2-Phenylethan-1-ol	$C_8H_{10}O$	122.16
107	$C_7H_6OH^+$	Benzaldehyde	C_7H_6O	106.12
117	$C_6H_{12}O_2H^+$	Ethyl butanoate	$C_6H_{12}O_2$	116.16
121	$C_8H_8OH^+$	Phenylacetaldehyde	C_8H_8O	120.15
135	$C_9H_{10}OH^+$		$C_9H_{10}O$	134.18
137	$C_{10}H_{17}^+$	1,8-Cineole	$C_{10}H_{18}O$	154.25

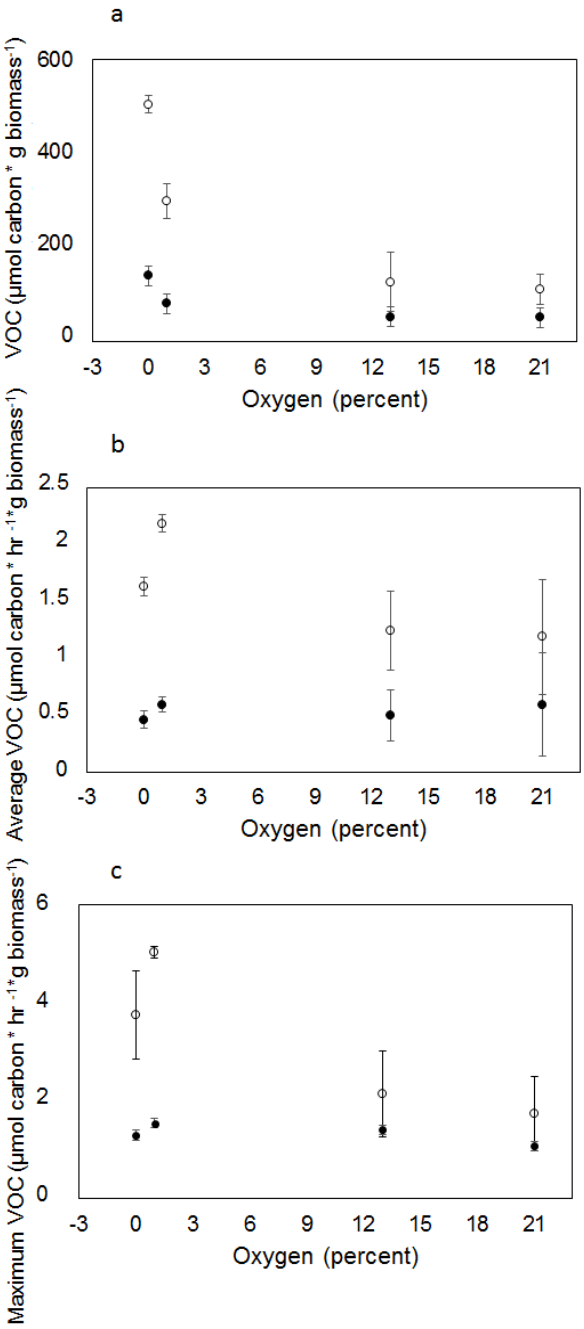


Figure 4-2. Panel a depicts the total specific production of carbon number four (C4) and higher volatile organic compounds (open circles), of which a portion are carbon number eight (C8) and higher (black-filled circles), during an experiment at four different oxygen conditions (average of biological duplicates and standard error) as measured by PTR-MS. Panel b depicts average specific production rates of C4 and C8 VOCs during an entire experiment. Panel c depicts the maximum specific production rates identified during each experiment.

The highest rates of C4 VOC production were found under microaerophilic conditions. Figure 4-2 shows that microaerophilic conditions gave the highest average specific VOC production rate (the moles of carbon produced during an entire experiment divided by the duration of the experiment) of C4 and higher compounds (2.2 +/- 0.074 μmol carbon * hr^{-1} * g $biomass^{-1}$). The highest maximum specific VOC production rate (of individual sampling points in each experiment) was also observed under microaerophilic conditions for both C4 and C8 VOCs as shown in Figure 4-2 (panel c). As part of their mutualistic relationship with their plant hosts, endophytic fungi may produce bioactive VOC suites to inhibit other organisms from invading the plant and to signal the plant to raise its own defenses (Porras-Alfaro & Bayman, 2011; Saikkonen et al. 1998). Endophytes like *Nodulisporium* isolate TI-13 experience microaerophilic conditions inside the tree and may produce the highest yields of bioactive VOCs under similar conditions (Strobel et al. 2008). No statistically significant trend was observed for C8 and higher compound average specific production rates as seen in Figure 4-2.

VOC Production through Culture Growth Phases

A plot of total VOC production rates over time followed a typical growth curve where production increased with decreasing oxygen conditions (See Figure 4-1), predominately due to the major product ethanol. Ethanol is produced as a primary metabolite, so a growth curve pattern was expected as seen in Figure 4-3 (Garraway and Evans, 1984). Most of the tentatively identified compounds tracked in this study showed

a similar production trend, such as phenylacetaldehyde shown as panel b of Figure 4-3. However, for two compounds (ethyl acetate and another ester at m/z 103), production was significantly higher at 1 % oxygen than any other condition tested around the start of stationary phase, which is the expected trend for secondary metabolites (Garraway and Evans, 1984). The modulated production rates seen in panels c and d of Figure 4-3 were also present in the biological replicates.

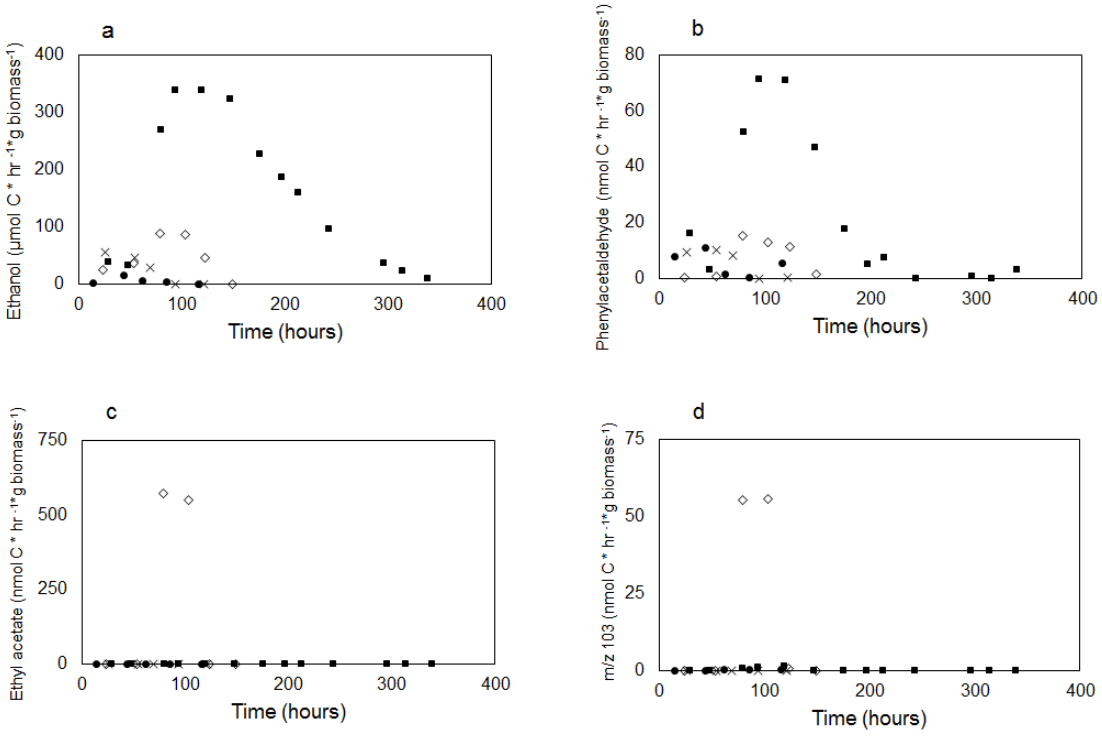


Figure 4-3. Specific production rates of ethanol (panel a), phenylacetaldehyde (panel b), ethyl acetate (panel c), and an ester at m/z 103 (panel d) at four different oxygen conditions as measured by PTR-MS. On each graph, data symbols are 21% oxygen (●), 13% oxygen (×), 1% oxygen (◇), and 0% oxygen (■) for a representative run of each.

Ethyl acetate is produced by acetyltransferases from ethanol and acetyl-CoA (Yoshioka & Hashimoto, 1981). Ehrlich pathway alcohols can be esterified in a similar

way, and some acetyltransferases act on multiple alcohols (Verstrepen et al. 2003a; Verstrepen et al. 2003b). The ion at 103 shows a similar pattern, but could not be clearly identified. However this ion was identified in previous fungal work as propanoic acid, 2-methyl- methyl ester (Ezra et al. 2004b), and this m/z ratio in PTR-MS is most likely an ester when produced by fungi . High oxygen concentrations, anoxic conditions, or the presence of ethanol or fatty acid related compounds in the medium can repress expression of some acetyltransferases and lower ester production in *Saccharomyces cerevisiae* (Plata et al. 2005; Verstrepen et al. 2003a; Yoshioka & Hashimoto, 1981), explaining the observation of maximum ester production at microaerophilic conditions. Endophytic fungal VOCs can inhibit microbial growth by disruption of cell membrane integrity and damaging DNA (Campos et al. 2015). These endophytic VOCs are synergistically toxic in combination, although esters are the most inhibitory individual class of compounds (Strobel et al. 2001). Interestingly, these experiments show that esters are the only metabolites with drastically modulated production rates at the tested oxygen conditions, likely due to production of some acetyltransferases only under microaerophilic conditions.

Selectivity

Selectivity, the total amount of gas phase VOC product in ppm C divided by the amount of respiratory CO_2 in ppm C, was determined for the four oxygen conditions (0, 1, 13, and 21 %) as shown in Figure 4-4. Selectivity increased by a factor of 49 times, to a maximum of 2.3 +/- 0.09 anoxically, again predominately due to ethanol production.

This high selectivity, slightly above the maximum theoretical yield of two for ethanol or volatile organic carbon metabolism, (Garraway and Evans, 1984; Hunt, 2016) is likely due to volatilization of ethanol from the liquid phase outpacing ethanol production. However, ethanol appeared to be the only major product of isolate TI-13 fermentation, and no acetate was identified. Other well-studied cellulosic fungi, such as *Fusarium oxysporum*, *Trichoderma reesei*, and *A. sarcoides*, produce significant amounts of acetate during fermentation, reducing ethanol yields (Chambergo et al. 2002;; Mallette, 2013; Panagiotou et al. 2005).

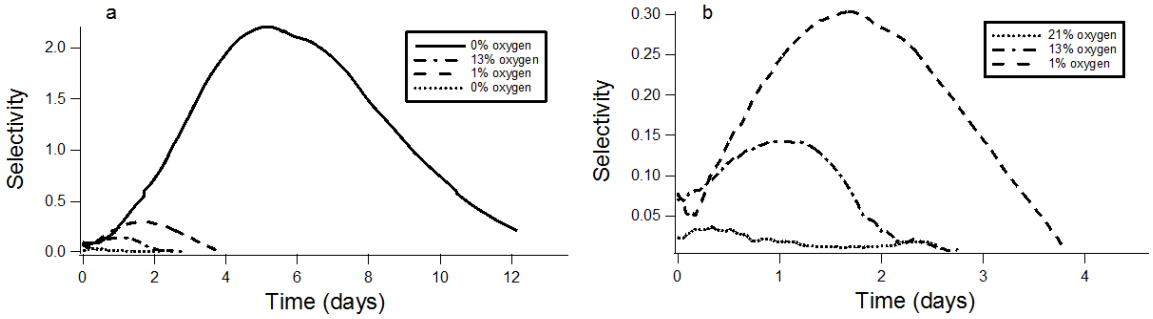


Figure 4-4. Selectivity, the amount of volatile organic carbon divided by respiratory CO_2 carbon, at all four oxygen concentrations. Panel a shows a representative run for each of the four oxygen conditions starting at the point the oxygen conditions were changed at approximately mid-exponential phase, and panel b shows just the three conditions containing oxygen.

While lowering oxygen concentrations significantly increased secondary metabolite production, the specific production rates of high value biofuel and flavor VOCs were low, at a maximum of 5.0 +/- 0.11 µmol carbon * hr^{-1} * g $biomass^{-1}$, for C4 and higher products. Although optimizing process conditions such as medium, temperature, and sparge rate may increase yields, genetic engineering is likely required to

achieve industrially viable production rates. The techniques used to identify and quantify VOCs used here could be combined with transcriptomics and metabolic modeling to identify target genes for genetic engineering (Lee et al. 2005; Stephanopoulos et al. 1998). Alternatively, *Nodulisporium* isolate TI-13 has several advantageous properties including cellulose utilization, VOC production, resistance to high concentrations of VOCs, and ethanol specific fermentation that could be further studied and engineered into other organisms.

Solid state reactors are currently routinely used to produce foods and enzymes, but with the advent of more sophisticated monitoring instrumentation and modeling tools, their industrial role is expected to expand (Thomas et al. 2013). To this end, a packed bed reactor to produce bioethanol, 55 m in length, was constructed in China with sweet sorghum as the substrate (Li et al. 2013). A similar design could be applied to the fungal production of VOCs which takes advantage of increase in ethanol and C4 and higher compound production at low oxygen conditions. Fungal inoculum and a waste cellulosic could enter in one end, and aeration would allow the accumulation of biomass. Further in the reactor, oxygen concentrations would decrease and production of ethanol and higher value VOCs would significantly increase. Such a reactor may provide an environmentally friendly biofuel and flavor compound production scheme.

Conclusions

Lower oxygen conditions significantly increased the production of ethanol and secondary metabolites in *Nodulisporium* isolate TI-13, with 23 times more VOCs produced under anoxic conditions than aerobically, predominately due to ethanol. Total production of C4 and higher VOCs was also highest anoxically. However, both average and maximum specific production rates of C4 VOCs were highest at 1 % oxygen (2.2 +/- 0.074 and 5.0 +/- 0.11 µmol carbon * hr^{-1} * g $biomass^{-1}$, respectively). The methods used here could be combined with other techniques to learn more about VOC producing pathways. This information could then be combined with genetic engineering to increase production.

Acknowledgements

This research was supported in part by the National Science Foundation (NSF) EFRI Program under Grant No. 0937613. Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF. Thank you to the Montana State University Center for Biofilm Engineering, the Chemistry and Biochemistry Department, and the Chemical and Biological Engineering Department for support. The authors would also like to thank Drs. Ross Carlson and Kris Hunt for sound technical advice.

Supplemental Material

Table 4-S1. Identification of volatile organic compounds produced by the endophytic fungus *Nodulisporium* isolate TI-13 grown in a solid state reactor by head space solid phase microextraction gas chromatography mass-spectrometry. Retention time, compound identification, and quality match are shown for exponential phase aerobic (1), mid-stationary phase anoxic (2) and late-stationary phase anoxic (3) conditions.

RT	**Compound**	**Quality Match Condition 1**	**Quality Match Condition 2**	**Quality Match Condition 3**
4.86	Heptane		70.5	
4.96	Acetaldehyde		93.4	88.7
6.28	2-Methylpropan-1-ol	90.5		
6.33	2-Methylpropanal	76.7	84.3	75.3
6.63	Methyl Acetate		84.6	
7.57	Ethyl Acetate	72.6	93.7	95.6
8.17	2-Methylbutanal	70.6		72.1
8.22	3-Methylbutanal	84.9	84.4	82.1
8.52	Ethanol	92.7	96.2	96.5
9.58	Pentanal		81.4	82.8
10.3	2-Methylpropyl acetate	88.0		
10.8	Ethyl butanoate	83.2		
10.8	Propan-1-ol		74.6	
11.8	4-Methylhexan-3-one			73.4
11.9	Hexanal			73.7
12.0	2-Methylpropan-1-ol		87.7	83.1
13.0	Ethyl pentanoate	77.9		85.7
13.2	Butan-1-ol	73.5		71.5
14.3	Heptan-2-one		72.8	
14.3	Heptanal		74.5	
14.9	1,8-Cineole	92.9		75.7
15.3	Ethyl hexanoate	72.9		
16.2	Hexyl acetate	74.3		80.5
16.6	Octanal		77.9	85.2
16.8	**3**-Hydroxybutan-2-one	84.7		
17.4	Ethyl heptanoate			77.3
17.7	6-Methyl-5-hepten-2-one		75.8	70.4
19.1	2-Butoxyethan-1-ol		73	73.0
19.5	Ethyl octanoate	84.3		88.8
20.4	Furan-2-carbaldehyde			74.1
21.7	Benzaldehyde	70.0	76.1	73.4

4-S1 Continued

23.4	5-Methyldihydrofuran-2(3H)-one		70.7	78.1
23.8	Phenylacetaldehyde	75.5	72.4	72.4
24.4	α-Terpineol			71.1
25.6	Methyl phenylacetate		81.6	86.4
26.0	Ethyl phenylacetate		74.6	71.0
29.0	2-Phenylethan-1-ol		77.8	81.8

Supplemental Text

Total volatile organic carbon (VOC) quantifications performed with proton transfer reaction-mass spectrometry (PTR-MS) were compared to the robust total VOC measurement taken with the platinum catalyst and sensitive CO_2 detector as previously described in Schoen et al. 2016. Recovery, defined as total PTR-MS measured carbon divided by platinum catalyst and CO_2 detector quantified carbon, was 0.954 +/- 0.0460 (average and standard error of 54 measurements) which is considered good agreement as discussed in Schoen et al. 2016 previously.

The carbon number of ions with higher than 2% of the total signal were assumed based on mass to charge ratio, previously identified endophytic fungal metabolites, and Head Space Solid Phase MicroExtraction Gas Chromatography-Mass Spectrometry (HS-SPME GC-MS). Several of the major ions have been consistently identified in previous PTR-MS fungal work as follows: ions m/z 47, m/z 65, m/z 93 as ethanol; m/z 33 and m/z 51 as methanol; m/z 45 as acetaldehyde; m/z 81 and m/z 137 as terpenes and terpenoids (Mallette et al. 2012; Schoen et al. 2016; Tomsheck et al. 2010). Terpenes and terpenoids appeared to be predominately 1,8-cineole based on the SPME data.

Ions m/z 41 and m/z 43 were examined using GC PTR-MS as previously described (Rogers et al. 2006). While there were fragments of higher alcohols at these mass to charge ratios, the largest contributions appeared to be from a C3 alcohol, identified as propan-1-ol in the SPME data. These ions were conservatively counted as C3 compounds in the data analysis.

M/z 57 was tentatively identified as 2-methylpropan-1-ol, a common volatile fungal product (Hazelwood et al. 2008; Roze et al. 2010). Additionally, 2-methylpropan-1-ol was identified in the SPME data and has previously been identified in fungal PTR-MS work (Ezra et al. 2004).

Similarly, m/z 71 was tentatively identified as 3-methylbutan-1-ol, another common volatile fungal product (Hazelwood et al. 2008; Roze et al. 2010). 3-methylbutan-1-ol has also been previously identified at this mass to charge ratio in fungal PTR-MS studies (Ezra et al. 2004; Mallette et al. 2012).

M/z 59 was assumed to be a C3 carbonyl because that was the only likely biogenic compound to produce this ion. Protonation of acetone or an isomer can produce this ion, but such a compound was not identified in the SPME data. However, m/z 59 has been suggested to be a C3 carbonyl in previous fungal PTR-MS work (Mallette et al. 2012), specifically acetone (Ezra et al. 2004).

M/z 61, 79, and 89 were tentatively identified as ethyl acetate. Ethyl acetate was identified in the SPME data, and these are its expected fragments (Buhr et al. 2002; Ezra et al. 2004; Inomata & Tanimoto, 2008).

M/z 75 was tentatively identified as methyl acetate. Methyl acetate was identified in the SPME data, and this was its expected molecular ion (Buhr et al. 2002; Ezra et al. 2004).

M/z 85 was assumed to be a C6 alcohol. M/z 85 was an expected fragment of C6 alcohols (Buhr et al. 2002), and 1-hexanol has previously been identified as a product of an endophytic fungus (Griffin et al. 2010).

M/z 99 was assumed to be $C_6H_{10}O$. Hexenals produce a fragment at this mass to charge ratio using PTR-MS (Lee, 2012). The compound that produces this ion was likely to be carbon number six or higher, so this was a conservative assumption.

While no compound could be identified at m/z 103, this ion was identified as the molecular ion of propanoic acid, 2-methyl, methyl ester ($C_5H_{10}O_2$) in previous fungal PTR-MS work (Ezra et al. 2004). This was also a plausible product of fungal metabolism (Hazelwood et al. 2008). This ion was not attributed to 1-hexanol as this compound was not expected to produce detectible amounts of the protonated molecule (Buhr et al. 2002).

M/z 105 was tentatively identified as 2-phenylethan-1-ol. M/z 105 was identified as a fragment of 2-phenylethan-1-ol in previous fungal PTR-MS work (Ezra et al. 2004; Mallette et al. 2012), and 2-phenylethan-1-ol was identified in the SPME data set.

M/z 107 was tentatively identified as benzaldehyde. M/z 107 has been identified as benzaldehyde in previous fungal PTR-MS work (Mallette et al. 2012), and benzaldehyde was identified in the SPME data set.

M/z 117 was tentatively identified as ethyl butanoate, as this was its major expected ion using PTR-MS (Buhr et al. 2002), and this compound was identified in the

SPME data set. It was also an expected fungal product through esterification of an Ehrlich pathway acid (Hazelwood et al. 2008).

M/z 121 was tentatively identified as phenylacetaldehyde. Phenylacetaldehyde was identified in the SPME data set, and its molecular ion was expected at m/z 121. While this mass to charge ratio was attributed to other compounds in previous fungal PTR-MS work (Tomsheck et al. 2010), these compounds were not identified in our SPME data set. Additionally, phenylacetaldehyde was a known fungal metabolic product (Hazelwood et al. 2008).

M/z 135 was tentatively identified as $C_9H_{10}O$ as a modified benzene structure, similar to the ion identified at 121, except with an additional CH_2. While no compounds expected to produce this ion were identified in the SPME data, this was a reasonable carbon assumption.

CHAPTER FIVE

PRODUCTION OF VOLATILE ORGANIC COMPOUNDS UNDER VARYING NITROGEN CONDITIONS BY *ASCOCORYNE SARCOIDES*

Contribution of Authors and Co-Authors

Author: Heidi R. Schoen

Contributions: Assisted in designing the study, collected data, analyzed data, and wrote the paper.

Co-Author: W. Berk Knighton

Contributions: Assisted in designing the study, analyzed data, discussed results and implications, and edited the manuscript.

Co-Author: Brent M. Peyton

Contributions: Assisted in designing the study, analyzed data, discussed results and implications, and edited the manuscript.

Manuscript Information Page

Heidi R. Schoen, W. Berk Knighton, Brent M. Peyton
Biotechnology and Bioengineering
Status of Manuscript:
__X__Prepared for submission to a peer-reviewed journal
_____Officially submitted to a peer-reviewed journal
_____Accepted by a peer-reviewed journal
_____Published in a peer-reviewed journal

VOLATILE ORGANIC COMPOUND PRODUCTION WITH VARYING NITROGEN CONDITIONS IN A SOLID STATE FUNGAL REACTOR

Heidi R. Schoen[1,2], W. Berk Knighton[3], Brent M. Peyton[1,2]*

1. Department of Chemical & Biological Engineering, Montana State University, 305 Cobleigh Hall, PO Box 173920 Bozeman, MT 59717
2. Center for Biofilm Engineering, Montana State University, 366 Barnard Hall, PO Box 173980, Bozeman MT 59717
3. Department of Chemistry and Biochemistry, Montana State University, 103 Chemistry and Biochemistry Building, PO Box 173400, Bozeman MT 59717

Abstract

Endophytic fungal production of volatile organic compounds (VOCs) and liquid phase metabolites was quantified with growth on five different nitrogen sources (asparagine, leucine, phenylalanine, methionine, and ammonium chloride). As measured in the reactor off-gas, the model VOC producing fungus *Ascocoryne sarcoides* produced 3.2 times more total VOCs (0.15 +/- 0.015 carbon mols), 6.2 times more carbon number four and higher VOCs (7.2 +/- 0.87 carbon mmols), and 5.4 times more carbon number eight and higher VOCs (9.7 +/- 2.5 carbon µmol) when grown on ammonium chloride than on the other nitrogen sources, including compounds such as 3-methylbutanol, 2-methylpropanol, and ethanol. However, in the liquid phase, 1.4 g/L of arabitol, a sweeter, were identified in the culture grown with phenylalanine as the nitrogen source.

Introduction

Petroleum reserves are limited and will become increasingly expensive to extract (Grigoriev et al., 2011). Both fuel and other petroleum derivatives, such as flavor compounds, will need to be produced in another manner (Grigoriev et al., 2011; Schwab et al., 2008). Lignocellulosic biomass has the greatest potential to replace oil, as it is renewable, cheaply produced, and available in vast quantities (Grigorier et al., 2011). Filamentous fungi can directly convert cellulosic biomass to biofuel and flavor compounds with little or no pretreatment (Grigorier et al., 2011). Some endophytic fungi produce volatile compounds that are similar to gasoline and diesel fuel currently produced from oil (Strobel et al., 2008). These higher carbon number compounds are more compatible with existing fuel and transportation systems than ethanol so are more desirable biofuel targets (Griffin et al., 2010).

Most of the biofuel and flavor compound produced by fungi are bioactive secondary metabolites (Keller et al., 2005). In filamentous fungi, production of many secondary metabolites is controlled by the global regulator, *laeA*, and the related velvet proteins, which modulate metabolic pathways based on environmental conditions and the stage of the fungal lifecycle (Bayram et al., 2008; Bok & Keller, 2004). Induction or changes in yield of secondary metabolites by altering culture conditions has been well documented, including different strains, temperatures, media, oxygen conditions, and co-culturing (Ahamed & Ahring, 2011; Bunge et al., 2008; Mallette et al., 2014; Roze et al., 2010).

At least four major pathway types are likely responsible for volatile organic carbon (VOC) production in the endophytic, filamentous fungus, *Ascocoryne sarcoides*. Some VOCs are closely related to central metabolism, such as fermentation products like ethanol and 2,3-butanediol or arabitol from the pentose phosphate pathway (Huang et al., 2007). A second mechanism is the modification of linoleic acid by lipoxygenases resulting in the formation of C8 alcohols and ketones, such as 1-octen-3-ol (Gianoulis et al., 2012; Wurzenberger & Grosch, 1984). A third route for hydrocarbon production is linked to fatty acid synthesis, producing products like terpenes and nonanoic acid (Quin et al., 2014; Shaw et al., 2015). Finally, the Ehrlich pathway, which allows the fungus to utilize amino acids as a nitrogen source, produces VOCs, such as 3-methylbutanol and 2-methyl propanoic acid (Hazelwood et al., 2008; Roze et al., 2010). Seven amino acids are reactants in the Ehrlich pathway by deamination, resulting in an α-keto acid, which is then decarboxylated into a fusel aldehyde. The aldehyde can then be oxidized to a fusel acid or reduced to a fusel alcohol (Hazelwood et al., 2008). Amino acid conversion to fuel alcohols and acids is performed predominately during exponential growth phase, and is therefore associated with primary metabolism (Griffin et al., 2010). Amino acid metabolism is linked to production of other secondary metabolites by *laeA* and the velvet proteins, making production of non-corresponding metabolites more likely (e.g. changing nitrogen source to an Ehrlich pathway associated amino acid will not only increase Ehrlich metabolites, but fatty acid pathway related products as well) (Roze et al., 2010).

The current study quantifies metabolite production by *A. sarcoides* in both the gas and liquid phases. VOCs were monitored throughout the growth phases of the model

VOC producing endophyte when grown on five nitrogen sources (asparagine, leucine, phenylalanine, methionine, and ammonium chloride) in a minimal glucose medium using proton transfer reaction-mass spectrometry for individual components as previously described by Schoen et al. (2016) with identifications confirmed using Head Space Solid Phase MicroExtraction Gas Chromatography-Mass Spectrometry. Liquid phase products were quantified at stationary phase using high performance liquid chromatography.

Materials and Methods

Microorganism

Ascocoryne sarcoides (NRRL 50072), the model VOC producing organism, was used (Gianoulis et al., 2012; Stinson et al., 2003; Strobel et al., 2008).

Growth Conditions

Inoculum cultures were generated by growing the fungus on potato dextrose agar until hyphae covered ≥60 % of the plate surface. A 20 % glycerol solution (10 mL) was added to each plate, scraped with a sterile glass rod, and the suspended fungal biomass solution was collected. The solution was mixed thoroughly and added to Microbank microbead vials and stored at -80 ˚C.

The composition of the inoculum medium was 30 g * L^{-1} glucose and 0.5 g * L^{-1} yeast extract. 250 mL of sterile filtered (0.22 µm) medium was added to 500 mL sterile baffled flasks. Two microbeads were added to each inoculum culture and grown for eight days at 140 rpm and 30 °C. All inoculum flasks were covered with sterile Kimguard

(Kimberly-Clark; Roswell, GA, USA) to allow gas exchange but prevent microbial contamination.

Growth medium had the following composition (g L^{-1}): 30 glucose, 0.05 yeast extract, 8.25 $NaH_2PO_4 \cdot 2H_2O$, 13.6 KH_2PO_4, 0.86 $MgSO_4 \cdot 7H_2O$, 0.28 $Ca(NO_3)_2 \cdot 4H_2O$, 0.006 $FeCl_3 \cdot 6H_2O$, 0.0075 $ZnSO_4 \cdot 7H_2O$, 0.18 KCl, 0.24 KNO_3, 0.0042 H_3BO_3, and 0.024 $MnCl_2 \cdot 4H_2O$. One of the following nitrogen sources was added: 34 mmol leucine, phenylalanine, methionine or ammonium chloride or 17 mmol asparagine. To avoid component precipitation, the medium was filter sterilized (0.2 µm), and the pH was adjusted to 4.5 using 1 N NaOH or 1 N HCl.

Fungal Reactor System

Air-tight, one liter baffled flasks were used as reactors. Reactors were shaken at 140 rpm at room temperature. Reactors, hydration flasks, and all tubing were autoclaved at 121 °C for 20 min before use. 250 mL of medium and 50 mg * L^{-1} of biomass, as determined by a modified Bradford protein assay, were added to each flask (Bradford, 1976). Biological triplicate experiments and an uninoculated control were run for each of the six nitrogen conditions (including a control condition where no nitrogen source was added to the medium).

Reactors were continuously sparged with 200 mL * min^{-1} of hydrated medical grade, clean compressed air in a setup similar to that previously described by Bunge et al., 2008. Reactor off-gas was then diluted with 400 mL * min^{-1} of medical grade air to

reduce condensation and pulled through the CO_2 detector and PTR-MS with the PTR-MS diaphragm pump as previously described (Schoen et al., 2016). The reactor off-gas flow path was alternated every three hours using an automated three-way valve to direct the gas either straight to the CO_2 detector to measure respiratory CO_2 only or through the VOC oxidizing platinum catalyst and then to the CO_2 detector, measuring CO_2 from both VOCs and fungal respiration. The PTR-MS sampled both gas steams that entered and bypassed the platinum catalyst once per day. Background CO_2 and VOCs measured for each tank of medical grade compressed air were subtracted from these values.

Biomass Dry Weight Analysis

Glass microfiber filters were dried in an oven at 80 ˚C for approximately 24 h. The filters were placed in a desiccator for one hour before being weighed. Fungal biomass from 10 mL culture samples was collected via vacuum filtration. The biomass containing filters were washed with approximately 20 mL of 30 ˚C deionized water, placed in an 80 ˚C oven for 24 h, transferred to a desiccator for one hour and then re-weighed to determine the mass of collected fungal culture.

High Performance Liquid Chromatography (HPLC) and Colormetric Assay

Supernatant samples were collected within 24 h of the culture reaching stationary phase. Samples were filtered (0.2 µm) to remove biomass and debris.

Glucose utilization and previously identified liquid phase metabolites were quantified in the liquid samples using an Agilent 1200 HPLC. Sample filtrate (20 µL)

was injected on an HPX-87H column (Bio-Rad) at 35°C, with a 0.005M H_2SO_4 mobile phase (0.6 mL min^{-1}). All concentrations were determined using a Refractive Index Detector and appropriate calibration curves.

A second HPLC method was used to quantify amino acid utilization as described by (Henderson et al., 2000). Briefly, the amino acids were derivatized with o-phthalaldehyde to create a fluorescent compound quantified by HPLC. Sample (50 µL) derivatized with o-phthalaldehyde buffered at pH 10.2 was injected into a Zorbax Eclipse-AAA column. Concentrations were determined using a diode array detector and appropriate calibration curves.

Ammonium chloride utilization was quantified colormetrically with the 2-phenylphenol method as described by (Rhine et al., 1998) with a PowerWave XS microplate reader (BioTek, Winooski, Vermont, USA) at an absorbance of 660 nm.

PTR-MS was used to provide compositional information about the VOCs in the reactor off-gas. PTR-MS uses H_3O^+ ions to protonate molecules (e.g. VOCs) with proton affinities greater than water (Lindinger et al., 1998). The singly charged ions are then typically detected as protonated molecules (ions with a mass-to-charge ratio, m/z, equal to the molecular weight plus 1 for the proton) by a quadrupole mass spectrometer (Lindinger et al., 1998). Carbon number was assumed for each mass to charge ratio based on HS-SPME GC-MS data, known fungal metabolites, and identification of endophytic fungal VOCs in previous studies (Luchner et al., 2012).

A multicomponent standard containing methanol, acetaldehyde, and alpha-pinene (Apel-Riemer Environmental Inc., Broomfield, CO, USA) was dynamically diluted to

determine PTR-MS sensitivity factors for those compounds. Ethanol calibration was performed separately using a permeation tube at a variety of humidities as described previously (Schoen et al., 2016). Sensitivity factors of 4.1, 17.4, 18.2, and 2.2 ncps (normalized counts per second) * $ppbv^{-1}$ for ethanol (m47, m65, m93), methanol (m33, m51), acetaldehyde (m45), and terpenes and terpenoids (m137) were used, respectively. The off-gas of the abiotic control and fungal cultures were both analyzed by PTR-MS, and VOCs measured in the abiotic control were subtracted from the fungal VOC production measurements. Concentrations of all other reported compounds were estimated using Equation 2:

$$[R] = \frac{I_{RH^+}}{\mathrm{ktz}\left(I_{\mathrm{m/z21}} * 500 + I_{\mathrm{m/z39}} * 250\right)} \quad (2)$$

where R is the concentration of the compound of interest, k is the reaction rate constant (here assumed to be $k = 2 \times 10^{-9}$ cm^3 $molecule^{-1}$ s^{-1}), t is the reaction time (Lindinger et al., 1998), and z is a mass dependent correction factor for transmission bias (Knighton et al., 2009; Steinbacher et al., 2004) determined for this PTR-MS. Ions representing two percent or greater of the total ion signal were quantified, with the exception of isotopes.

Head Space Solid Phase Microextraction Gas Chromatography Mass Spectrometry (HS-SPME GC-MS)

HS-SPME GC-MS was performed (Montana State University Mass Spectrometry Facility) as previously described (Griffin et al., 2010; Mallette et al., 2012) to assist in assignment of identities to PTR-MS ions. Briefly, a SPME fiber (divinylbenzene/carboxen on polydimethylsiloxane; Supelco; Bellefonte, PA, USA) was conditioned at 240 °C for 15 min before being exposed to the headspace of the liquid sample for 35 min. The exposed fiber was inserted into a modified GC injection port operated at 240 °C. The Zebron ZB-WAX (30 m × 0.25 mm ID × 0.50 μm) GC column (Phenomenex; Torrance, CA, USA) temperature was held at 30 °C for 2 min and then ramped to 230 °C at 7 °C min^{-1}. Compounds were identified via library comparison using the National Institute of Standards and Technology (NIST) database. All compounds reported had quality matches of at least 70; direct manual comparisons were made to spectra of known compounds when there were multiple high quality matches.

Results

Volatile Organic Compound Production

The endophytic fungus, *Ascocoryne sarcoides*, was grown on a minimal glucose medium with five different nitrogen sources and monitored for production of Ehrlich pathway products and other metabolites. Proton transfer reaction-mass spectrometry (PTR-MS) quantified individual volatile organic compounds (VOCs) in the reactor off-

gas (See Table 5-1 for tentative identifications of quantified ions). Total VOCs, carbon number four (C4) and higher VOCs (gasoline range), and carbon number eight (C8) and higher VOCs (diesel range) groupings were quantified (U.S. Department of Energy, 2013). Tentative identification of PTR-MS ions was assisted using HS-SPME GC-MS as shown in Supplemental Table 5-S1. As shown in Figure 5-1, ammonium chloride had the highest production of all groupings VOCs, with 3.2 times more total VOCs (0.15 +/- 0.015 carbon mols), 6.2 times more carbon number four and higher VOCs (7.2 +/- 0.87 carbon mmols), and 5.4 times more carbon number eight and higher VOCs (9.7 +/- 2.5 carbon µmol). Phenylalanine had the second highest total VOC production followed by leucine, methionine, and asparagine. Leucine and phenylalanine had the second highest C4 VOC production, followed by methionine and asparagine. Finally, leucine, asparagine, and phenylalanine produced similar amounts of C8 VOCs, with methionine producing the lowest production of C8 VOCs of the nitrogen sources.

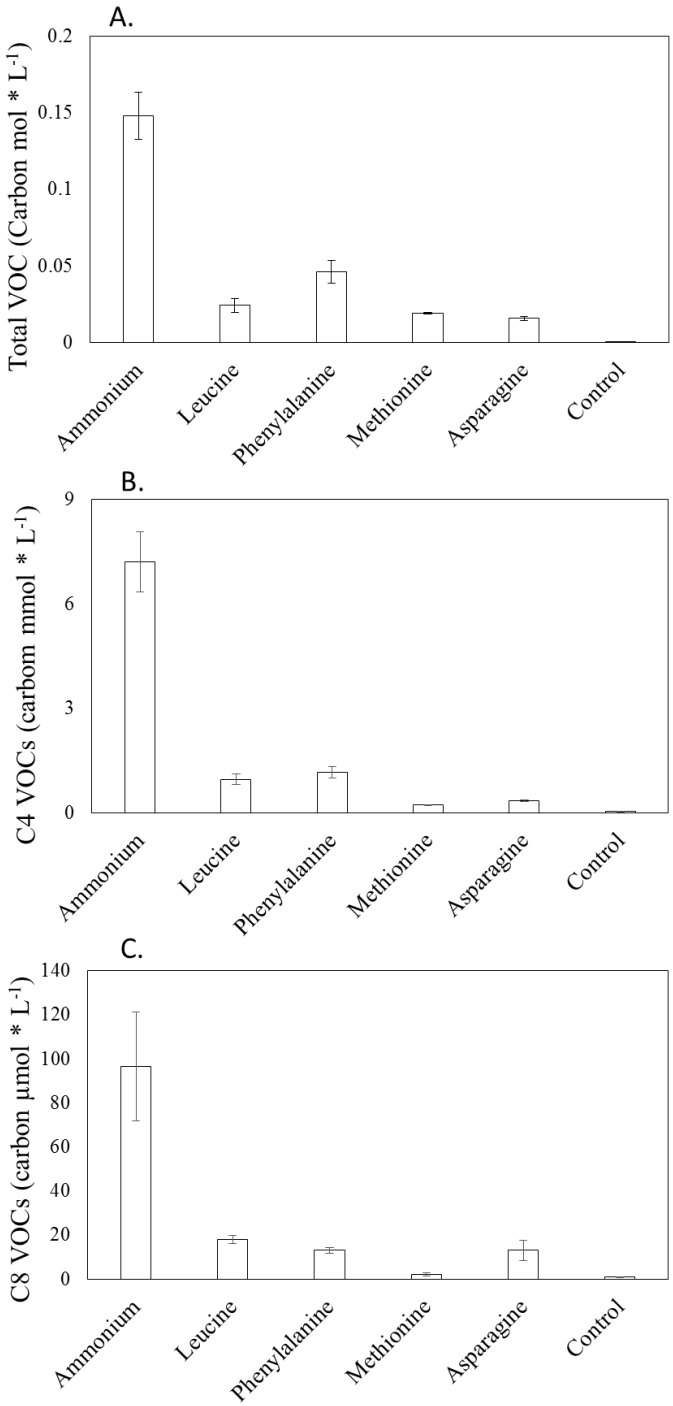


Figure 5-1. *Ascocoryne sarcoides* total VOC production per liter of culture as the average and standard error of three biological triplicates on each of the five nitrogen sources tested, as well as a control with no nitrogen source. Measurements were taken with PTR-MS.

Table 5-1. Ions quantified by PTR-MS. Ions representing two percent or greater of the total ion signal were quantified. The table provides tentative ion and compound identities based on HS-SPME GC-MS data, known fungal metabolites, and identification of endophytic fungal VOCs in previous studies. No compounds were clearly identified for m/z 95 and 135.

Ion	Tentative Ion Identification	Tentative Compound Identification	Formula	MW
33	CH_4OH^+	Methanol	CH_4O	32.04
41	$C_3H_5^+$	Propan-1-ol	C_3H_8O	60.10
43	$C_3H_7^+$	Propan-1-ol	C_3H_8O	60.10
45	$C_2H_4OH^+$	Acetaldehyde	C_2H_4O	44.05
47	$C_2H_6OH^+$	Ethanol	C_2H_6O	46.07
49	CH_4SH^+	Methanethiol	CH_4S	48.11
51	$CH_4OH^+(H_2O)$	Methanol	CH_4O	32.04
57	$C_4H_9^+$	2-Methylpropan-1-ol	$C_4H_{10}O$	74.12
59	$C_3H_6OH^+$	Acetone	C_3H_6O	58.08
61	$C_2H_5O_2^+$	Ethyl Acetate	$C_4H_8O_2$	88.11
63	$C_2H_6OH^+(H_2O)$	Acetaldehyde	C_2H_4O	44.05
		Dimethyl Disulfide	$C_2H_6S_2$	94.19
65	$C_2H_6OH^+(H_2O)$	Ethanol	C_2H_6O	46.07
69	$C_5H_9^+$	3-Methylbutanal	$C_5H_{10}O$	86.13
71	$C_5H_{11}^+$	3-Methylbutan-1-ol	$C_5H_{12}O$	88.15
73	$C_4H_8OH^+$	2-Methylpropanal	C_4H_8O	72.11
81	$C_6H_9^+$	Monoterpene	$C_{10}H_{18}O$	154.25
87	$C_5H_{10}OH^+$	3-Methylbutanal	$C_5H_{10}O$	86.13
	$C_5H_{10}OH^+$	2-Pentanone	$C_5H_{10}O$	86.13
89	$C_4H_8O_2H^+$	Ethyl Acetate	$C_4H_8O_2$	88.11
93	$H^+(C_2H_6O)_2$	Ethanol	C_2H_6O	46.07
95	$C_7H_{11}^+$			
105	$C_8H_8^+$	2-Phenylethan-1-ol	$C_8H_{10}O$	122.16
107	$C_7H_6OH^+$	Benzaldehyde	C_7H_6O	106.12
121	$C_8H_8OH^+$	Phenylacetaldehyde	C_8H_8O	120.15
135	$C_9H_{10}OH^+$		$C_9H_{10}O$	134.18
137	$C_{10}H_{17}^+$	Monoterpene	$C_{10}H_{18}O$	154.25

Volatile Organic Compound Profiles

Figure 5-2 shows that the ammonium chloride C4 VOC profile had a growth curve type shape that lags slightly behind the growth curve as shown in Supplemental Figure 5-S1. Ammonium chloride produced significantly more C4 VOCs than the other

nitrogen sources at most time points, with the exception of leucine early in the experiment. Early C4 VOCs from leucine were attributed to high initial Ehrlich pathway compound production, such as 3-methylbutanol and 3-methylbutanal, which were likely created as leucine was used as a nitrogen source in association with growth. Leucine produced the highest production rates of C4 VOCs initially, but production plateaued around the beginning of stationary phase. In contrast, phenylalanine C4 VOC production lagged significantly behind its growth curve (See Supplemental File 2 for all dry weight and PTR-MS data), and increased significantly during stationary phase. The methionine C4 VOC profile was similar in shape to the leucine profile, with lower production rates. The asparagine C4 VOC profile was similar in shape to phenylalanine, but again with lower production rates.

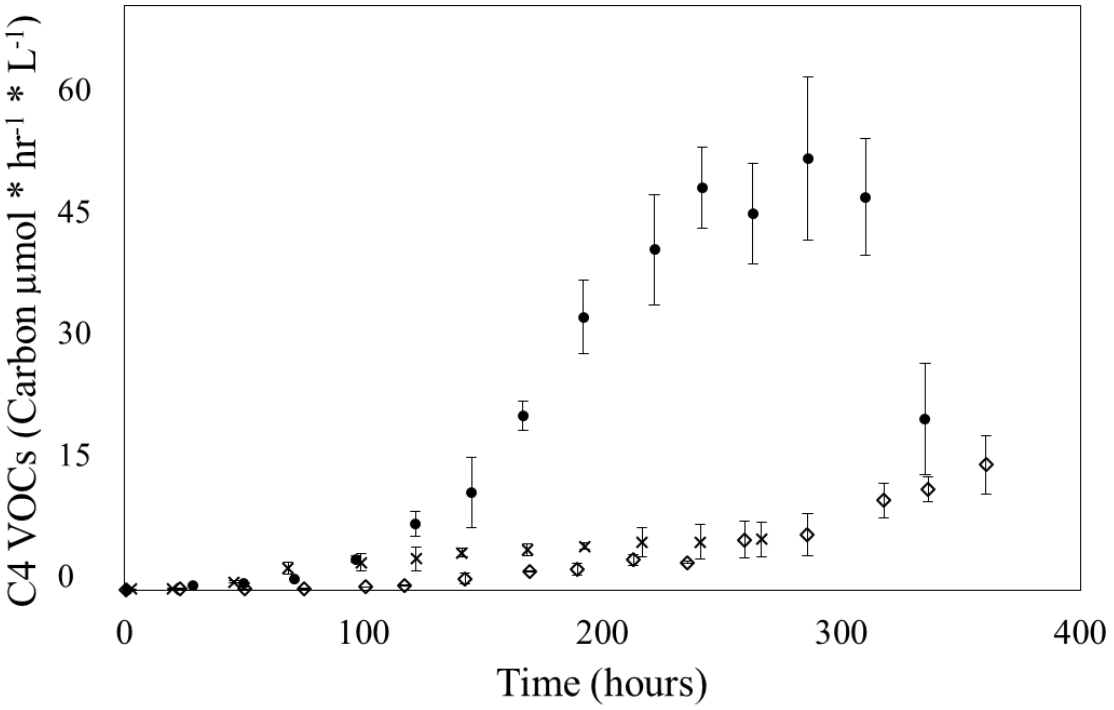


Figure 5-2. Carbon number four and higher VOC production rates (average and standard error of biological triplicates) as measured by PTR-MS from *Ascocoryne sarcoides* grown on ammonium chloride (closed circles), phenylalanine (open diamonds), and leucine (x) as nitrogen sources.

Figure 5-3 shows 3-methylbutanol, the leucine Ehrlich pathway alcohol product, had the highest gas-phase concentrations throughout time for the ammonium chloride experiments. Both the ammonium chloride and leucine profiles had growth curve like shapes, which for leucine was expected as leucine was used as a nitrogen source to support growth and its carbon backbone was degraded to VOCs. Many of the ammonium chloride profiles had similar shapes. With ammonium chloride as the nitrogen source, 3-methylbutanol is a secondary metabolite, as it is a bioactive compound not associated with growth. However the ammonium chloride 3-methylbutanol profile, as well as most of the other individual ammonium chloride VOC profiles, unexpectedly had a growth

curve like shape. The plots for phenylalanine and asparagine showed a significant increase in 3-methylbutanol in association with the start of stationary phase. These were the expected profile shapes for 3-methylbutanol as a secondary metabolite in these cultures.

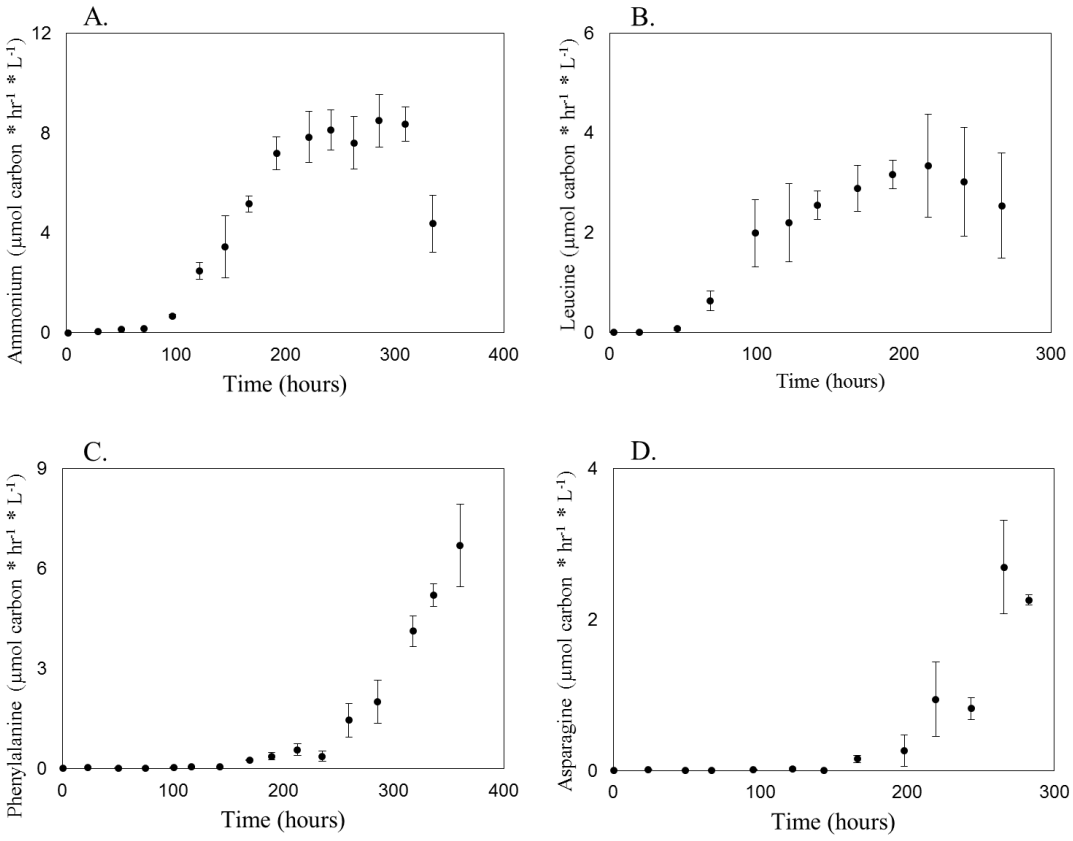


Figure 5-3. 3-methylbutanol production as measured by PTR-MS from *Ascocoryne sarcoides* cultures grown on ammonium chloride (panel a), leucine (panel b), phenylalanine (panel c), and asparagine (panel d).

Liquid Metabolites

Ehrlich pathway products and other secondary metabolites were also examined in the liquid phase using high performance liquid chromatography. Glucose and nitrogen consumption were also measured. Table 5-2 shows a substantial amount of arabitol, 9.15 +/- 1.39 mmol, was produced in the phenylalanine experiment with a yield of 0.081 g/g glucose. Arabitol is a sweetner and was listed as one of the 12 top value added chemicals from biomass based on its precursor value (Werpy & Petersen, 2004). This culture also produced benzyl alcohol, 0.713 +/- 0.60 mmol, and phenylethanol, 1.86 +/- 0.17 mmol, which are both biofuel and flavor compounds.

The expected aldehyde, alcohol, and acid products in the gas and liquid phases of the three amino acids that feed into the Ehrlich pathway, leucine, phenylalanine, and methionine, accounted for less than 50 % of the nitrogen consumed. Likely the amino acid backbones were being turned into other products within the cell.

Table 5-2. Compounds quantified using high performance liquid chromatography from *Ascocoryne sarcoides* grown in liquid culture on five different nitrogen sources. Each value is from the beginning of stationary phase, based on dry weight.

	Phenylalanine		Methionine		Ammonium Chloride		Asparagine		Leucine	
Compound	% used	std error	% used	std error	% used	std error	% used	std error	% used	std error
Glucose	57.2	3.9	16.1	2.3	50.5	1.1	45.0	1.2	39.3	3.2
Nitrogen	22.8	4.4	16.1	2.8	44.4	0.39	65.2	4.9	24.6	0.73
Compound	mmol	std error	mmol	std error	mmol	std error	mmol	std error	mmol	std error
Succinate	0.328	0.030	0.440	0.009	0.200	0.010	0.263	0.021	0.132	0.015
Lactate	0.447	0.020	0.131	0.010	0.787	0.016	0.291	0.041	1.36	0.029
Formate	0.678	0.020	0.128	0.021	0.825	0.033	Trace		0.968	0.193
Acetate	0.165	0.004	3.90	0.310	3.43	0.273	0.606	0.403	0.127	0.115
Arabitol	9.15	1.39	0.832	0.127	1.57	0.372	3.36	0.510	6.86	1.04
Ethanol	6.60	1.29	2.43	0.753	4.09	0.341	4.12	1.29	2.87	0.257
1-Propanol	0.685	0.081	Trace		0.354	0.015			0.174	0.007
Benzyl Alcohol	0.713	0.060								
Phenylethanol	1.86	0.174			0.124	0.011				
Methylthiopropanoic acid			0.107	0.005						
Methylthiopropanol			0.110	0.010						
3-Methylbutan-1-ol					Trace				0.181	0.051
3-Methylbuatnoic acid									Trace	

Table 5-2 shows that phenylalanine utilized more glucose than the other nitrogen sources, although ammonium chloride and asparagine had higher titers and asparagine had a significantly faster growth rate (See Supplemental Figures 5-S2 and 5-S3). Production of the major quantified phenylalanine product, arabitol , is associated with high osmotic pressure (Kelly & Budd, 1991). Alternatively, Table 5-2 also shows asparagine had the highest percentage of nitrogen used. The asparagine carbon backbone can be assimilated into the TCA cycle (Boer et al., 2007), and asparagine had the highest growth rates and biomass titers. However, no increase in fatty acid related or any other VOCs or liquid secondary metabolites was seen in asparagine cultures compared to the ammonium chloride or phenylalanine experiments, although VOC profiles were similar in shape to the cultures grown with phenylalanine. Finally, *A. sarcoides* showed limited growth and VOC production on methionine, likely because the sulfur compounds produced were inhibitory. Two additional peaks were observed by HPLC. A peak with a large area was observed at 21 minutes in the leucine experiment samples, and smaller peaks were seen at the same retention time in all of the samples from experiments with a nitrogen source. Similarly, a large peak was observed at 38 minutes in the phenylalanine samples, with smaller peaks at the same retention time in the other samples from experiments with nitrogen sources. Efforts that did not yield the identities of these peaks are described in the supplemental section.

Discussion

The endophytic fungus, *A. sarcoides*, produced the highest concentrations of volatile organic compounds when grown on ammonium chloride, even though multiple other nitrogen sources tested were reactants for VOCs. Here production of secondary metabolites started earlier and had higher specific production rates with ammonium chloride as the nitrogen source. Secondary metabolism, as well as parts of amino acid metabolism, are controlled in many filamentous fungi by the global regulator, *laeA*, and the related velvet proteins, which is known to modulate production of secondary metabolites based on environmental conditions (Bayram et al., 2008; Bok & Keller, 2004). Activation of these regulating complexes likely resulted in higher VOC production than increased reactants in the experiments performed here. The VOC suites produced by the endophyte *A. sarcoides* are bioactive and may act directly to inhibit other organisms or signal the plant host to raise its own defenses (Porras-Alfaro & Bayman, 2011; Saikkonen et al., 1998; Strobel et al., 2008). Endophytic fungi isolated from the branches of trees, such as *A. sarcoides*, likely encounter ammonium within the plant host when it is fixed by endophytic bacteria (Doty et al., 2016). *A. sarcoides* exposure to ammonium may be the standard environment within the tree, or high levels of ammonium locally fixed by endophytic bacteria may activate production of VOCs by the fungus to protect its niche.

The fungus *A. sarcoides* is a promising source of metabolites to serve as biofuel, flavor, and industrial precursor compounds. Optimization of process conditions may

increase production of valuable metabolites like arabitol, benzyl alcohol, and phenylethanol, while utilizing smaller concentrations of amino acids. Alternatively, metabolic engineering may increase yields. Previously, metabolic engineering has been used to increase intracellular amino acid biosynthesis and subsequent products (Connor et al., 2010). *A. sarcoides* is also an excellent candidate for rational metabolic engineering as it has a well annotated genome and metabolic model (Hunt 2016; Gianoulis et al., 2012).

Acknowledgements

This research was supported in part by the National Science Foundation (NSF) EFRI Program under Grant No. 0937613. Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NSF. Thank you to the Montana State University Center for Biofilm Engineering, the Chemistry and Biochemistry Department, and the Chemical and Biological Engineering Department for support. The authors would also like to thank Drs. Ross Carlson and Kris Hunt for sound technical advice.

Supplemental Material

Two large peaks of unknown identity were observed in the HPLC chromatograms. Based on refractive index detector (RID) peak area, the large leucine peak (retention time 21 minutes) concentration was estimated as roughly 3 g/L (referred to as mystery peak A). Mystery peak B had a large area in the phenylalanine chromatogram of approximately 1 g/L (retention time 38 minutes). No corresponding significant increases were seen in the total VOC data or any ions in the PTR-MS, leading to the conclusion that these unknowns are likely not volatile and may be acids. Head space solid phase microextraction gas chromatography-mass spectrometry (SPME GC-MS) also did not identify any large peaks matching the identity of the unknown ions. The identity of the peaks did not match any expected acid, alcohol, or aldehyde Ehrlich pathway products based on measuring the retention times of standards on HPLC.

Further efforts to identify the peaks included liquid phase direct injection on the GC-MS under similar conditions as described above for both the leucine and phenylalanine experiment samples. Only ethanol was identified in these runs. Next, liquid phase SPME GC-MS of the leucine and phenylalanine samples was performed. No peaks matching the identity of the mystery compounds were identified. Additionally, a derivatization method for identifying acids and amides was performed on the leucine and phenylalanine samples. Amino acids such as isoleucine and valine were substantially more abundant that any metabolites, and no clear potential matches were identified for mystery peaks A and B.

The mystery peak fractions were then collected from HPLC. The timing of the fraction collections was determined by the difference in retention times between the ultraviolet light (UV) detector and the RID, which are in series. The fraction samples were collected immediately following the UV detector at the inlet line to the RID (although sample was collected for 30 seconds before and after expected elution). Liquid phase SPME GC-MS was performed on these fractionated samples. Then liquid phase SPME GC-MS using a polar fiber (85 µm polyacrylate), as opposed to the nonpolar fiber previously utilized, was performed on the fractionated samples. In the mystery peak A fraction, the polar fiber identified a very large peak with a NIST database quality match of 86 for 2,4-diisocyanato-1-methyl-benzene. However, this compound is not readily soluble in water and is not mystery peak A.

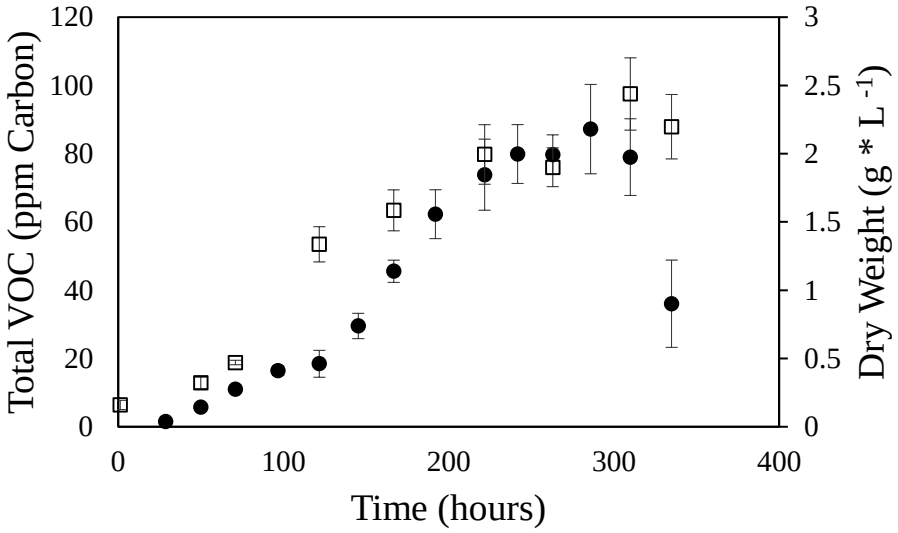
Additionally, 1H 13C NMR was performed at Pacific Northwest National Lab on both the full phenylalanine and leucine samples and the HPLC fractions containing mystery peaks A and B. The full sample NMR identified arabitol as a significant product, but this testing has of yet been unsuccessful in identifying mystery peaks A and B.

Supplemental Table 5-S1. Identification of VOCs produced by the endophytic fungus *Ascocoryne sarcoides* by head space solid phase microextraction gas chromatography mass-spectrometry. Retention time, compound identification, and quality match are shown for each of the five nitrogen sources tested (asparagine [1], ammonium chloride [2], methionine [3], phenylalanine [4], and leucine [5]) and the control [6] with no nitrogen source.

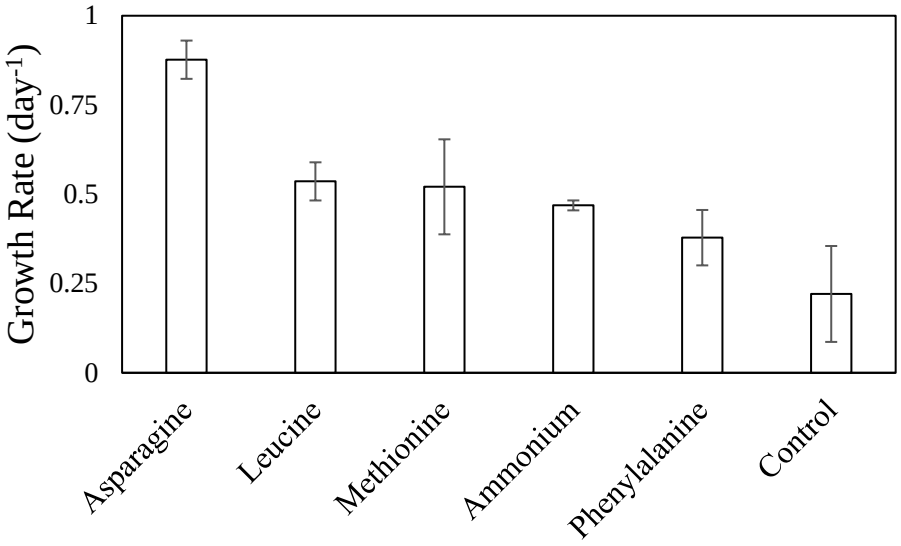
RT	Compound	1	2	3	4	5	6
4.8	Methanethiol			91.6			
4.9	Propane, 2-ethoxy-2-methyl-		82.9	83.0	85.6	83.6	76.3
5.0	Acetaldehyde		72.9			70.3	
6.3	Propanal, 2-methyl		78.6				
6.4	Acetone	83.3		84.0	88.2		87.5
7.0	Furan, tetrahydro-2,2,5,5-tetramethyl-		72.4			90.0	
7.6	Ethyl Acetate	95.3	96.2	85.3	96.5	92.2	
7.9	2-Butanone				75.4		
8.2	Butanal, 3-methyl		73.2		81.1	82.7	
8.4	Isopropyl Alcohol						79.2
8.5	Ethanol	96.3	95.5	97.0	95.5	93.5	
9.6	1,3-Dioxolane, 2,4,5-trimethyl-		84.8				
9.6	2-Pentanone			79.3	74.7		73.6
10.3	Acetic acid, 2-methylpropyl ester		86.5				
10.8	1-Propanol			90.5		84.3	
11.8	3-Hexanone, 4-methyl-			71.6	71.6		
11.9	Disulfide, dimethyl			98.5			
11.9	1-Propanol, 2-methyl	92.2	94.3	88.4	88.5	93.5	
13.4	2H-Pyran, 2-ethoxytetrahydro-	73.1	90.2		79.0		
14.6	1-Butanol, 3-methyl	71.2	72.3			77.2	
14.8	2-Heptanone, 4-methyl						70.3
15.6	2-Heptanone, 4,6-dimethyl-		75.4		87.6		
16.2	Propanoic acid, 2-oxo-, ethyl ester	73.3	82.8		78.8		
16.7	2-Butanone, 3-hydroxy	72.1	77.2		77.2	76.0	
17.7	5-Hepten-2-one, 6-methyl-		76.3	80.7		76.6	
18.8	Nonanal				71.7		
20.0	2-Butoxyethyl acetate	72.1		73.4			
20.1	Acetic acid	95.1		96.9	90.2	98.1	
21.7	Pentanoic acid, 2-hydroxy-4-methyl-, ethyl ester					80.1	
21.8	Benzaldehyde	74.7		74.4	70.3	75.5	
22.2	Propanoic acid, 2-methyl-	89.7		74.4		91.0	
23.8	Benzenaceldehyde				73.6		
23.9	Butanoic acid, 3-methyl-					93.0	
24.5	Formic acid, phenylmethyl ester				79.0		

Table 5-S1 Continued

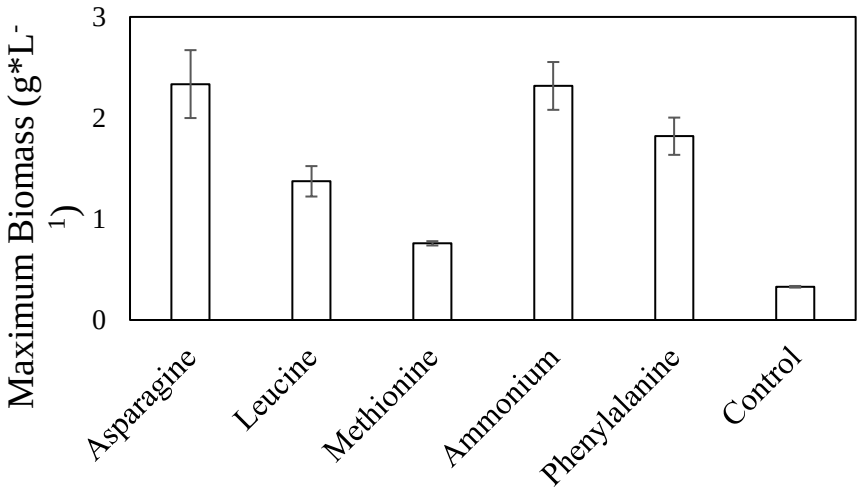
25.4	1-Propanol, 3-(methylthio)-			85.7			
27.2	Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester	78.4		74.8	73.4		
27.7	Benzyl Alcohol				73.4		
27.8	2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	71.6					
29.1	Phenylethyl Alcohol	77.9		70.2	80.0	73.0	
31.5	Nonanoic acid					78.1	



Supplemental Figure 5-S1. Total VOC production (closed circles) as measured by the platinum catalyst and CO_2 detector and cell dry weight (open squares) with ammonium chloride as the nitrogen source as a representative experiment. Data are the average of three biological replicates and standard error.



Supplemental Figure 5-S2. *Ascocoryne sarcoides* specific growth rate and standard error of three biological replicates grown on five different nitrogen sources and a control with no nitrogen source.



Supplemental Figure 5-S3. *Ascocoryne sarcoides* maximum biomass by dry weight and standard error of three biological replicates grown on five different nitrogen sources and a control with no nitrogen source.

CHAPTER SIX

CONCLUSIONS & FUTURE WORK

Research Summary

This dissertation identifies and quantifies biofuel and flavor relevant compounds produced by endophytic fungi under different culture conditions, as well as provides a new analytical method to quantify total volatile organic carbon. Titers, growth rates, and volatile organic products of three *Nodulisporium* isolates from tropical regions were examined, in hopes of identifying more industrially relevant properties than temperate forest fungus, *Ascocoryne sarcoides*. Based on the production of multiple high value products, isolate TI-13 was further explored in solid state for production of volatile organic compounds (VOC) at varying oxygen conditions. A new analytical method for measuring total VOC in the gas phase was created to quantify the complex mixture of headspace VOCs produced by the fungus. Finally, the research returned to quantifying *A. sarcoides* VOC and liquid secondary metabolite production with varying nitrogen sources, since this fungus has an annotated genome as well as a metabolic model to help understand the changing concentrations of potential flavor and biofuel products. Overall this work was devoted to understanding the activation of secondary metabolite production under different culture conditions.

i. Identify Titers, Growth Rates, and VOC Products of Three *Nodulisporium* Isolates at Varying pH and Temperature Conditions

Titer, growth rate, and VOC production were examined at the pH conditions 4, 5, 6, and 7 for each of the three isolates. All strains had the highest titers and fastest growth rates at pH 6. Additionally, temperatures of 27, 30, 33 and 37 °C at pH 6 were investigated. Titers were highest for EC and TI at 33 °C at 15 +/- 0.43 and 13 +/- 0.58 g/L respectively. Titer was highest for CO at 30 °C at 19 +/- 1.1 g/L. These titers are significantly higher than that of 5 g/L observed at optimal temperature and pH for *A. sarcoides* (Mallette, 2013). In previous studies, *A. sarcoides* had a maximum growth rate at room temperature of 2.1 +/- 0.84 day $^{-1}$ (Mallette, 2013) but lower maximum specific growth rates were observed at 33 °C for CO and TI (1.4 +/- 0.025 and 1.6 +/- 0.084 day $^{-1}$ respectively) and 30 °C for EC (1.2 +/- 0.12 day $^{-1}$).

VOCs with longer carbon chain lengths were identified at 27 °C, which is favorable because longer chain length VOC are more energy dense biofuels that can replace a larger portion of gasoline. Smaller carbon chain length products, including fermentation products, were identified at 37 °C. VOCs related to fatty acid metabolism especially had a strong trend of increased carbon chain length with decreased temperature for all three strains. Overall, fewer products were identified at lower pH.

The identification of VOCs of increasing carbon length with decreasing temperature has not been seen before. One explanation is that the increase in chain length observed here may be due to temperature dependent kinetics of catabolic and anabolic fatty acid reactions (Kates & Baxter, 1962). Differences in cell membrane properties as a function of temperature, including transporter expression, could more readily allow

excretion of longer chain metabolites from the cells (Kawahara et al. 2016; Liu et al. 2011). Alternatively, many of the VOCs detected are bioactive and their production may have evolved to support endophyte ecological functions. The temperature dependence of VOC carbon chain length in this study may be controlled by global regulators, which modulate secondary metabolism in filamentous fungi (Bok & Keller, 2004).

ii. Quantification of VOCs from *Nodulisporium* Isolate TI-13 Grown on Solid State

Quantification of the complex mixtures of VOCs produced by fungi can be difficult (Mallette, 2013; Morath et al. 2012), so an additional method to accurately quantify total VOC production was developed. A platinum catalyst that completely and quantitatively oxidized VOCs to CO_2 was used in conjunction with a sensitive CO_2 detector. By alternating the reactor off-gas through and around the catalyst, a near continuous record of both total VOC production and respiratory CO_2 was produced from a non-homogeneous solid state reactor system. The accurate total VOC measurement can be used for real-time process monitoring, combined with the respiratory CO_2 measurement for carbon balances, or compared to other less accurate quantification systems to help identify discrepancies in the system.

In the model system solid state system, PTR-MS was utilized to quantify the individual VOCs in the reactor off-gas. The carbon in these individual compounds was summed so that the PTR-MS measurements could be compared to the accurate total VOC quantification found with the platinum catalyst. Here the PTR-MS carbon accounted for 94% of the platinum catalyst measurement, which is considered excellent agreement. The

verification performed in this study and others (Baasandorj et al. 2015; Veres et al. 2010) shows the platinum catalyst system is accurate to within +/- 5%, which is more accurate than other available VOC measurement methods like GC-MS and PTR-MS. Issues like compounds that are not readily quantified by PTR-MS (e.g. alkanes) and ions composed of multiple compounds with different sensitivity factors can lead to inaccurate total VOC quantification. The robust total VOC measurement provided by the platinum catalyst and CO_2 detector can help identify these issues.

iii. Evaluate VOC Production by *Nodulisporium*
Isolate TI-13 under Varying Oxygen Conditions

In this study, oxygen conditions were modulated, since they have been shown to produce variations in the amounts and types of secondary metabolites produced by other fungi (Mallette, 2013). Additionally, fungi may produce more secondary metabolites in solid state than in liquid culture (Krishna, 2005), so TI-13 was grown in a solid state reactor that was continuously spared was 0, 1, 13, or 21 % oxygen. The highest total VOC production was at 0 % (2.5 +/- 0.031 g), with decreasing production with increasing oxygen conditions. Additionally, the highest total production of volatile compounds with carbon chain lengths of C4 and longer was at 0 % oxygen (40 +/- 13 mg). The highest specific production rates of these C4 and longer products were obtained at 1% oxygen, of 47 +/- 0.22 µg/(g biomass*hr). The increased yields found in this first quantitative study of C4 bioactive compounds under microaerophilic conditions may be due to ecological reasons; the endophytic isolate TI-13 used in this study likely exists under microaerophilic conditions within the tree host (Strobel et al. 2008). Production of

bioactive VOCs under microaerophilic conditions may aid the plant host in defending against microbial and insect pathogens.

For most individual compounds, including ethanol and 3-methyl 1-butanol, production increased from aerobic to anoxic conditions. However, for two compounds, ethyl acetate and an ester at m/z 103, production increased dramatically under microaerophilic conditions. Genetic engineering may allow increased production of these proteins under desired conditions.

iv. Measure VOC Production by
A. sarcoides under Varying Nitrogen Conditions

Growth rate, titer, gas phase VOC production, and liquid secondary metabolites were measured with varying nitrogen source in a minimal medium. Ammonium chloride was investigated as a base condition as well as four amino acids. Three of the amino acids (methionine, leucine, and phenylalanine) feed into the Ehrlich pathway, which allows fungi to utilize amino acids as nitrogen sources and produces flavor- and biofuel-relevant compounds as byproducts (Hazelwood et al. 2008; Roze et al. 2011). Asparagine, which cannot be used in the Ehrlich pathways, was tested as an additional control.

Ammonium chloride had the highest total (0.0066 +/- 0.00068 g/L*hr) and C4 and longer (3.8E-04 +/- 4.4E-5 g/L*hr) VOC yields of the nitrogen sources examined. This was unexpected, as the Ehrlich pathway amino acids produced VOCs as a direct result of nitrogen utilization, and ammonium chloride utilization pathways do not directly result in any VOCs. In the cultures with ammonium chloride as the nitrogen source, products

included ethanol, acetaldehyde, ethyl acetate, phenylethyl alcohol, and 3-methyl 1-butanol. Ammonium chloride may induce transcription of global regulators (Bok & Keller, 2004) which can impact production of fungal secondary metabolites. Endophytic bacteria produce ammonia (Gayathri et al. 2010), and excess ammonium may induce production of bioactive secondary metabolites to inhibit bacterial growth.

In this research, temperature, pH, oxygen, and nitrogen source modulate the types and amounts of VOCs produced by the endophytic fungi studied. Much of the increased detection of C4 and longer VOCs would not be predicted based on culture conditions. Instead, the culture conditions likely activated individual promotors and global regulators (Bok & Keller, 2004; Verstrepen et al. 2003a) that may have evolved for ecological reasons to increase production of VOCs. Despite increases seen across many conditions, yields of high value VOCs were still much lower than would be industrially relevant. However, genetic modification has often been required for filamentous fungi to produce industrially relevant yields of products (Punt et al. 2002). Experiments similar to those conducted with PTR-MS here could be combined with transcriptomics and metabolic modeling (Lee et al. 2005; Stephanopoulos et al. 1998) to efficiently genetically engineer a fungal strain that produces high yields of valuable C4 and longer VOCs. These and other future work ideas are discussed below.

Future work

i. Increased Production and Applicability of Fungal VOCs and Secondary Metabolites

While production of valuable VOC products were increased by a factor of 4.7 in the studies presented here, production needs to be increased by 50 fold to be of interest to industry. The enzymes that produce some high value VOCs, such as octane, have not yet been identified (Gianoulis et al. 2012). Additionally, some reactions steps may be catalyzed by multiple enzymes, not all of which have been identified, such as those resulting in 3-methyl 1-butanol acetate (Verstrepen et al. 2003b). Transcriptomics, combined with PTR-MS measurements and modulating process conditions, could help identify these enzymes. A knowledge of these VOC producing genes, in coordination with metabolic modeling (Lee et al. 2005; Stephanopoulos et al. 1998), could allow genetic engineering (Nødvig et al. 2015), of a strain like TI-13 to overexpress specific products. Alternatively, these pathways could be placed in a species, such as *Saccharomyces cerevisiae*, which is already used widely in industry.

In coordination with genetically engineered increases in production of VOCs, mycofumication, the fungal VOC inhibition of other organisms, should be considered. Fungal VOCs work synergistically to inhibit microorganism (Alpha et al. 2015; Strobel et al. 2001), so over expression of just one, or a random mixture, may not have the same effect as the original mixture of compounds. To eliminate this potential dilemma, the mode of action of synergistic VOC mixtures should be further explored. Similar to the experiment performed in *E. coli* (Campos et al. 2015), other bacteria, yeast, and

filamentous fungi could be exposed to natural and artificial mixtures of fungal VOCs to monitor which VOCs inhibit or kill varying organisms. These bioactive VOCs appear to impact the cell membrane and damage DNA (Campos et al. 2015). Understanding which compounds, or mixtures of compounds, are most bioactive against the intended target can help rationally design genetic modifications to fungi so desired VOC mixtures can be produced at higher concentrations. Different mixtures, fungal or manmade, could be designed to be more active against specific targets, while keeping toxicity towards humans low.

Finally, the mechanism of VOC resistance in VOC producing fungi should be studied. Increasing production of bioactive VOCs may inhibit the organism producing them, as artificially created mixtures have been shown to do previously (Strobel et al. 2001). While increasing aeration rates of such processes may help reduce self-inhibition (Freeman et al. 1993), increased production of compounds which have limited ability to impact the producing fungus should be chosen. To further investigate this, VOC concentrations within the cell, versus outside, should be explored to identify compounds which may cause intracellular toxicity. Compounds which readily permeate the cell or are actively transported may be preferentially overexpressed, and active transporters could be overexpressed as well.

In another vein, bioactive fungal VOCs (Ezra et al. 2004a; Mends et al. 2012; Strobel et al. 2001) could be screened for activity against biofilms. Fungal VOCs may inhibit formation of biofilms. Additionally, fungal VOCs may penetrate biofilm EPS and inhibit or kill formed biofilms. However, if a combination of bioactive fungal VOCs that

inhibits biofilm formation and has low toxicity to humans, it could be used in coordination with debridement to treat chronic biofilm wounds in humans, or in industry to prevent fouling, or in other applications.

ii. Applications of Monitoring VOCs

Monitoring VOCs could also be applied to other microbial systems. Initial production of VOCs, such as acetaldehyde by *A. sarcoides* and TI-13, could be identified for multiple species as a marker of metabolic activity and likely impending growth before changes in optical density (Bunge et al. 2008) or respiratory CO_2 can be detected. Monitoring these early growth marker VOCs in species across the tree of life could provide fundamental information about lag phase or low cell number metabolic activities. Initial and ongoing VOC monitoring, using a platinum catalyst and sensitive CO_2 detector system, could be used in high value fermentations, such as production of active pharmaceutical ingredients, or long research studies, where quick recognition of a failed run could save substantial money or time. For some processes, PTR-MS may be required to identify specific volatile compounds produced to provide more meaning monitoring.

Monitoring off-gas volatiles with the PTR-MS may also be employed to understand biofilms, which are inherently heterogenous. Volatile metabolites could be identified using SPME GC-MS. Quantifying off-gas VOCs during biofilm formation, growth, and stationary phase could provide additional information about their physiology. Volatiles could also be monitored during the addition of anti-biofilm agents to better understand their activity and biofilm resistance. VOC monitoring may also help identify volatile quorum sensing metabolites, such as farnesol or 1-octen-3-ol in fungi (Cottier &

Mühlschlegel, 2011; Hornby et al. 2001), of pure and mixed biofilm cultures. Finally, the PTR-MS could be used to monitor VOCs released from chronic wounds to identify biomarkers. The VOC data could help identify specific metabolisms being used by organisms in the wound, as well as their developmental state. Monitoring VOCs before and during chronic wound treatments is a non-invasive method that could lead to more tailored approaches to care. The VOC profiles could be linked to the most effective wound care strategy, hopefully leading to faster and more complete chronic wound healing. In an initial study comparing *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms using PTR-MS, *S. aureus* biofilms had substantially more of the tentatively identified compounds acetaldehyde (m/z 45), isoprene (m/z 69), and 2-pentanone (m/z 87). *P. aeruginosa* biofilms had 6 times more methanethiol (m/z 49) and 7 times more dimethyl sulfide (m/z 63).

iii. Consolidated Processing Evaluations

Future work could involve further evaluation of isolate TI-13 as a consolidated bioprocessing organism, one that can directly turn cellulosic feedstocks into biofuel and flavor compounds. In addition to small amounts of high value longer chain flavor and biofuel compounds, this isolate produces significant amounts of ethanol. To further evaluate this organism, yields of cellulases could be identified, as well as oxygen requirement for cellulase production. Cellulase expression is downregulated under anoxic conditions in the model organism *Trichoderma reesei* (Rautio et al. 2006), but because of the natural microaerophilic environment, TI-13 may produce cellulases at lower oxygen concentrations. Additionally, carbon balances could be performed in liquid phase to

directly identify ethanol yields. While ethanol production alone may not be economically feasible, it value as a byproduct may reduce the yields necessary of C4 and longer fuel and flavor compounds necessary to create a profitable process.

To evaluate the feasibility of such a system, a large packed bed reactor, similar to the one described by (Li et al. 2013) could be modeled with TI-13 producing ethanol and higher value products. Such a reactor could be packed with an agricultural byproduct widely and cheaply available in the U.S., such as beet pulp, yard waste, or corn stover. In such a reactor, air would be continuously sparged from the entrance along with the addition of TI-13 inoculum and the carbon source. Down the reactor, oxygen would be depleted and the fungus would have a reduced growth rate but substantially higher yields of ethanol and longer chain VOCs. A high gas flow rate would remove the inhibitory volatiles from the fungi and hopefully increase their production. A slow rotation rate of around 2/day would allow the fungus access to more substrate but have limited impact on mycelia. A better understanding of substrate utilization, ethanol yields, and cellulase production by TI-13 could allow a prediction of the C4 and longer compound yields required for an industrially viable process.

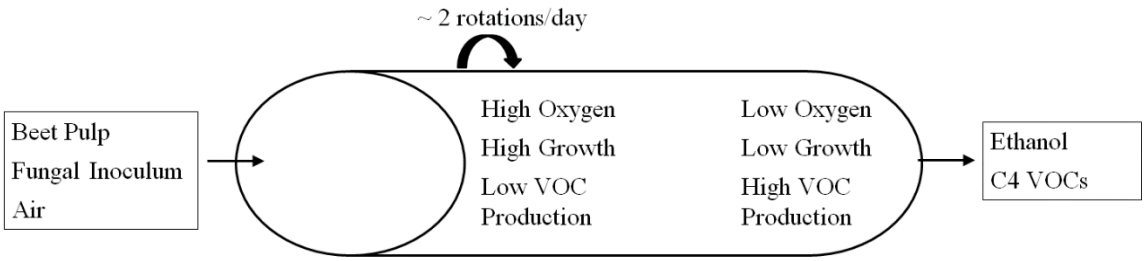


Figure 6-1. A conceptual packed bed reactor the conversion of agricultural byproduct, here beet pulp, to VOCs including ethanol and smaller amounts of longer chain hydrocarbons by a cellulosic fungus.

Similar evaluations could be performed on novel thermophilic fungal isolates from Yellowstone. These organisms grow quickly at 50 °C on cellulose and produce potential flavor and biofuel VOCs. The higher production temperatures could help reduce the energy required to cool a large solid state reactor. Additionally, the higher temperatures would increase volatility of longer carbon number diesel fuel relevant compounds, such as sesquiterpenes.

iv. Excretion of biofuels and other VOC products

Examining VOC products in the medium of fungal cultures at varying temperatures identified longer carbon chain length products at lower temperatures. It is unknown if this finding represents production of these compounds or their excretion from the cell. Cells could be lysed under different conditions to identify the types and amounts of volatile compounds within the cells versus outside the cells. Transporters in the cell walls could be examined to see if different types or amounts of transporters are expressed under

different conditions. Similarly, the types of lipids in the plasma membrane may change under different culture conditions, changing the ability of VOCs to readily pass. This information could be helpful in genetically engineering a VOC excreting consolidated bioprocessing organism.

Similarly, an area of study with algae is the excretion of lipids from the cell. A major cost of biofuel production with algae is harvesting and separating the lipids (Kawahara et al. 2016a; Liu et al. 2011). Excretion eliminates these steps and could dramatically reduce the cost of algae biofuels. The data presented in this dissertation suggest that changes in temperature, and possibly other process conditions, may have dramatic impact on excretion of fatty acid related compounds. A combination of genetic engineering and modified process conditions could significantly increase yields of extracellular lipid products.

REFERENCES

Abrahão, M.R.E., Molina, G., Pastore, G.M. 2013. Endophytes: Recent developments in biotechnology and the potential for flavor production. *Food Research International*, **52**(1), 367-372.

Ahamed, A., Ahring, B.K. 2011. Production of hydrocarbon compounds by endophytic fungi *Gliocladium* species grown on cellulose. *Bioresource Technology*, **102**(20), 9718-9722.

Ali, H.K.Q., Zulkali, M. 2011. Design aspects of bioreactors for solid-state fermentation: a review. *Chemical and Biochemical Engineering Quarterly*, **25**(2), 255-266.

Alpha, C.J., Campos, M., Jacobs-Wagner, C., Strobel, S.A. 2015. Mycofumigation by the volatile organic compound-producing fungus *Muscodor albus* induces bacterial cell death through DNA damage. *Applied and Environmental Microbiology*, **81**(3), 1147-1156.

Ammann, C., Spirig, C., Neftel, A., Steinbacher, M., Komenda, M., Schaub, A. 2004. Application of PTR-MS for measurements of biogenic VOC in a deciduous forest. *International Journal of Mass Spectrometry*, **239**(2-3), 87-101.

Atsumi, S., Hanai, T., Liao, J.C. 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature*, **451**(7174), 86-89.

Baasandorj, M., Millet, D.B., Hu, L., Mitroo, D., Williams, B.J. 2015. Measuring acetic and formic acid by proton-transfer-reaction mass spectrometry: sensitivity, humidity dependence, and quantifying interferences. *Atmospheric Measurement. Techniques*, **8**(3), 1303-1321.

Barker, B.M., Kroll, K., Vödisch, M., Mazurie, A., Kniemeyer, O., Cramer, R.A., 2012. Transcriptomic and proteomic analyses of the *Aspergillus fumigatus* hypoxia response using an oxygen-controlled fermenter. *BMC Genomics*, **13**(1), 1-25.

Bayram, Ö., Krappmann, S., Ni, M., Bok, J.W., Helmstaedt, K., Valerius, O., Braus-Stromeyer, S., Kwon, N.-J., Keller, N.P., Yu, J.-H. 2008. VelB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science*, **320**(5882), 1504-1506.

Ben Akacha, N., Gargouri, M. 2015. Microbial and enzymatic technologies used for the production of natural aroma compounds: Synthesis, recovery modeling, and bioprocesses. *Food and Bioproducts Processing*, **94**, 675-706.

Berger, R. 2009. Biotechnology of flavours—the next generation. *Biotechnology Letters*, **31**(11), 1651-1659.

Biasioli, F., Gasperi, F., Yeretzian, C., Märk, T.D. 2011. PTR-MS monitoring of VOCs and BVOCs in food science and technology. *TrAC Trends in Analytical Chemistry*, **30**(7), 968-977.

Boer, V.M., Tai, S.L., Vuralhan, Z., Arifin, Y., Walsh, M.C., Piper, M.D., de Winde, J.H., Pronk, J.T., Daran, J.M. 2007. Transcriptional responses of *Saccharomyces cerevisiae* to preferred and nonpreferred nitrogen sources in glucose-limited chemostat cultures. *FEMS Yeast Research*, **7**(4), 604-20.

Bok, J.W., Keller, N.P. 2004. LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryotic Cell*, **3**(2), 527-35.

BP. 2015. Statistical Review of World Energy June 2015. Retrieved from: http://biomasspower.gov.in/document/Reports/BP%20statistical%20review-2015.pdf

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**(1–2), 248-254.

Brakhage, A.A. 2013. Regulation of fungal secondary metabolism. *Nature Reviews Microbiology*, **11**(1), 21-32.

Bunge, M., Araghipour, N., Mikoviny, T., Dunkl, J., Schnitzhofer, R., Hansel, A., Schinner, F., Wisthaler, A., Margesin, R., Märk, T.D. 2008. On-Line Monitoring of Microbial Volatile Metabolites by Proton Transfer Reaction-Mass Spectrometry. *Applied and Environmental Microbiology*, **74**(7), 2179-2186.

Crespo, E., Cristescu, S.M., de Ronde, H., Kuijper, S., Kolk, A.H.J., Anthony, R.M., Harren, F.J.M. 2011. Proton Transfer Reaction Mass Spectrometry detects rapid changes in volatile metabolite emission by *Mycobacterium smegmatis* after the addition of specific antimicrobial agents. *Journal of Microbiology Methods*, **86**(1), 8-15.

Campos, M., Jacobs-Wagner, C., Strobel, S.A. 2015. Mycofumigation by the volatile organic compound-producing fungus *Muscodor albus* induces bacterial cell death through DNA damage. *Applied and Environmental Microbiology*, **81**(3), 1147-1156.

Chambergo, F.S., Bonaccorsi, E.D., Ferreira, A.J., Ramos, A.S., Ferreira, J.R., Abrahao-

Neto, J., Farah, J.P.S., El-Dorry, H. 2002. Elucidation of the metabolic fate of glucose in the filamentous fungus *Trichoderma reesei* using expressed sequence tag (EST) analysis and cDNA microarrays. *The Journal of Biological Chemistry*, **277**(16), 13983-13988.

Connor, M.R., Cann, A.F., Liao, J.C. 2010. 3-Methyl-1-butanol production in *Escherichia coli*: random mutagenesis and two-phase fermentation. *Applied Microbiology and Biotechnology*, **86**(4), 1155-1164.

Cottier, F., Mühlschlegel, F.A. 2011. Communication in fungi. *International Journal of Microbiology*, **2012**, 351832.

Couriol, C., Amrane, A., Prigent, Y., 2001. A new model for the reconstruction of biomass history from carbon dioxide emission during batch cultivation of *Geotrichum candidum*. *Journal of Bioscience and Bioengineering*, **91**(6), 570-575.

Daisy, B.H., Strobel, G.A., Castillo, U., Ezra, D., Sears, J., Weaver, D.K., Runyon, J.B. 2002. Naphthalene, an insect repellent, is produced by *Muscodor vitigenus*, a novel endophytic fungus. *Microbiology*, **148**(Pt 11), 3737-3741.

de Gouw, J., Warneke, C., Karl, T., Eerdekens, G., van der Veen, C., Fall, R. 2003. Sensitivity and specificity of atmospheric trace gas detection by proton-transfer-reaction mass spectrometry. *International Journal of Mass Spectrometry*, **223–224**, 365-382.

Demain, A.L. 1986. Regulation of secondary metabolism in fungi. *Pure and Applied Chemistry*, **58**(2).

Desgranges, C., Vergoignan, C., Georges, M., Durand, A. 1991. Biomass estimation in solid state fermentation I. Manual biochemical methods. *Applied Microbiology and Biotechnology*, **2**: 200-205.

Doty, S.L., Sher, A.W., Fleck, N.D., Khorasani, M., Bumgarner, R.E., Khan, Z., Ko, A.W., Kim, S.H., DeLuca, T.H. 2016. Variable nitrogen fixation in wild *Populus*. *PLoS One*, **11**(5), e0155979.

Edwards, N.T., Hanson, P.J. 1996. Stem respiration in a closed-canopy upland oak forest. *Tree Physiology*. **4**, 433–439.

Eerdekens, G., Ganzeveld, L., Vilà-Guerau de Arellano, J., Klüpfel, T., Sinha, V., Yassaa, N., Williams, J., Harder, H., Kubistin, D., Martinez, M. 2009. Flux estimates of isoprene, methanol and acetone from airborne PTR-MS

measurements over the tropical rainforest during the GABRIEL 2005 campaign. *Atmospheric Chemistry and Physics*. **13**, 4207-4227.

Ezra, D., Hess, W.M., Strobel, G.A. 2004a. New endophytic isolates of *Muscodor albus*, a volatile-antibiotic-producing fungus. *Microbiology*, **150**(12), 4023-4031.

Ezra, D., Jasper, J., Rogers, T., Knighton, B., Grimsrud, E., Strobel, G. 2004b. Proton transfer reaction-mass spectrometry as a technique to measure volatile emissions of Muscodor albus. *Plant Science*, **166**(6), 1471-1477.

Freeman, A., Woodley, J.M., Lilly, M.D. 1993. *In situ* product removal as a tool for bioprocessing. *Nature Biotechnology*, **11**(9), 1007-1012.

Garraway, M.O., Evans, R.C. 1984. Fungal nutrition and physiology. Wiley.

Gayathri, S., Saravanan, D., Radhakrishnan, M., Balagurunathan, R., Kathiresan, K. 2010. Bioprospecting potential of fast growing endophytic bacteria from leaves of mangrove and salt-marsh plant species. *Indian Journal of Biotechnology*, **9**(4), 397-402.

Gianoulis, T.A., Griffin, M.A., Spakowicz, D.J., Dunican, B.F., Sboner, A., Sismour, A.M., Kodira, C., Egholm, M., Church, G.M., Gerstein, M.B. 2012. Genomic analysis of the hydrocarbon-producing, cellulolytic, endophytic fungus *Ascocoryne sarcoides*. *PLoS Genetics*, **8**(3), e1002558-e1002558.

Griffin, M.A., Spakowicz, D.J., Gianoulis, T.A., Strobel, S.A. 2010. Volatile organic compound production by organisms in the genus *Ascocoryne* and a re-evaluation of myco-diesel production by NRRL 50072. *Microbiology*, **156**(Pt 12), 3814-3829.

Gillies, B., Yamazaki, H., Armstrong, D.W. 1987. Production of flavor esters by immobilized lipase. *Biotechnology Letters*, **9**(10), 709-714.

Grigoriev, I.V., Cullen, D., Goodwin, S.B., Hibbett, D., Jeffries, T.W., Kubicek, C.P., Kuske, C., Magnuson, J.K., Martin, F., Spatafora, J.W. 2011. Fueling the future with fungal genomics. *Mycology*, **2**(3), 192-209.

Guo, B., Wang, Y., Sun, X., Tang, K. 2008. Bioactive natural products from endophytes: a review. *Applied Biochemistry and Microbiology*, **44**(2), 136-142.

Hasunuma, T., Okazaki, F., Okai, N., Hara, K.Y., Ishii, J., Kondo, A. 2013. A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. *Bioresource Technology*, **135**, 513-522.

Hazelwood, L.A., Daran, J.-M., van Maris, A.J., Pronk, J.T., Dickinson, J.R. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology*, **74**(8), 2259-2266.

Henderson, J., Ricker, R., Bidlingmeyer, B., Woodward, C. 2000. Rapid, accurate, sensitive, and reproducible HPLC analysis of amino acids. *Agilent Technologies*.

Hibbett, D.S., Ohman, A., Glotzer, D., Nuhn, M., Kirk, P., Nilsson, R.H. 2011. Progress in molecular and morphological taxon discovery in fungi and options for formal classification of environmental sequences. *Fungal Biology Reviews*, **25**(1), 38-47.

Holker, U., Hofer, M., Lenz, J. 2004. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied Microbiology and Biotechnology*, **64**(2), 175-86.

Hornby, J.M., Jensen, E.C., Lisec, A.D., Tasto, J.J., Jahnke, B., Shoemaker, R., Dussault, P., Nickerson, K.W. 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Applied and Environmental Microbiology*, **67**(7), 2982-2992.

Huang, W.-Y., Cai, Y.-Z., Hyde, K.D., Corke, H., Sun, M. 2007. Endophytic fungi from *Nerium oleander* L (*Apocynaceae*): main constituents and antioxidant activity. *World Journal of Microbiology and Biotechnology*, **23**(9), 1253-1263.

Hung, R., Lee, S., Bennett, J.W. 2015. Fungal volatile organic compounds and their role in ecosystems. *Applied Microbiology and Biotechnology*, **99**(8), 3395-405.

Hunt, K.A., 2016. The stoichiometry of nutrient and energy transfer: from organelles to organisms. Ph.D. Thesis. Montana State University at Bozeman.

Hyde, K., Soytong, K. 2008. The fungal endophyte dilemma. *Fungal Diversity*, **33**, 163-173.

Jager, W., Nasel, B., Nasel, C., Binder, R., Stimpfl, T., Vycudilik, W., Buchbauer, G. 1996. Pharmacokinetic studies of the fragrance compound 1,8-cineol in humans during inhalation. *Chemical Senses*, **21**(4), 477-80.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., Piechulla, B. 2009. Bacterial volatiles and their action potential. *Applied Microbiology and Biotechnology*, **81**(6), 1001-12.

Kajos, M.K., Rantala, P., Hill, M., Hellén, H., Aalto, J., Patokoski, J., Taipale, R., Hoerger, C.C., Reimann, S., Ruuskanen, T.M., Rinne, J., Petäjä, T. 2015. Ambient measurements of aromatic and oxidized VOCs by PTR-MS and GC-MS: intercomparison between four instruments in a boreal forest in Finland. *Atmospheric Measurement Techniques*, **8**, 4453-4473.

Kates, M., Baxter, R.M. 1962. Lipid composition of mesophilic and psychrophilic yeasts (*Candida* species) as influenced by environmental temperature. *Canadian Journal of Biochemistry and Physiology*, **40**, 1213-27.

Kawahara, A., Sato, Y., Saito, Y., Kaneko, Y., Takimura, Y., Hagihara, H., Hihara, Y. 2016. Free fatty acid production in the cyanobacterium *Synechocystis* sp. PCC 6803 is enhanced by deletion of the cyAbrB2 transcriptional regulator. *Journal of Biotechnology*, **220**, 1-11.

Keller, N.P., Turner, G., Bennett, J.W. 2005. Fungal secondary metabolism - from biochemistry to genomics. *Nature Reviews Microbiology*, **3**(12), 937-47.

Kelly, D.J.A., Budd, K. 1991. Polyol metabolism and osmotic adjustment in the mycelial Ascomycete *Neocosmospora vasinfecta* (E. F. Smith). *Experimental Mycology*, **15**(1), 55-64.

Knighton, W.B., Fortner, E.C., Midey, A.J., Viggiano, A.A., Herndon, S.C., Wood, E.C., Kolb, C.E. 2009. HCN detection with a proton transfer reaction mass spectrometer. *International Journal of Mass Spectrometry*, **283**(1–3), 112-121.

Krings, U., Berger, R.G. 1998. Biotechnological production of flavours and fragrances. *Applied Microbiology and Biotechnology*, **49**(1), 1-8.

Krishna, C. 2005. Solid-state fermentation systems—an overview. *Critical Reviews in Biotechnology*, **25**(1-2), 1-30.

Lee, S.Y., Lee, D.-Y., Kim, T.Y. 2005. Systems biotechnology for strain improvement. *Trends in Biotechnology*, **23**(7), 349-358.

Leffingwell and Associates. 2014. Flavor & Fragrance Industry Leaders. Retrieved from: http://www.leffingwell.com/top10.htm.

Li, S., Li, G., Zhang, L., Zhou, Z., Han, B., Hou, W., Wang, J., Li, T. 2013. A demonstration study of ethanol production from sweet sorghum stems with advanced solid state fermentation technology. *Applied Energy*, **102**, 260-265.

Liao, S., Wang, F., Wu, T., Pan, W. 2016. Crude oil price decision under considering emergency and release of strategic petroleum reserves. *Energy*, **102**, 436-443.

Lindinger, W., Hansel, A., Jordan, A. 1998. On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) medical applications, food control and environmental research. *International Journal of Mass Spectrometry and Ion Processes*, **173**(3), 191-241.

Liu, X., Sheng, J., Curtiss III, R. 2011. Fatty acid production in genetically modified cyanobacteria. *Proceedings of the National Academy of Sciences*, **108**(17), 6899-6904.

Luchner, M., Gutmann, R., Bayer, K., Dunkl, J., Hansel, A., Herbig, J., Singer, W., Strobl, F., Winkler, K., Striedner, G. 2012. Implementation of proton transfer reaction-mass spectrometry (PTR-MS) for advanced bioprocess monitoring. *Biotechnology and Bioengineering*, **109**(12), 3059-69.

Lynd, L.R., van Zyl, W.H., McBride, J.E., Laser, M. 2005. Consolidated bioprocessing of cellulosic biomass: an update. *Current Opinion in Biotechnology*, **16**(5), 577-83.

Mallette, N., M. Pankratz, E., E. Parker, A., A. Strobel, G., C. Busse, S., P. Carlson, R., M. Peyton, B. 2014. Evaluation of cellulose as a substrate for hydrocarbon fuel production by *Ascocoryne sarcoides*; (NRRL 50072). *Journal of Sustainable Bioenergy Systems*, **04**(01), 33-49.

Mallette, N.D. 2013. Volatile fuel & organic compound production by *Ascocoryne sarcoides*. *Ph.D. Thesis.* Montana State University at Bozeman.

Mallette, N.D., Knighton, W.B., Strobel, G.A., Carlson, R.P., Peyton, B.M. 2012. Resolution of volatile fuel compound profiles from *Ascocoryne sarcoides*: a comparison by proton transfer reaction-mass spectrometry and solid phase microextraction gas chromatography-mass spectrometry. *AMB Express*, **2**(1), 23.

Mends, M.T., Yu, E., Strobel, G.A., Riyaz-Ul-Hassan, S., Booth, E., Geary, B., Sears, J., Taatjes, C.A., Hadi, M.Z. 2012. An endophytic *Nodulisporium* sp. producing volatile organic compounds having bioactivity and fuel potential. *Journal of Petroleum & Environmental Biotechnology*, **3**, 117.

Meyer, V. 2008. Genetic engineering of filamentous fungi — Progress, obstacles and future trends. *Biotechnology Advances*, **26**(2), 177-185.

Morath, S.U., Hung, R., Bennett, J.W. 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal Biology Reviews*, **26**(2-3), 73-83.

Mukherjee, P.K., Horwitz, B.A., Singh, U.S., Mukherjee, M., Schmoll, M. 2013. Trichoderma: Biology and Applications. CABI.

Ng, C., Jung, M.-y., Lee, J., Oh, M.-K. 2012. Production of 2,3-butanediol in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. *Microbial Cell Factories* **1**, 1–14.

Nigg, J., Strobel, G., Knighton, W.B., Hilmer, J., Geary, B., Riyaz-Ul-Hassan, S., Harper, J.K., Valenti, D., Wang, Y. 2014. Functionalized para-substituted benzenes as 1,8-cineole production modulators in an endophytic *Nodulisporium* species. *Microbiology*, **160**, 1772–1782.

Nødvig, C.S., Nielsen, J.B., Kogle, M.E., Mortensen, U.H. 2015. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PloS one*, **10**(7), e0133085.

Pandey, A. 2003. Solid-state fermentation. *Biochemical Engineering Journal*, **13**(2), 81-84.

Panagiotou, G., Villas-Bôas, S.G., Christakopoulos, P., Nielsen, J., Olsson, L., 2005. Intracellular metabolite profiling of *Fusarium oxysporum* converting glucose to ethanol. *Journal of Biotechnology*, **115**(4), 425-434.

Pino, J.A., Marbot, R., Vázquez, C. 2004. Volatile Flavor Constituents of *Karanda* (*Carissa carandas* L.) Fruit. *Journal of Essential Oil Research*, **16**(5), 432-434.

Plata, C., Mauricio, J.C., Millán, C., Ortega, J.M. 2005. Influence of glucose and oxygen on the production of ethyl acetate and isoamyl acetate by a *Saccharomyces cerevisiae* strain during alcoholic fermentation. *World Journal of Microbiology and Biotechnology*, **21**(2), 115-121

Porras-Alfaro, A., Bayman, P. 2011. Hidden fungi, emergent properties: endophytes and microbiomes. *Annual Reviews in Phytopathology*, **49**, 291-315.

Punt, P.J., van Biezen, N., Conesa, A., Albers, A., Mangnus, J., van den Hondel, C. 2002. Filamentous fungi as cell factories for heterologous protein production. *Trends in Biotechnology*, **20**(5), 200-206.

Quin, M.B., Flynn, C.M., Schmidt-Dannert, C. 2014. Traversing the fungal terpenome. *Natural Product Reports*, **31**(10), 1449-1473.

Rautio, J.J., Smit, B.A., Wiebe, M., Penttilä, M., Saloheimo, M. 2006. Transcriptional monitoring of steady state and effects of anaerobic phases in chemostat cultures of the filamentous fungus *Trichoderma reesei*. *BMC Genomics*, **7**, 247-247.

Rhine, E., Mulvaney, R., Pratt, E., Sims, G. 1998. Improving the Berthelot reaction for determining ammonium in soil extracts and water. *Soil Science Society of America Journal*, **62**(2), 473-480.

Rodriguez-Bustamante, E., Sanchez, S. 2007. Microbial production of C13-norisoprenoids and other aroma compounds via carotenoid cleavage. *Critical Reviews in Microbiology*, **33**(3), 211-30.

Rodriguez, R.J., White, J.F., Jr., Arnold, A.E., Redman, R.S. 2009. Fungal endophytes: diversity and functional roles. *New Phytologist*, **182**(2), 314-30.

Romano, A., Capozzi, V., Spano, G., Biasioli, F. 2015. Proton transfer reaction-mass spectrometry: online and rapid determination of volatile organic compounds of microbial origin. *Applied Microbiology and Biotechnology*, **9**, 3787-95.

Roze, L.V., Chanda, A., Laivenieks, M., Beaudry, R.M., Artymovich, K.A., Koptina, A.V., Awad, D.W., Valeeva, D., Jones, A.D., Linz, J.E. 2010. Volatile profiling reveals intracellular metabolic changes in *Aspergillus* parasiticus: veA regulates branched chain amino acid and ethanol metabolism. *BMC Biochemistry*, **11**, 33.

Saikkonen, K., Faeth, S.H., Helander, M., Sullivan, T.J. 1998. FUNGAL ENDOPHYTES: A Continuum of Interactions with Host Plants. *Annual Review of Ecology and Systematics*, **29**(1), 319-343.

Saini, J.K., Saini, R., Tewari, L. 2015. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: concepts and recent developments. *3 Biotech*, **5**(4), 337-353.

Sánchez, Ó.J., Cardona, C,A. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology*, **99**(13), 5270-5295.

Schmidberger, T., Gutmann, R., Bayer, K., Kronthaler, J., Huber, R. 2014. Advanced online monitoring of cell culture off-gas using proton transfer reaction mass spectrometry. *Biotechnology Progress*, **2**, 496-504.

Schoen, H.R., Peyton, B.M., Knighton, W.B. 2016. Rapid total volatile organic carbon quantification from microbial fermentation using a platinum catalyst and proton transfer reaction-mass spectrometry. *AMB Express*, **6**(1), 90.

Schwab, W., Davidovich-Rikanati, R., Lewinsohn, E. 2008. Biosynthesis of plant-derived flavor compounds. *Plant J*, **54**(4), 712-32.

Shaw, J.J., Berbasova, T., Sasaki, T., Jefferson-George, K., Spakowicz, D.J., Dunican, B.F., Portero, C.E., Narváez-Trujillo, A., Strobel, S.A. 2015. Identification of a

fungal 1, 8-cineole synthase from *Hypoxylon* sp. with specificity determinants in common with the plant synthases. *Journal of Biological Chemistry*, **290**(13), 8511-8526.

Singer, W., Gutmann, R., Dunkl, J., Hansel, A. 2009. PTR-MS Technology for process monitoring and control in biotechnology. *JPAC: Process Analytical Chemistry,* **11**(1), 1–4.

Singhania, R.R., Patel, A.K., Soccol, C.R., Pandey, A. 2009. Recent advances in solid-state fermentation. *Biochemical Engineering Journal*, **44**(1), 13-18.

Steinbacher, M., Dommen, J., Ammann, C., Spirig, C., Neftel, A., Prevot, A.S.H. 2004. Performance characteristics of a proton-transfer-reaction mass spectrometer (PTR-MS) derived from laboratory and field measurements. *International Journal of Mass Spectrometry*, **239**(2–3), 117-128.

Stephanopoulos, G., Aristidou, A.A., Nielsen, J. 1998. Metabolic Engineering: Principles and Methodologies. Elsevier Science.

Stinson, M., Ezra, D., Hess, W.M., Sears, J., Strobel, G. 2003. An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. *Plant Science*, **165**(4), 913-922.

Stockfors, J. 2000. Temperature variation and distribution of living cells within tree stems: implications for stem respiration modeling and scale-up. *Tree Physiology*, **15**, 1057–1062.

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., Schuhmacher, R. 2010. Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. *Journal of Microbiological Methods*, **81**(2), 187-193.

Strobel, G.A. 2014. Methods of discovery and techniques to study endophytic fungi producing fuel-related hydrocarbons. *Natural Product Reports*, **31**(2), 259-272.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C. 2001. Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. *Microbiology*, **147**(11), 2943-2950.

Strobel, G.A., Knighton, B., Kluck, K., Ren, Y., Livinghouse, T., Griffin, M., Spakowicz, D., Sears, J. 2008. The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum* (NRRL 50072). *Microbiology*, **154**(Pt 11), 3319-28.

Strobel, G.A., Spang, S., Kluck, K., Hess, W.M., Sears, J., Livinghouse, T. 2008. Synergism among volatile organic compounds resulting in increased antibiosis in *Oidium* sp. *FEMS Microbiology Letters*, **283**(2), 140-5.

Suryanarayanan, T.S. 2013. Endophyte research: going beyond isolation and metabolite documentation. *Fungal Ecology*, **6**(6), 561-568.

Suutari, M., Rintamaki, A., Laakso, S. 1997. Membrane phospholipids in temperature adaptation of *Candida utilis*: alterations in fatty acid chain length and unsaturation. *Journal of Lipid Research*, **4**, 790–794.

Thomas, L., Larroche, C., Pandey, A. 2013. Current developments in solid-state fermentation. *Biochemical Engineering Journal*, **81**, 146-161.

Tomsheck, A.R., Strobel, G.A., Booth, E., Geary, B., Spakowicz, D., Knighton, B., Floerchinger, C., Sears, J., Liarzi, O., Ezra, D. 2010. *Hypoxylon* sp., an endophyte of *Persea indica*, producing 1, 8-cineole and other bioactive volatiles with fuel potential. *Microbial Ecology*, **60**(4), 903-914.

U.S. Department of Energy. 2013. Fuel properties comparison. Retrieved from: http://www.afdc.energy.gov/fuels/fuel_properties.php.

U.S. Environmental Protection Agency Office of Prevention, Pesticides and Toxic Substances (U.S. EPA). 1994. Reregistration eligibility decision (R.E.D.) Facts: limonene. EPA 738-R- 94-034. Retrived from: http://www.epa.gov/oppsrrd1/REDs/factsheets/3083fact.pdf. Accessed 1 Oct 2014.

Veres, P., Gilman, J., Roberts, J., Kuster, W., Warneke, C., Burling, I., Gouw, J.d. 2010. Development and validation of a portable gas phase standard generation and calibration system for volatile organic compounds. *Atmospheric Measurement Techniques*, **3**(3), 683-691.

Verstrepen, K.J., Derdelinckx, G., Dufour, J.-P., Winderickx, J., Thevelein, J.M., Pretorius, I.S., Delvaux, F.R. 2003a. Flavor-active esters: Adding fruitiness to beer. *Journal of Bioscience and Bioengineering*, **96**(2), 110-118.

Verstrepen, K.J., Van Laere, S.D., Vanderhaegen, B.M., Derdelinckx, G., Dufour, J.-P., Pretorius, I.S., Winderickx, J., Thevelein, J.M., Delvaux, F.R. 2003b. Expression levels of the yeast alcohol acetyltransferase genes ATF1, Lg-ATF1, and ATF2 control the formation of a broad range of volatile esters. *Applied and Environmental Microbiology*, **69**(9), 5228-5237.

Werpy, T., Petersen, G. 2004. Top Value Added Chemicals from Biomass: Volume I -- Results of Screening for Potential Candidates from Sugars and Synthesis Gas. ; National Renewable Energy Lab., Golden, CO (US).

Wurzenberger, M., Grosch, W. 1984. The formation of 1-octen-3-ol from the 10-hydroperoxide isomer of linoleic acid by a hydroperoxide lyase in mushrooms (*Psalliota bispora*). *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, **794**(1), 25-30.

Xiao, Z., Wang, X., Huang, Y., Huo, F., Zhu, X., Xi, L., Lu, J.R. 2012. Thermophilic fermentation of acetoin and 2, 3-butanediol by a novel Geobacillus strain. *Biotechnology for Biofuels*, **5**(1), 1.

Xu, Q., Singh, A., Himmel, M.E. 2009. Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. *Current Opinions in Biotechnology*, **20**(3), 364-71.

Yegneswaran, P.K., Gray, M.R., and Thompson, B.G. 1990. Kinetics of CO2 hydration in fermentors: pH and pressure effects. *Biotechnology and Bioengineering*, **36**, 92–96.

Yoshioka, K., Hashimoto, N. 1981. Ester formation by alcohol acetyltransferase from brewers' yeast. *Agricultural and Biological Chemistry*, **45**(10), 2183-2190.

Zhi-Lin, Y., Yi-Cun, C., Bai-Ge, X., Chu-Long, Z. 2012. Current perspectives on the volatile-producing fungal endophytes. *Critical Reviews in Biotechnology*, **32**(4), 363-73.