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Lipid Accumulation by Rhodococcus Rhodochrous

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Lipid accumulation by *Rhodococcus rhodochrous*

By

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Lipid accumulation by *Rhodococcus rhodochrous*

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Oleaginous microbes can accumulate over 20% of their cell dry weight as lipids that are stored as intracellular energy reserves. The characterization of other oleaginous bacteria creates opportunities for the development of alternative feedstocks and technologies. *Rhodococcus rhodochrous* is a gram-positive bacterium known for its biodegradation capabilities, but little is known about its ability to accumulate lipids. As *R. rhodochrous* is capable of degrading hydrocarbon gasses and other aromatics, this study aims to investigate any associated lipid production during the conversion of waste and nontraditional carbon sources, such as model lignocellulosic inhibitors. Lignocellulosic biomass is the most abundant and renewable organic material in the world and is composed of cellulose, hemicellulose, and lignin, which can be pretreated to release sugars from the complex, and often recalcitrant, lignin polymer for microbial fermentation. *R. rhodochrous* was cultivated with various carbon sources, including glucose, xylose, acetic acid, furfural, phenol, vanillic acid, hydroxybenzoic acid, and propane. The results suggest that *R. rhodochrous* can survive in the presence of these compounds, achieving almost 7g/L cell dry weight after 168 hours and still accumulate up to 40-50% of cell dry weight as lipid in glucose supplemented media. Furthermore, the

aromatic compounds are undetected after 48 hours indicating that *R. rhodochrous* was able to tolerate these compounds and accumulate lipids. Fatty acid methyl ester profiles show a prevalence of palmitic and oleic methyl esters. Overall, these studies are contributing to a better understanding and characterization of another oleaginous *Rhodococcus* species, *Rhodococcus rhodochrous*.

DEDICATION

This work is dedicated to the everlasting spirit of my friend and officemate, Rachel Ianni, whose strength inspires perseverance and whose smile keeps me laughing when life gets tricky. Officemates for life.

This work is also dedicated to the memory of Michael Bradley, a farmer who saw potential when others saw a long day's work and an uncle who always saw big dreams in a very curious little girl.

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TABLE OF CONTENTS

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER

I. INTRODUCTION AND LITERATURE REVIEW 1

1.1 The need for alternative fuels 1
1.1.1 Oil Market Volatility and Energy Security 1
1.1.2 Environmental Concerns 5
1.2 The Current Status of Biofuels 7
1.2.1 The Renewable Fuel Standard 7
1.2.2 The Generations of Biofuels 8
1.2.3 The Lignocellulosic Challenge 10
1.3 Biodiesel 13
1.4 Oleaginous Microbes 15
1.4.1 Microbial Lipids 16
1.4.2 Lipid Synthesis in Oleaginous Yeasts and Bacteria 16
1.4.3 *Rhodococcus opacus* as a Model Oleaginous Bacterium 20
1.4.4 *Rhodococcus rhodochrous*: Potential as Oleaginous Bacterium 21
1.5 Research Significance and Purpose 22
1.6 References 25

II. DRAFT GENOME SEQUENCE OF *RHODOCOCCUS RHODOCHROUS* STRAIN ATCC 21198 34

2.1 Abstract 34
2.2 Draft Genome Sequence of *Rhodococcus rhocochrous* Strain ATCC 21198 34
2.3 Acknowledgements 36
2.4 References 38

III. LIPID ACCUMULATION BY *RHODOCOCCUS RHODOCHROUS* GROWN ON GLUCOSE 39

3.1 Abstract 39
3.2 Introduction 40
3.3 Materials and Methods 42
3.3.1 Culture Conditions 42
3.3.2 Analysis 43
3.3.3 Transmission Electron Microscopy (TEM) 45
3.4 Results and Discussion 46
3.4.1 Effect of Glucose Concentration on Lipid Accumulation 46
3.4.2 Characterization of Lipid Accumulation 49
3.4.3 Fatty Acid Methyl Esters Analysis 54
3.5 Conclusion 56
3.6 Acknowledgements 57
3.7 References 58

IV. LIPID ACCUMULATION BY *RHODOCOCCUS RHODOCHROUS* GROWN IN MODEL LIGNOCELLULOSIC SUGARS AND INHIBITORS 61

4.1 Abstract 61
4.2 Introduction 62
4.3 Methods 64
4.3.1 Culture Conditions 64
4.3.2 Inhibitor Growth Conditions 65
4.3.3 Analyses 66
4.4 Results and Discussion 67
4.4.1 Glucose and Xylose Affect Growth and Lipid Accumulation 67
4.4.2 The Effect of Acetic Acid and Furfural on Growth and Lipid Accumulation 71
4.5 Conclusions 76
4.6 References 77

V. THE EFFECTS OF MODEL AROMATIC LIGNIN COMPONENTS ON GROWTH AND LIPID ACCUMULATION OF *RHODOCOCCUS RHODOCHROUS* 80

5.1 Abstract 80
5.2 Introduction 81
5.3 Materials and Methods 84
5.3.1 Culture and Experimental conditions 84
5.3.1.1 Shake-Flask Conditions 84
5.3.1.2 Fermenter Conditions 85
5.3.2 Analyses 85
5.3.3 Lipid Analysis 86

5.3.4 Phenol, HBA, and VA Analysis 86
5.3.5 Protein Analysis 88
5.4 Results and Discussion 90
5.4.1 Phenol reduces, but does not inhibit growth and lipid accumulation of *R. rhodochrous* 90
5.4.2 Lipid analysis and FAME profiles of *R. rhodochrous* grown in phenol 93
5.4.3 Proteomic analysis of *R. rhodochrous* grown in phenol 94
5.4.4 VA and HBA do not inhibit growth or lipid accumulation when *R. rhodochrous* is grown in the presence of glucose 108
5.5 Conclusions 113
5.6 Acknowledgements 114
5.7 References 115

VI. EVALUATION OF PROPANE AS A CARBON SOURCE FOR GROWTH AND LIPID ACCUMULATION BY *RHODOCOCCUS RHODOCHROUS* 119

6.1 Abstract 119
6.2 Introduction 119
6.3 Methods 121
6.3.1 Culture Conditions 121
6.3.2 Experimental Set-up 121
6.3.3 Experimental Set-up for Lipid analysis 124
6.3.3.1 Growth analysis 125
6.3.3.2 Lipid analysis 125
6.4 Results and Discussion 126
6.5 Acknowledgements 132
6.6 References 133

VII. CONCLUSIONS 135

LIST OF TABLES

3.1 Comparison of parameters related to biomass and lipid production among different oleaginous species grown on various substrates. 51

4.1 Fatty acid methyl ester profile (% w/w) of *R. rhodochrous* grown in glucose, xylose, or a mixture of glucose and xylose. 70

5.1 Fatty acid methyl ester profile (% w/w) of *R. rhodochrous* grown in glucose (Glu), phenol (Phe) or a mixture of glucose and phenol (GP). 96

5.2 Proteins of *R. rhodochrous* cultured in glucose, phenol, and glucose with phenol. 97

6.1 *R. rhodochrous* cell dry weight (g/L; CDW) and percent (g/g) lipid of CDW of prescription bottle agar and broth cultures grown on glucose or propane as a sole carbon source. 130

LIST OF FIGURES

1.1 Primary energy consumption by source and sector in 2013. 3

1.2 Pretreatment of lignocellulosic biomass. 12

1.3 Schematic of lipid accumulation in oleaginous microbes. 18

1.4 Schematic of glycolysis in response to a high C:N ratio. 19

3.1 Effects of glucose on lipid growth and lipid accumulation of *R. rhodochrous*. 48

3.2 Growth and lipid accumulation by *R. rhodochrous* in fermenter cultivation. 50

3.3 Transmission electron microscopy images of *R. rhodochrous*. 52

3.4 Thin layer chromatography of lipid extract from *R. rhodochrous* fermenter culture (RR). 53

3.5 FAME profile over time of *R. rhodochrous* grown in shake flasks supplemented with 10 (top), 20 (middle), or 40g/L (bottom) of glucose. 55

3.6 Percent FAMEs based on lipid extract of *R. rhodochrous* grown for 144 hours in 20 g/L in a BioFlo410 fermenter. 55

4.1 Chemical structures of acetic acid and furfural. 63

4.2 *R. rhodochrous* cell dry of shake flask cultures supplemented with glucose, xylose, or a mixture of glucose and xylose. 68

4.3 Glucose or xylose concentration of *R. rhodochrous* shake flask cultures. 68

4.4 Percent lipid accumulation by *R. rhodochrous* grown in glucose or xylose supplemented shake flask cultures. 69

4.5 *R. rhodochrous* cell dry weight of shake flask cultures with glucose supplemented with acetic acid, furfural, or a mix of acetic acid and furfural. 72

4.6 Glucose concentrations of *R. rhodochrous* shake flask cultures supplemented with acetic acid, furfural, or a mix of acetic acid and furfural (•). 73

4.7 Percent lipid accumulation by *R. rhodochrous* grown in inhibitor-supplemented shake flask cultures. 73

4.8 FAME profile of *R. rhodochrous* cultures at 72H grown in model lignocellulosic inhibitors, acetic acid and furfural. 74

4.9 *R. rhodochrous* growth and lipid accumulation in the presence of inhibitors in fermenter cultivation. 75

5.1 Chemical structures of phenol, vanillic acid, and 4-hydroxybenzoic acid. 82

5.2 Growth and lipid accumulation of *R. rhodochrous* grown in phenol-supplemented media. 92

5.3 *R. rhodochrous* cell dry weight and percent lipid accumulation of shake-flask cultures supplemented with model lignin compounds. 110

5.4 Fatty acid methyl ester profile (% w/w) of *R. rhodochrous* grown in model lignin compounds. 111

5.5 *R. rhodochrous* cell dry weight, percent lipid accumulation and pH of 3L fermentations supplemented with glucose and VA, HBA, or VA and HBA. 112

6.1 *R. rhodochrous* starter cultures were grown in Sorkhoh media with propane as the sole carbon source. 122

6.2 *R. rhodochrous* cultures were flushed with a gas mixture of approximately 65% propane, 30% oxygen, and 5% carbon dioxide for 1 minute. 123

6.3 *R. rhodochrous* was grown on agar surfaces in prescription bottles flushed with a propane gas mixture as the sole carbon source. 127

6.4 *R. rhodochrous* total cell dry weight (g; CDW) and percent (g/g) lipid of CDW of prescription bottle cultures grown on propane as a sole carbon source. 128

6.5 Absorbance (595nm) of *R. rhodochrous* prescription bottle broth cultures grown on propane or 20g/L glucose as a sole carbon source. 129

6.6 *R. rhodochrous* was grown on agar surfaces in prescription bottles supplemented with glucose or flushed with a propane gas mixture as the sole carbon source. 130

6.7 FAME profile of *R. rhodochrous* grown on propane as a sole carbon source.....132

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

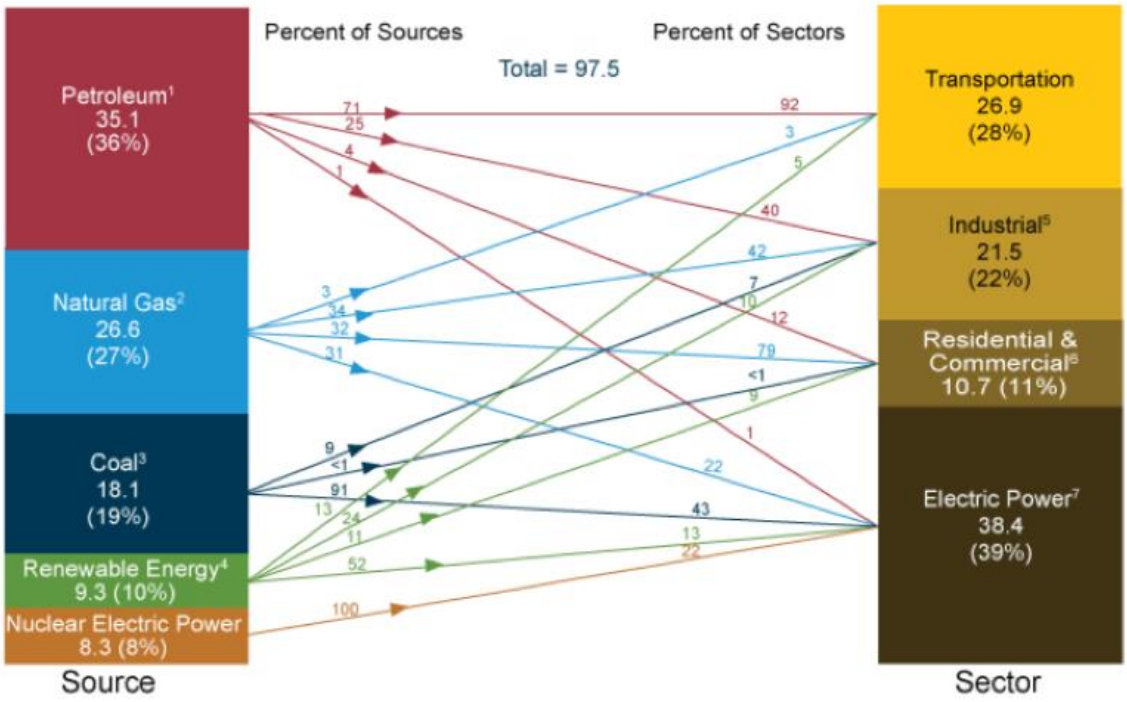
1.1 The need for alternative fuels

Energy security, environmental concerns, and unstable oil prices have been the driving trifecta of demand for alternative fuels in the United States. Our dependence on energy resources, often from unstable oil-producing countries has created political insecurities and concerns. As we try to gain energy security, unconventional oil becomes more common, flooding the market, and causing the major downshift of the usual unstable oil prices. Meanwhile, consumption of fossil fuels and the consequent CO_2 emissions have driven disruptions in the Earth's atmosphere and are presumed to be responsible for global climate change. While the significance of each of these three factors may fluctuate with global politics or new technologies, transportation energy will remain the prominent focus of multi-disciplined research.

1.1.1 Oil Market Volatility and Energy Security

Petroleum is one of the major energy sources consumed in the United States. Seventy-one percent of petroleum supply is fed directly to the transportation sector, supplying over 90% of transportation's liquid fuel (Figure 1.1). Some scientists have predicted that we will run out of petroleum in the next 50 years, as we are consuming fossil fuels at an alarming rate (Soetaert & Vandamme, 2009). While this doomsday argument may increase the urgency for alternatives to conventional petroleum reserves,

unconventional drilling, such as hydraulic fracturing and horizontal drilling, have made harder-to-get oil possible; thereby increasing the US's oil production tremendously. Hydraulic fracturing, or fracking, involves pumping water, sand, and potentially hazardous chemicals into gas shale (deep underground layers of rock) to release oil and natural gas (Conti et al., 2014a). Recent advances in horizontal drilling have enabled drillers to extract even more gas and oil from miles of shale instead of just a small section. This newfound abundance of oil has impacted global oil prices and triggered uncertainty in the future of biofuel research and commercialization.



Endnotes:

[1]Does not include biofuels that have been blended with petroleum---biofuels are included in "Renewable Energy."

[2]Excludes supplemental gaseous fuels.

[3]Includes less than -0.1 quadrillion Btu of coal coke net imports.

[4]Conventional hydroelectric power, geothermal, solar/PV, wind, and biomass.

[5]Includes industrial combined-heat-and-power (CHP) and industrial electricity-only plants.

[6]Includes commercial combined-heat-and-power (CHP) and commercial electricity-only plants.

[7]Electricity-only and combined-heat-and-power (CHP) plants whose primary business is to sell electricity or electricity and heat, to the public. Includes 0.2 quadrillion Btu of electricity net imports not shown under "Source."

Note: Primary energy in the form that it is first accounted for in a statistical energy balance, before any transformation to secondary or tertiary forms of energy (for example, coal is used to generate electricity).

*Sum of components may not equal total due to independent rounding.

Figure 1.1 Primary energy consumption by source and sector in 2013. Units are in quadrillion Btu. Source EIA, 2014.

In 2015 the US is poised to be one of the largest oil producers in the world with a predicted May 2015 output at its highest since 1972 and imports at its lowest since 1969 (EIA, 2015b). This increase in production coincides with the collapse of oil prices, from $107/barrel to below $50/barrel that occurred in the fall of 2014 and continued into 2015

(Bloomberg, 2015). Supply and demand as well as global instabilities drive the price of oil. As oil production increased, demand declined, particularly in the US, where more fuel-efficient vehicles and better driving practices (less miles, public transportation, etc.) caused an 8% decrease in gasoline consumption (Bloomberg, 2015). In addition to the oil usage in the US, largely driven by the increase in unconventional drilling, the Organization for Petroleum Exporting Countries (OPEC), which controls 40% of the market, has not limited their production, causing a buildup of inventory with a decline in demand (EIA, 2015b). Historically, Saudi Arabia, OPEC's largest producer, would cut some of its oil production to stabilize the market. Now, however analysts indicate that OPEC is threated by the US's shale oil dominating the market and would rather take the price cuts than relinquish market control to the US or other Persian Gulf producers (Lane, 2015; McGee, 2014; Mirhaydari, 2015; Solomon & Said, 2014; Waldman, 2015).

Oil production and thereby oil prices, are also affected by geopolitical instabilities. In 2008, energy security was one of several driving factors of the development of alternative fuels that would allow the US to be self-sufficient in energy production (Lopes, 2015). Energy security can also pertain to mitigating disruptions in oil supplies. In January 2015, non-OPEC supply was disrupted by over a half million barrels per day due to conflicts in South Sudan, Syria, and Yemen (EIA, 2015a). Furthermore, OPEC production in Libya, Angola, Algeria, and Kuwait declined and production in Iraq and Iran is expected to be disrupted by the increasing activity and threat of the Islamic State of Iraq and the Levant (EIA, 2015a). According to the Truman National Security Project, clean energy development is critical to reducing our dependence on a volatile

global oil market and mitigating the national security threat of climate change (TSBB, 2014).

1.1.2 Environmental Concerns

Drilling practices and petroleum-related emissions have led to serious environmental concerns. Shale drilling can access natural gas and oil with a lower carbon footprint (Lopes, 2015) and in displacing/replacing coal plants, shale has also contributed to reducing CO_2 emissions in the US (Carey, 2012). More recently scientists with the US Geological Survey (USGS) have observed "induced earthquakes" from the fluid-injection methods of shale drilling in areas of the US that have historically been geologically stable (Ellsworth, 2013; McGarr et al., 2015). For example, in 2014, the rate of 3 magnitude or greater earthquakes was greater in Oklahoma than in California (McGarr et al., 2015; McNamara et al., 2015). Induced earthquakes in the US may be a result of the deep disposal of wastewater or fluids related to oil and gas (Rubinstein & Mahani, 2015). New regulations for wastewater disposal, better fault monitoring systems and data sharing will help to improve our understanding of the role of fracking and induced earthquakes (McNamara et al., 2015).

Another environmental concern associated with unconventional drilling is groundwater contamination. A study in 2013 found that methane concentrations in shallow residential drinking-water wells were 6 times higher than in homes farther away from the drill site (Jackson et al., 2013). Some European countries have chosen not to use shale gas until these environmental concerns are addressed (Patel & Viscusi, 2013). The US Environmental Protection Agency (EPA) released a draft of a three-year study on the impact of fracking on drinking water and found that while there is potential for fracking

activities to impact drinking water resources, there was no evidence that fracking had a widespread impact as the number of instances were small compared to the amount of active fracking wells (EPA, 2015). The EPA is still investigating safe disposal methods for the large volumes of wastewater produced with shale gas extraction.

Fossil fuel emissions and wild fires have contributed to the increase in atmospheric CO_2 concentrations (Peters et al., 2007). Oceans act as a CO_2 sink, absorbing almost 26% of CO_2 emissions (SCOPE, 2015). As CO_2 concentrations rise and oceans are increasingly absorbing CO_2, water surface temperature increases and ocean acidification occurs, thereby potentially reducing the capacity of the Earth to mitigate rising CO_2 emissions (Le Quere et al., 2010; SCOPE, 2015). A recent study by Feldman et al. (2015) concluded that the greenhouse effect was indeed due to anthropogenic emissions. Observed evidence showed that rising CO_2 concentrations affect the Earth's surface energy and are a direct result of anthropogenic climate change and variations in photosynthetic activity (Feldman et al., 2015). The International Energy Agency issued its World Energy Outlook report predicting that long-term global temperatures will increase by 3.6°C (IEA, 2014). To prevent the widespread effects of global climate change, the International Panel on Climate Change recommends limiting temperature increase to 2°C, which means that the world cannot emit over 1,000 gigatons of CO_2 after 2014 (Conti et al., 2014b; IEA, 2014). As emissions are not likely to be reduced to zero, even with these recommendations, further alternatives such as cleaner burning fuels are necessary to mitigate the onset of global climate change.

1.2 The Current Status of Biofuels

1.2.1 The Renewable Fuel Standard

The Energy Policy Act of 2005 authorized the first Renewable Fuel Standard (RFS), which would mandate a minimum volume of biofuels for national transportation use (EPAct, 2005). In 2007 the Energy Independence and Security Act (EISA) was established to further reduce energy dependence and increase national security, to stimulate the economy by creating jobs, and to initiate research for producing clean, renewable, and efficient fuels (EISA, 2007). The EISA revised RFS1 and established RFS2, which mandated that 36 billion gallons of renewable fuel be produced by 2022, including 15 billion gallons of corn-based ethanol, 16 billion gallons of cellulosic biofuels, and 1 billion gallons of biodiesel (EISA, 2007). The US Environmental Protection Agency regulates the RFS through renewable identification numbers, compliance standards, and waivers for industries producing total renewable fuels, including advanced biofuels, cellulosic biofuels, and biomass-based diesel (Schnepf & Yacobucci, 2013). However, there has been significant instability of the RFS policy, including a delay by the EPA in setting the 2014 renewable fuel volume obligations. The Biotechnology Industry Organization (BIO) cites that this policy instability has scared off capital investments and cost 80,000 jobs that would have been created by the industry (BIO, 2014). Furthermore, an estimated 21 million metric tons of CO_2 equivalent, equal to 4.4 million additional cars on the road, is the difference between a consistent RFS and policy instability (BIO, 2014). The American Petroleum Institute (API) argues that the RFS is a broken policy, largely citing the blend wall (market's ability to absorb the amount of biofuels produced) and its effect on supply disruptions and cost increases

(API, 2015). A study by BIO concludes that in the 10-year span of the RFS, US carbon emissions have been reduced by 589.33 million metric tons (equal to over 124 million cars on the road) and almost 1.9 billion barrels of foreign oil have been displaced (BIO, 2014). The RFS began as a catalyst of the US's advancement of renewable fuels and is now a highly debated policy with significant implications for the future of the biofuels industry.

1.2.2 The Generations of Biofuels

In response to the increasing need for biofuels and the establishment of the RFS, new technologies, processes, and renewable biological sources are being rapidly developed. Generations are used to chart the evolution of the biofuels industry as research and commercialization progress. Conventional biofuels, also known as first generation, include processes that are already producing biofuels on a commercial scale, such as sugar and starch based ethanol and plant oil biodiesel (IEA, 2015). The benefits of first generation biofuels include economic and social security as well as environmental benefits (Naik et al., 2010). A popular example of first generation biofuels is bioethanol. Bioethanol is generally produced from the fermentation of sucrose from either sugarcane or sugar beets or from the fermentation of sugars derived from the enzymatic digestion of corn starch (Granda et al., 2007). While the use of ethanol for transportation fuel is not new, the growth of ethanol production has significantly increased, tripling yields between 2000-2007 (Granda et al., 2007).

However, to be a viable alternative to fossil fuels, Hill et al. proposed that the fuel should have better environmental benefits, provide a net energy gain, and be economically competitive (Hill et al., 2006). After years of research on the implications

of first generation biofuels, competition for land and water used for food production (food vs. fuel) is often listed as the primary disadvantage (Graham-Rowe, 2011; Naik et al., 2010; Sims et al., 2010). The worldwide demand for both food and transportation fuel challenge the significance of food-based biofuels (Hill et al., 2006; Tao & Aden, 2009). For example, in 2007 the US planted over 90 million acres of corn, but 30% was used for ethanol production and not for food (Graham-Rowe, 2011). This land use displacement increased food prices and spurred land use competition in an attempt to satisfy national energy demands (Hill et al., 2006; Tao & Aden, 2009). In 2015, as food production is estimated to require a 60% increase by 2050, the Food and Agricultural Organization (FAO) has shifted its focus from a "food vs fuel" debate to a "food and fuel debate", citing both as priorities for the future (UN, 2015).

Advanced biofuels include the later generations, which are still in the research and development or pilot phase. Second generation biofuels, or advanced biofuels, are produced from non-food biomass in an effort to mitigate the food vs. fuel debate created by first generation biofuels. These biofuels are produced in a more sustainable way, which some claim is truly carbon neutral or carbon negative toward its impact on CO_2 emissions (Naik et al., 2010). Lignocellulosic biomass is the feedstock for second generation biofuels and consists of crop byproducts or energy crops, such as switchgrass and energy cane. Energy crops require less fertilizer inputs and grow on marginal lands, resulting in a greater environmental benefit (Hill et al., 2006). Furthermore, although competing with food for land use, lignocellulosic biomass energy yields (GJ/ha) are expected to be greater than if first generation crops were grown on this same land (Sims et al., 2010). In 2007 the US, along with the private sector, allocated $385 million to the

US Department of Energy Biomass Program to research and develop several large-scale ethanol demonstration plants (Sims et al., 2010). Despite the large investments, several technical challenges remain towards the cost-effective and efficient utilization of lignocellulosic biomass.

Other advanced biofuel generations include third generation biofuels that produce a feedstock as well as a fuel, such as algae, and fourth generation biofuels that consist of electrofuels (Lane, 2010). These generations are still in the very early stages of technology development and have high capital costs. Second generation technologies are just now emerging as commercially viable, as POET-DSM Advanced Biofuels opened the first corn stover cellulosic ethanol plant in the US in September 2014 (Thorp et al., 2015). The successful development and implementation of advanced biofuels using non-food biomass could provide 27% of the total US transport fuel by 2050 while decreasing CO_2 emissions by 20% or 2.1 Gt (IEA, 2011).

1.2.3 The Lignocellulosic Challenge

Lignocellulosic biomass is the most abundant and renewable organic material in the world (Claassen et al., 1999). Examples of lignocellulosic biomass include wood, grass, forestry waste and agricultural residues (Palmqvist & Hahn-Hagerdal, 2000). The advantages of lignocellulosic residues over food crops are that lignocellulosic material does not compete with food economics and does not require extensive environmental resources. The lignocellulosic complex is composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose account for 70% of dry cellulosic material, which makes biomass ideal feedstocks for biofuels as the sugars derived from cellulosic

material can be used as substrate for microbial fermentation (Claassen et al., 1999; Franden et al., 2009; Palmqvist & Hahn-Hagerdal, 2000; Pandey & Kim, 2011).

Several pretreatment methods have been developed to free the cellulose, which consists of glucose units, and hemicellulose, which consists of pentose sugars, mainly xylose (Figure 1.2 ; Hu & Ragauskas, 2012; Mosier et al., 2005). As a result of lignocellulosic pretreatment, inhibitors such as acids, furans, and phenolic compounds are present in lignocellulosic hydrolysate and can affect fermentation productivity (Palmqvist & Hahn-Hagerdal, 2000). While cellulose and hemicellulose can be used to produce various bioproducts, lignin is a complex polyaromatic structure that is often recalcitrant to most bacteria and therefore limited in its use as a renewable and valuable resource.

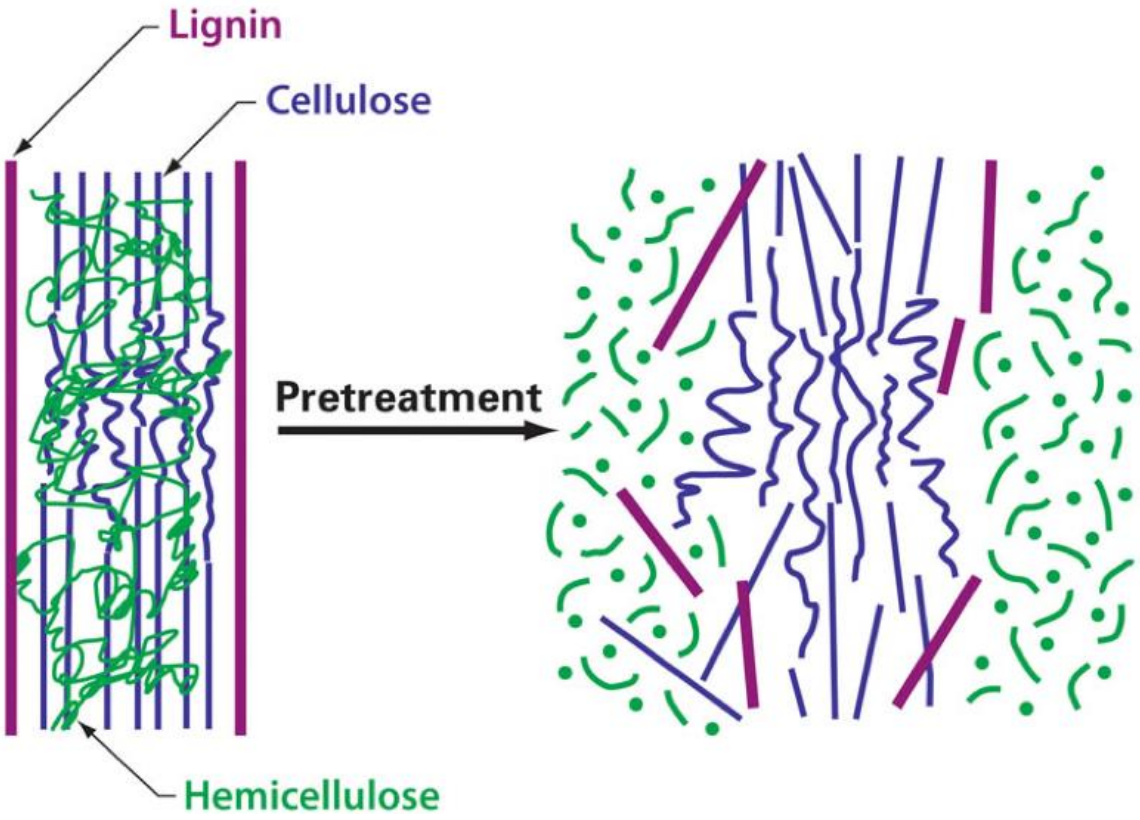


Figure 1.2 Pretreatment of lignocellulosic biomass.

The lignocellulosic complex is disrupted, releasing cellulose, hemicellulose, and lignin. Figure adapted from Mosier et al. 2005.

Furfural and acetic acid have been shown to significantly impact the yield of microbial fermentations, which is more widely documented for ethanol-producing microbes. As concentrations of acetic acid, formic acid, and levulinic acid increased, yeast ethanol yield decreased (Larsson et al., 1998). Acetate and furfural decreased the growth rates of *Zymomonas mobilis*, an ethanol-producing bacterium (Franden et al., 2009). Even if inhibitors do not affect ethanol yields, the growth rates and ethanol production rates can be inhibited by furans and aromatic compounds present in hydrolysates (Klinke et al., 2004). Thus, the challenge of using lignocellulosic biomass

for microbial conversion to a product lies in the ability of an organism to utilize pentose and hexose sugars while surviving in the presence of chemical inhibitors.

Lignin accounts for 10-25% of lignocellulosic material and the global paper industry alone produces almost 50 million tons of lignin per year (Gosselink et al., 2004). Only 2% of lignin is used commercially while the remainder is usually burned for energy; however, conversion of lignin offers potential for the microbial production of alternative fuels or bioproducts (Kosa & Ragauskas, 2012; Zakzeski et al., 2010). Lignocellulosic pretreatment while freeing sugars for fermentation, also degrades lignin to lignin oligomers and phenolic derivatives, such as vanillic acid, trans-p-coumaric acid and 4-hyroxybenzoic acid (Palmqvist & Hahn-Hagerdal, 2000; Saadia & Ashfaq, 2010). Degraded lignin fractions are often discarded due to lack of use and are ultimately found in industrial wastewaters (Saadia & Ashfaq, 2010; Upadhyaya et al., 2013; Wells et al., 2015). Alternatively, as cellulosic biomass refineries will produce more lignin than can be used for power (by burning), researchers are interested in transforming the excess lignin to value-added products, such as carbon fiber, plastics, and other chemicals currently being made from petroleum (Ragauskas et al., 2014).

1.3 Biodiesel

Biodiesel is a diesel fuel alternative made from renewable sources of triacylglycerides (TAGs) commonly found in vegetable oils and animal fats (Ma & Hanna, 1999; Tao & Aden, 2009). Transesterification is the common method in the US to make biodiesel by reacting a fat or oil with an alcohol to form fatty acid methyl or ethyl esters (FAMEs/FAEEs) and glycerol (Bart et al., 2010; Ma & Hanna, 1999). Ethanol or methanol are the most frequently used alcohols for transesterification due to their low

cost and quick reactions and a catalyst can also be used to improve the rate and yield of the reaction (Ma & Hanna, 1999). Different feedstock oils can affect the properties of the final biodiesel product, such as the cetane number, oxidative stability, and viscosity, which can ultimately have positive or negative impacts on biodiesel performance (Ciolkosz, 2009).

Soybean biodiesel, one of the most predominant alternative fuels in the US, reduces several air pollutants as well as greenhouse gases by 41% compared to diesel (Hill et al., 2006). Biodiesel also produces 93% more usable energy than its required input of fossil fuels compared to corn ethanol, which only has a 25% net gain of usable energy (Hill et al., 2006). Despite the growing interest in biodiesel, it is not economically viable as almost 85% of its cost lies in feedstock costs (Canakci & Sanli, 2008; Pinzi et al., 2013). Furthermore, biodiesel from edible oils, such as vegetable oils (soybean, sunflower, palm, etc.), is blamed for the increase in food prices and the abundant agricultural inputs for crop cultivation (Leiva-Candia et al., 2014). However, other researchers argue that soybeans are nitrogen fixers, requiring less agricultural inputs and are grown primarily for its protein with oil as a by-product (Granda et al., 2007). Soybean biodiesel is a promising fuel alternative, but feedstock costs and availability (Tao & Aden, 2009), fertile land requirements, and agricultural input are cost-limiting factors in its production (Hill et al., 2006). Therefore, alternative feedstocks and fuel sources must be studied.

Non-edible feedstock alternatives for biodiesel include waste frying oils, animal fats, algae oil, and oils from microorganisms. Waste frying oil and animal fats have had some success as a feedstock for biodiesel, but the high content of free fatty acids results

in biodiesel with high viscosity (Pinzi et al., 2013). Algae, yeasts, and bacteria can accumulate oil during optimal cultivation conditions and have several advantages over plant oils. These organisms have faster growth rates and are generally not affected by seasonal changes (Li et al., 2008). Challenges to microbial oil production remain in understanding the fatty acid metabolism (Kosa & Ragauskas, 2011; Lennen & Pfleger, 2013; Meng et al., 2009), metabolic engineering (Fischer et al., 2008; Lee et al., 2010; Shi et al., 2011) recovery of biomass and bioproducts (Grima et al., 2003), and scale-up concerns (Brigham et al., 2011; Cuellar et al., 2013).

1.4 Oleaginous Microbes

While biodiesel is most commonly extracted from TAGs stored in plants or animal fats, prokaryotes can also accumulate storage lipids and some species can synthesize TAGs by utilization of organic compounds (Alvarez & Steinbuchel, 2002; Holder et al., 2011). Historically, the appeal of microbial oils was due to a need for an alternative to plant oils and specific polyunsaturated fatty acids for human consumption (Wynn & Ratledge, 2005). The economics of most microbial oil has affected the feasibility of industrial production, but with the advancement of fermentation technology, microbial oil (primarily from fungi and yeast) can be produced in quantities equivalent to acres of agricultural land (Alvarez & Steinbuchel, 2002; Wynn & Ratledge, 2005). Microbial oils offer the unique advantage of being cultivated in a controlled environment, which results in consistency and reproducibility in biodiesel production (Fortman et al., 2008).

1.4.1 Microbial Lipids

Oleaginous microbes are those that accumulate more than 20 percent of their biomass as lipid (Wynn & Ratledge, 2005). These lipids, which can range from 20% to over 70% of the cell's biomass, are usually TAGs and stored intracellularly as a reserve supply of carbon and energy (Alvarez & Steinbuchel, 2002; Wynn & Ratledge, 2005). TAGs are water-insoluble triesters of glycerol with fatty acids that have a higher caloric value than carbohydrates and proteins, thus providing an efficient energy reserve, as TAGs yield much more energy when oxidized (Alvarez & Steinbuchel, 2002). In general, TAG accumulation occurs after synthesis of phospholipids during the exponential growth phase and when cellular growth is impaired during the stationary phase under excess carbon and limited nitrogen conditions (Ratledge & Wynn, 2002a). Several studies have shown that lipid accumulation in oleaginous microbes occurs after nitrogen exhaustion in the medium (Alvarez et al., 1996; Alvarez & Steinbuchel, 2002; Gouda et al., 2008; Kurosawa et al., 2010; Packter & Olukoshi, 1995; Silva et al., 2010). Phosphorus limitation has also been shown to influence lipid accumulation in oleaginous yeasts grown in high C:P ratios (Gill et al., 1977) and in high C:P ratios on nitrogen-rich substrate (Wu et al., 2010).

The carbon source for cell growth and TAG accumulation can vary, which results in variability of the fatty acid composition (Alvarez & Steinbuchel, 2002). This suggests potential advantageous opportunities for microbial oil production in the biofuel industry.

1.4.2 Lipid Synthesis in Oleaginous Yeasts and Bacteria

The fundamental requirement for lipid accumulation is a slow growth rate of the cells that will allow excess carbon to be assimilated faster than it can be converted for

growth. The slow growth rate implies nutrient limitation and therefore stationary phase has been reached. During stationary phase, cells are still metabolically active and can produce secondary metabolites (those not required for growth).

Mechanisms of lipid accumulation have been well studied in yeasts. The first identifiable metabolic event following nitrogen limitation was observed in yeast in the late 1970s and has been extensively reviewed (Ratledge & Wynn, 2002b). As nitrogen is depleted, intracellular adenosine monophosphate (AMP) concentration decreases immediately before lipid accumulation begins. As nitrogen is depleted, AMP deaminase (a nitrogen scavenging enzyme) activity increases, causing a decrease in AMP (Figure 1.3, triangle 1). AMP is essential for isocitrate dehydrogenase (ICDH) to catalyze the reaction of isocitrate to 2-oxoglutarate. As AMP concentration decreases, ICDH activity also decreases (Figure 1.3, triangle 2). Isocitrate equilibrates with citrate by the enzyme aconitase (Figure 1.3, triangle 3). The citrate is transported out of the mitochondria into the cell where it is cleaved by ATP:citrate lyase (ACL) to oxaloacetate and acetyl-CoA (Figure 1.3, triangle 4). ACL was the first identified enzyme that influences the different metabolic activity exhibited between oleaginous yeast and nonoleaginous yeast. Acetyl-CoA can then be used for fatty acid synthesis and TAG accumulation, assuming the proper enzymes are available. Wynn and Ratledge have also shown malic enzyme (ME), which converts malate to pyruvate and NADPH, to be of importance in achieving high concentrations of NADPH for fatty acid synthesis and lipid accumulation (Ratledge, 2002; Wynn & Ratledge, 2005).

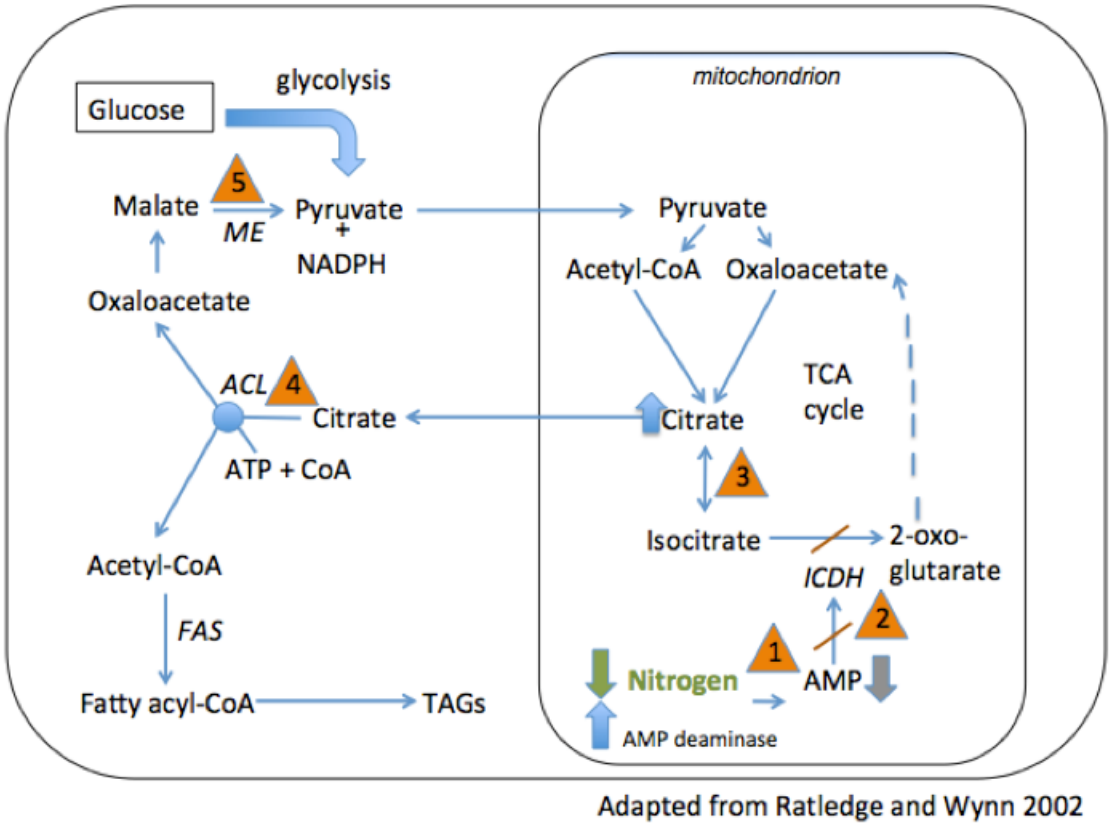


Figure 1.3 Schematic of lipid accumulation in oleaginous microbes. Adapted from Ratledge and Wynn 2002.

While nitrogen limitation initiates a metabolic shift to favor TAG accumulation in oleaginous microbes, excess glucose (in a high C:N ratio) must still be assimilated (Figure 1.4). A key enzyme in glycolysis is phosphofructokinase (PFK), which can be inhibited by high concentrations of citrate. As previously described, nitrogen depletion ultimately results in an increase of citrate. However, phosphofructokinase can form a stable complex with NH_4 and remain active so that carbon can still be assimilated (Wynn et al., 2001). Furthermore, pyruvate kinase is regulated by citrate to ensure a continuous flow of carbon to pyruvate (Evans & Ratledge, 1984).

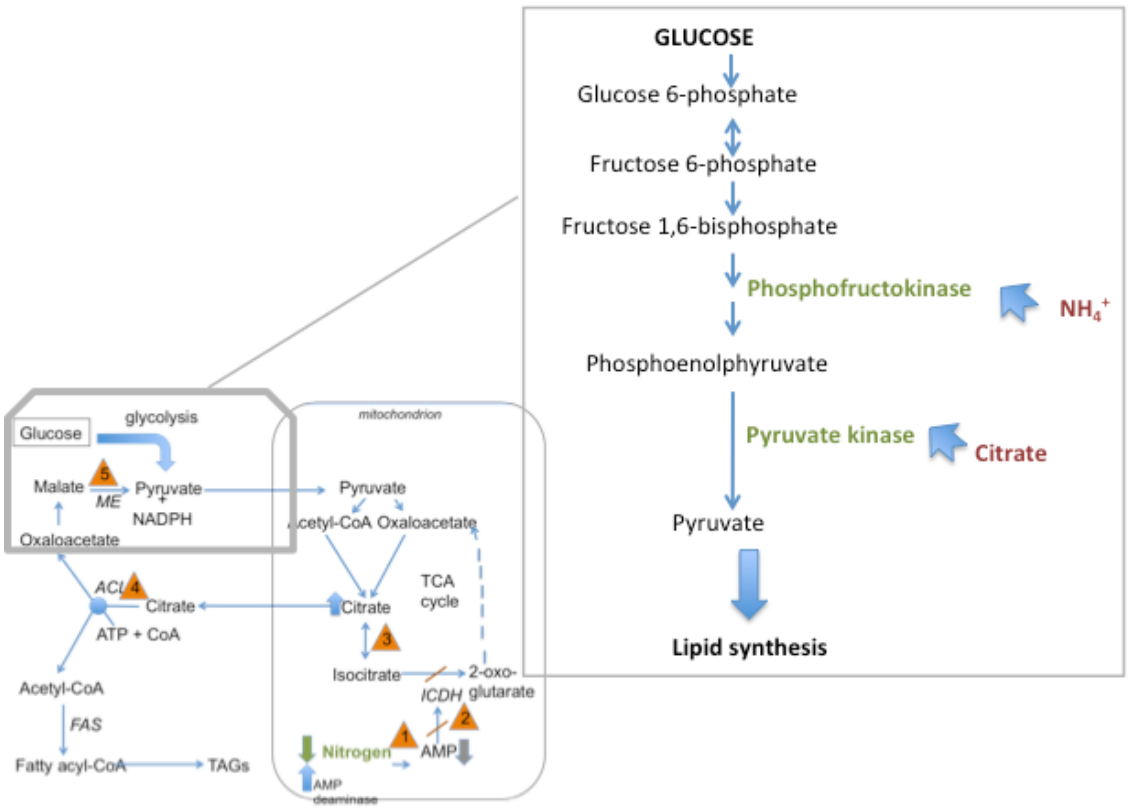


Figure 1.4 Schematic of glycolysis in response to a high C:N ratio.

Similarly, when nutrient limitation occurs in some bacteria, cell growth is halted and lipid accumulation can occur (Alvarez & Steinbuchel, 2002). TAG accumulation occurs predominantly in stationary phase (Alvarez et al., 2000) as fatty acid biosynthesis shifts from phospholipids for cell proliferation to TAG storage (Alvarez & Steinbuchel, 2002). Diacylglycerol acyltransferase (DGAT) has been shown to be a key enzyme in TAG biosynthesis (Alvarez et al., 2000; Davila Costa et al., 2015). Research to identify other key enzymes involved in bacterial lipid biosynthesis is still ongoing (Davila Costa et al., 2015).

1.4.3 *Rhodococcus opacus* as a Model Oleaginous Bacterium

Bacteria belonging to the gram-positive Actinobacteria phylum, such as *Streptomyces*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Dietzia*, or *Gordonia*, can synthesize and accumulate TAGs (Alvarez & Steinbuchel, 2002). Of these bacteria, some *Rhodococcus* species have been characterized for TAG accumulation (Alvarez et al., 2008; Alvarez & Steinbuchel, 2002; Hernandez et al., 2013; Silva et al., 2010) and have been shown to accumulate up to 80% of cellular biomass as lipids (Alvarez et al., 1996).

Rhodococcus opacus PD630, the first *Rhodococcus* species to be labeled oleaginous, can accumulate variable amounts of TAGs in relation to dry cell weight when grown on different substrates: 76% with gluconate; 38% with hexadecane; and 87% with olive oil (Alvarez et al., 1996). Lipids are stored within inclusion bodies that form intracellularly in *R. opacus* PD630. These inclusions are characterized as having a definite shape, suggesting the presence of a surface membrane as well as the associated proteins involved in inclusion formation (Alvarez et al., 1996). TAGs accumulate intracellularly during periods of starvation, ensuring survival in rapidly changing, harsh environments (Alvarez & Steinbuchel, 2002). Furthermore, inexpensive feedstocks like organic wastes or agro-industrial waste from carob, orange, and sugarcane molasses can be used for TAG accumulation in *R. opacus* PD630 (Gouda et al., 2008). High density cell cultivation of *R. opacus* PD630 obtained high TAG concentrations in bioreactors when sugar beet molasses and sucrose were used as substrates (Voss & Steinbuchel, 2001). TAG accumulation from growth on a variety of carbon sources (Alvarez & Steinbuchel, 2002; Holder et al., 2011) make *R. opacus* a potential candidate for biotechnological application (Gouda et al., 2008; Voss & Steinbuchel, 2001), while its

catabolic versatility has made *Rhodococcus* species popular in bioremediation (Larkin et al., 2005; Martinkova et al., 2009). The potential to couple the utilization of diverse substrates with TAG accumulation in *R. opacus* offers a promising approach to alternative fuels.

1.4.4 *Rhodococcus rhodochrous*: Potential as Oleaginous Bacterium

Rhodococcus rhodochrous was first described in 1891 (Overbeck, 1891; Zopf, 1891) as a generic term for a group of similar strains that were ultimately reclassified as *Mycobacterium rhodochrous* (Gordon, 1966) and then *Rhodococcus rhodochrous* (Goodfellow & Alderson, 1977). *Rhodococcus rhodochrous* is a red pigment-forming *Rhodococcus* species most notably used for the industrial production of acrylamide from acrylonitrile (Kobayashi & Shimizu, 1998; Yamada & Kobayashi, 1996). Because of a diverse metabolism, the degradative capability of *R. rhodochrous* that can utilize polychlorinated biphenyls, hydrocarbons, and other aromatic compounds as a sole carbon source has been extensively reviewed (Martinkova et al., 2009).

R. rhodochrous contains mycolic acids, which has enabled this bacterium to be utilized as a model for studying toxic glycolipids produced by other *Mycobacterium, Nocardia,* and *Rhodococcus* species (de Almeida & Ioneda, 1989; Ioneda & de Almeida, 1991). Neutral plus fatty acids, total glycolipids, and high polar lipids isolated from *R. rhodochrous* after growth on glucose, galactose, or mannose and were shown to be differentially toxic in the mouse model (de Almeida & Ioneda, 1989). *R. rhodochrous* showed significant growth in glucose (with nitrogen) after 120 hours and produced 6% diethyl ether-soluble lipids (de Almeida & Ioneda, 1989). Sorkhoh et al. characterized lipids of *R. rhodochrous* after growth on dodecane and glucose (Sorkhoh et al., 1990). As

R. rhodochrous is a hydrocarbon degrader, more total lipids were observed from growth on dodecane than glucose and included the lipid classes of sterols and diacylglycerophosphocholines (Sorkhoh et al., 1990). *R. rhodochrous* has also been shown to produce fatty acids during total mineralization of hexadecane and octadecane (Rodgers et al., 2000). These studies have shown that diversity in hydrocarbons used as carbon sources impact the production and accumulation of lipids in *R. rhodochrous.*

Rhodococcus rhodochrous ATCC 21198, originally named *Nocardia paraffinica*, is patented for its utilization of gaseous hydrocarbons, including propane, ethane, and butane (Tanaka et al., 1973). Previous research has shown that *Rhodococcus* species grown on propane are induced for co-metabolic degradation of vinyl chloride. Babu and Brown (1984) identified a new propane oxygenase involved in propane metabolism by *R. rhodochrous* ATCC 21198 and more recently, vinyl chloride degradation by a *R. rhodochrous* propane oxygenase was also shown (French, 2000). Despite the widespread use of *R. rhodochrous* in degradation research, the understanding of its ability for lipid accumulation is limited.

1.5 Research Significance and Purpose

R. rhodochrous lipid accumulation for the purpose of FAMEs and biodiesel production is not well characterized. *R. rhodochrous* has been shown to produce lipids when grown on glucose and dodecane substrates (Sorkhoh et al., 1990). The ability for *R. rhodochrous*, a well-known hydrocarbon degrader, to use glucose as a substrate for lipid accumulation will provide insight into the potential capabilities of *R. rhodochrous* as an oleaginous microbe. The first aim of this dissertation was to test the hypothesis that *R. rhodochrous* accumulates lipids, such as triglycerides, intracellulary when grown in

glucose-supplemented media. The objectives were (1) to visually determine whether *R. rhodochrous* can accumulate intracellular lipids using transmission electron microscopy and (2) to quantitatively characterize lipid accumulation by *R. rhodochrous* using a Bligh and Dyer lipid extraction method (Bligh & Dyer, 1959) coupled with thin-layer chromatography. We also obtained the genomic sequence of *R. rhodochrous* ATCC 21198, which provided insight towards its lipid accumulation capabilities.

The versatile metabolism of *R. rhodochrous* allows for degradation of several environmental contaminants, persistent compounds, and potentially lignin-derived chemicals. *R. rhodochrous* has been successfully applied to bioremediation efforts, but to our knowledge lipid accumulation for biofuel production has not been characterized. The aim of this research was to combine these applications, while producing a usable product, with the specific intention of displacing the current costly feedstocks for biodiesel production. The second aim of this dissertation was to test the hypothesis that *R. rhodochrous* tolerates model lignocellulosic hydrolysate inhibitors and utilizes phenolic substrates, including model lignin compounds for lipid accumulation. The objectives were to investigate the growth and lipid accumulation by *R. rhodochrous* grown in (1) acetic acid and furfural, (2) phenol, and the (3) model aromatic lignin compounds, vanillic acid and 4-hydroxybenzoic acid. We also performed a proteomic analysis on *R. rhodochrous* grown in phenol to gain further insight towards the enzymatic activity in aromatic substrate degradation and lipid accumulation.

As natural gas is concurrent with petroleum, oil companies will burn off the natural gas in the process of oil production. Not only does the flaring of natural gas add to the already critically high number of atmospheric greenhouse gases, but flaring also

wastes a potentially valuable substrate. The ability of *R. rhodochrous* to use gaseous hydrocarbons would allow for an alternative to gas flaring. Furthermore, if utilization of gases can be coupled with growth and lipid accumulation by *R. rhodochrous*, these lipids can be used for dietary supplements or alternative fuel production. Therefore, the third aim of this dissertation was to test the hypothesis that *R. rhodochrous* utilizes gaseous hydrocarbons for lipid accumulation. This study was a preliminary, proof-of-concept study to determine if lipid accumulation occurs when *R. rhodochrous* is grown on propane.

This work was performed with funding under the directives of the Environmental Protection Agency (EPA) Science To Achieve Results Fellowship. Characterization of lipid accumulation by *Rhodococcus rhodochrous* addresses the EPA mission of promoting sustainable and livable communities by: (1) helping to fulfill society's energy demand by using oleaginous microbes as an alternative biodiesel feedstock; (2) using waste as a substrate for microbial lipid accumulation repurposes potential environmental contaminants into energy resources; and thereby (3) cleaning-up a community while establishing a sustainable fuel alternative.

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CHAPTER II

DRAFT GENOME SEQUENCE OF *RHODOCOCCUS RHODOCHROUS* STRAIN ATCC 21198

Shields-Menard, SA., SD Brown, DM Klingeman, K Indest, D Hancock, JJ Wewalwela, WT French, and JR Donaldson. 2014. Draft Genome Sequence of *Rhodococcus rhodochrous* strain ATCC 21198. Genome Announcements. 13;2(1).

2.1 Abstract

Rhodococcus rhodochrous is a Gram-positive red-pigmented bacterium commonly found in the soil. The draft genome sequence for *R. rhodochrous* strain ATCC 21198 is presented here to provide genetic data for a better understanding of its lipid accumulation capabilities.

2.2 Draft Genome Sequence of *Rhodococcus rhocochrous* Strain ATCC 21198

Rhodococcus rhodochrous is a red pigment-forming Gram-positive bacterium belonging to the Actinobacteria phyla and the Nocaridaceae family. This metabolically diverse bacterium has been most notably used for the industrial production of acrylamide from acrylonitrile and in the bioremediation of hydrocarbons, polychlorinated bisphenyls, and other aromatic compounds (Yamada and Kobayashi 1996, Kobayashi and Shimizu 1998, Larkin, Kulakov et al. 2005, Martinkova, Uhnakova et al. 2009). Other members of the Actinobacteria, such as *Streptomyces*, *Nocardia*, *Rhodococcus*, and *Gordonia* have

been shown to synthesize and accumulate triacylglycerols (TAGs;(Alvarez and Steinbuchel 2002)). The potential for *R. rhodochrous* to utilize a variety of carbon sources for accumulation of TAGs is of interest due to the increasing global need for alternative biofuels. Despite the widespread use of *R. rhodochrous* in degradation research, the understanding of its ability for lipid accumulation is limited.

A draft genome sequence was generated for strain ATCC 21198 to develop a better understanding of the lipid accumulation capabilities of *R. rhodochrous*. The isolate of *R. rhodochrous* ATCC 21198 was purchased from American Type Culture Collection (ATCC). Genomic DNA was isolated from frozen cell pellets using the MoBio PowerSoil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) modified with the addition of lysozyme.

Sequence data was generated using an Illumina MiSeq instrument (Quail, Smith et al. 2012) according to the manufacturer's instructions, using a paired-end approach with an approximate insert library size of 400 bp and read lengths of 250 bp, The CLC Genomics Workbench (version 6.5) was used to trim and filter reads for quality sequence data and subsequent assembly. The draft genome sequence for *R. rhodochrous* ATCC 21198 strain is represented by 161 DNA contigs with an estimated genome size of ~6.4Mb and G+C DNA content of 70.2%. The average sequence depth coverage across the genome was ~214 times the genome size, which was annotated as described previously (Brown, Klingeman et al. 2012) for 6,039 predicted protein-encoding gene models.

In response to an environment with little nitrogen and excess carbon, oleaginous microbes will store carbon as lipid (Alvarez and Steinbuchel 2002, Ratledge 2002). *Rhodococcus* species have been shown to produce large amounts of lipid in the form of TAGs due to high activity of the enzyme, acyl-CoA:Diacylglycerol acyltransferase (DGAT; (Alvarez, Alvarez et al. 2008). In this draft sequence of *R. rhodochrous*, there are several predicted wax ester synthase/diacylglycerol acyltransferase genes showing sequence identity to other *Rhodococcus* species. Other putative fatty acid and TAG synthesis genes were also discovered in the *R. rhodochrous* genome such as acetyl-CoA carboxylase, acyl carrier proteins, 1-Acyl-G3P acyltransferase, glycerol kinase, and glycerol-3-phosphate dehydrogenase (Kosa and Ragauskas 2011). Several cytochrome P450-like enzymes were predicted in the genome of *R. rhodochrous* as well as the associated genes, flavodoxin and feredoxin. Other predicted genes related to degradation mechanisms include oxygenases, dioxygenases, and a putative aromatic degradation protein. The *R. rhodochrous* draft genome offers insight into the potential metabolic capabilities of this organism and will help to facilitate further studies.

This draft genome sequence has been deposited in GenBank under accession number AZHI00000000. The version described in this paper is version AZHI01000000. For strain and DNA requests contact T. F.

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CHAPTER III

LIPID ACCUMULATION BY *RHODOCOCCUS RHODOCHROUS* GROWN ON GLUCOSE

Shields-Menard, SA. M Amirsadeghi, B Sukhbaatar, E Revallame, R Hernandez, JR Donaldson, and WT French. 2015. Lipid accumulation by *Rhodococcus rhodochrous* grown on glucose. Journal of Industrial Microbiology and Biotechnology. DOI 10.1007/s10295-014-1564-7

3.1 Abstract

Biodiesel is an alternative fuel made from costly vegetable oil feedstocks. Some microorganisms can accumulate lipids when nutrients are limited and carbon is in excess. *Rhodococcus rhodochrous* is a gram-positive bacterium most often used in bioremediation or acrylamide production. The purpose of this study was to investigate and characterize the lipid accumulation capabilities of *R. rhodochrous*. Shake flasks and a large-scale fermentation were used to cultivate *R. rhodochrous* in varying concentrations of glucose. *R. rhodochrous* achieved almost 50 % of dry cell mass as lipid when grown in 20 g/L of glucose. Wax esters and triglycerides were identified in *R. rhodochrous* lipid extract. The transesterified extractables of *R. rhodochrous* consisted of mostly palmitic (35 %) and oleic (42 %) acid methyl esters. This study shows *R. rhodochrous* to be an oleaginous bacterium with potential for application in alternative fuels.

3.2 Introduction

Alternative fuels are necessary to fulfill the increasing demand for sustainable liquid fuel sources. As fossil fuels are expected to remain a dominant source of energy, sustainable fuels are being used to displace the fossil fuels used for transportation (IEA 2012). Despite developments in corn ethanol and lignocellulosic ethanol, the issues of land use, chemical input, and food competition persist (Tao and Aden 2009, Graham-Rowe 2011). Biodiesel is an alternative to diesel fuel that is made from the transesterification of triglycerides, commonly found in animal fat and vegetable oils (Ma and Hanna 1999). Currently, the most common biodiesel feedstock is soybean oil, which accounts for almost 70 % of the total cost of biodiesel and uses acres of arable land that is subject to a volatile climate (Hill, Nelson et al. 2006, Tao and Aden 2009, Shi, Valle-Rodriguez et al. 2011).

Microbial oils are an alternative, which could use lignocellulosic mono-sugars as substrate to serve as a reliable, climate-independent source of oil (Fortman, Chhabra et al. 2008, Li, Du et al. 2008, Graham-Rowe 2011). These lignocellulosics, such as energycane, switchgrass, and giant miscanthus, are advantageous because they require minimal inputs and are more resistant to changes in weather (Graham-Rowe 2011). This lignocellulosic biomass must be pretreated to release sugars for fermentation by microorganisms. While pretreatment releases sugars, it also produces microbial growth inhibitors such as acetic acid, furfural, and phenolic lignin compounds (Palmqvist and Hahn-Hagerdal 2000). Not all microbes can tolerate these conditions and lipid yields may be affected. Therefore, identifying novel oleaginous microbes is essential to

improving the use of diverse substrates, such as lignocellulosic material, for microbial lipid accumulation.

While the oleaginous capabilities of yeasts are well characterized, the potential of oleaginous bacteria are still being investigated. Microbes accumulating over 20 % of its biomass as lipid are considered to be oleaginous microbes (Wynn and Ratledge 2005). Generally, this lipid accumulation occurs in stationary phase when a key nutrient (e.g. nitrogen) is limited and a carbon source is in excess (Alvarez and Steinbuchel 2002, Ratledge 2002). Perhaps the most well-known oleaginous bacterium, *Rhodococcus opacus,,* has been shown to accumulate almost 80 % of its biomass as triglycerides (Alvarez, Mayer et al. 1996). Other oleaginous bacteria include *Mycobacterium*, *Streptomyces*, and *Acinetobacter* species as well as other species of *Rhodococcus* (Alvarez and Steinbuchel 2002).

Microbial lipid accumulation has been observed in bacteria belonging to the actinomycetes group, frequently cultivated on gluconate, glucose, and other sugar substrates or hydrocarbons (Alvarez and Steinbuchel 2002). *Rhodococcus rhodochrous* is a red-pigmented, gram-positive, soil bacterium also belonging to the Actinomycetes clade. Due to its ability to utilize a range of aromatic compounds and hydrocarbons, including hydrocarbon gasses, *R. rhodochrous* is also used in bioremediation efforts (Martinkova, Uhnakova et al. 2009). A hydrocarbon-degrading strain of *R. rhodochrous* isolated from Kuwait was grown in 10g/L of glucose and had a total lipid content of 1.1 %, compared to 4.1% when grown in 10g/L of dodecane (Sorkhoh, Ghannoum et al. 1990). Therefore, the limitations of using pure glucose as a carbon source involve not only cost and sustainability, but glucose might not be the ideal substrate for the organism

to achieve maximum lipid yield (i.e. hydrocarbons could produce higher yields). The purpose of the initial shake flask experiments was to investigate the ability for *R. rhodochrous* to accumulate lipids and to identify the concentration of glucose for optimum lipid yield.

As interest in renewable fuels increases, oleaginous microbes are at the forefront of multi-disciplined research. Advantages of microbial oil include less land-use, controlled cultivation environment, and consistent yields (Alvarez and Steinbuchel 2002, Wynn and Ratledge 2005, Fortman, Chhabra et al. 2008). These advantages make microbial oil a favorable alternative to crop or animal derived biodiesel feedstocks. The characterization of other oleaginous bacteria creates opportunities for the development of alternative feedstocks and technologies. Despite the widespread use of *R. rhodochrous* in industry, the understanding of its ability for lipid accumulation is not well known. This research seeks to provide *R. rhodochrous* with the designation of oleaginous as it investigates its lipid accumulation abilities in an effort to offer a unique approach to addressing the need for alternative fuels.

3.3 Materials and Methods

3.3.1 Culture Conditions

R. rhodochrous, obtained from the American Type Culture Collection (#21198), was maintained in nutrient broth or on nutrient agar (Fisher Scientific, Pittsburg, Pennsylvania) and incubated at 30°C. Inoculums were prepared by transferring lawns of *R. rhodochrous* grown for 5 days on nutrient agar to phosphate buffer saline or an aliquot of sterile Sorkhoh medium (Sorkhoh et al 1990). The medium was supplemented with 10g/L (C:N of 27:1), 20 g/L (C:N of 54:1) or 40g/L (C:N of 108:1) of glucose for shake

flask experiments and 20 g/L of glucose for the fermenter experiment. The cultures were incubated in baffled flasks at 30°C in a shaking incubator at 125 rpm (New Brunswick Scientific Model I26, Edison, New Jersey). The shake flask experiments were conducted in triplicates and the experimental mean and standard deviation were calculated for all gravimetric and sugar analyses. Repeated measures ANOVA were performed using the Statistical Package for the Social Sciences (SPSS) software when necessary and significance was determined when $p<0.05$.

R. rhodochrous was grown in a 14L BIOFLO 410 fermenter (New Brunswick Scientific, Edison, NJ) with a working volume of 7L. The pH of the culture was uncontrolled but monitored using the systems pH probe. Temperature was maintained at 30°C and agitation was set to 300 rpm (Mondala et al. 2012). Antifoam 204 concentrate (0.01 % v/v; Sigma-Aldrich, St. Louis, MO) was aseptically added via syringe filter. Air filtered through a 0.45um HEPA vent filter (Whatman, Kent, UK) was bubbled through the culture at 1vvm (volume of air per volume of media per minute; Mondala et al. 2012). The inoculum was prepared as previously described for the shake-flask experiments. Samples were taken as described below every 12 hours until 48 hours and then daily until 168 hours. The average of two samples per time point was reported.

3.3.2 Analysis

Culture samples (50 mL) were collected every 24 hours and centrifuged at 4500 rpm for 10 minutes. The supernatant was removed and used for pH (Accumet pH meter) and sugar measurements and the pellet was frozen at -20°C. Glucose concentrations were measured for all samples in triplicate using an YSI 2900 Biochemistry Analyzer (YSI

Incorporated Life Sciences, Yellow Springs, Ohio). The experimental mean and standard deviation is reported for all shake flask experiments. Residual nitrate in the supernatant was determined using the manufacturer's methods of an ICS 3000 ion chromatography system (Dionex Corp., Sunnyvale, CA) equipped with an IonPac AS16 anion exchange analytical column (250 x 4mm), AG16 guard column (50 x 4mm), and a conductivity detector. Averages and standard deviations of triplicate injections of one sample are reported for nitrate results.

Lipids were extracted from a freeze-dried pellet using a modified Bligh and Dyer (Bligh and Dyer 1959) method as described by Revellame et al. (2012). The extract was filtered through glass wool into a tared amber vial. The solvent was removed using a TurboVap LV (Caliper Life Sciences, Hopkinton, MA, USA) at 50°C and a constant flow of nitrogen gas at a rate of 15psi for 50 minutes. The recovered extract was weighed to determine the gravimetric lipid yield (mean and standard deviation of triplicate flasks are reported) . The extractables were then transesterified into fatty acid methyl esters (FAME) and analyzed by three injections of one sample using an Agilent 6890N gas chromatograph with a flame-ionization detector (GC-FID; Agilent Technologies Inc., Wilmington, Delaware) with a Zebron ZB-FFAP column (30 m x 0.25 mm, film thickness 0.25 um). Means and standard deviations of triplicate injections of one sample are reported. Oven temperature was 50-250°C with a rate of 10°C per minute. Helium was used as the carrier gas with a flow rate of 1.5 mL per minute and the detector temperature was 260°C. The instrument was calibrated using a standard solution containing known concentration of C9-C24 FAMEs (purchased from Sigma) by taking in

account the response factor of internal standard. 1, 3- dichlorobenzene was used as an internal standard.

The composition of the lipid extract from fermenter samples was evaluated using thin-layer chromatography (TLC) as described previously (Revellame, Hernandez et al. 2012). The dried lipid extracts were reconstituted in 1mL of chloroform and applied to a 20 x 20 cm glass-backed Analtech Uniplate™ pre-coated with 250 um silica gel-G (Sigma-Aldrich, St. Louis, MO, USA). Drummond microcaps® disposable pipettes (Fisher Scientific, Pittsburg, PA, USA) were used to apply samples (20uL) and standards (30uL). Standards of phospholipid mixture, mono-,di-, and triglycerides, and palmityl palmitate (Sigma-Aldrich, St. Louis, MO, USA) were used to represent lipid classes of interest. Plates were developed in either 94/6 (v/v) n-hexane/diethyl ether or 85/15/2 (v/v/v n-hexane/diethyl ether/acetic acid and then sprayed with a 10 % (w/v) cupric sulfate in 8 % (v/v) phosphoric acid to visualize bands. The plates were allowed to dry and then charred in a 150°C oven.

3.3.3 Transmission Electron Microscopy (TEM)

Lipid accumulation by *R. rhodochrous* was visualized using TEM images of samples taken during fermentation. A sample of 2mL of culture was pelleted to remove the media and fixed in 2.5 % glutaraldehyde in 0.1 M Na-Cacodylate buffer at pH 7.2. Fixed samples were rinsed with 0.1 M Na-Cacodylate buffer and then post fixed in buffered 2 % osmium tetraoxide. Samples were rinsed once more in water, *en bloc* stained with 2 % aqueous uranyl acetate, dehydrated in a graded ethanol series, and embedded in Spurr's resin as similarly described by Alvarez et al (Alvarez, Mayer et al. 1996). Ultra-thin sections were cut with a Reichert-Jung Ultracut 3 ultra-microtome and

stained with uranyl acetate and lead citrate. Stained sections (two sections per sample) were viewed on a JEOL 1230 120kV TEM.

3.4 Results and Discussion

3.4.1 Effect of Glucose Concentration on Lipid Accumulation

For this study, three different concentrations of glucose were used to investigate the effect of glucose on the lipid accumulation abilities of *R. rhodochrous*. Glucose was exhausted in the 10g/L glucose treatment by 48 hours whereas in the 20 g/L and 40g/L treatments, glucose concentrations were reduced to 6.5g/L and 25.8g/L, respectively (Figure 3.1A). Nitrate was consumed in all treatments after 48 hours of incubation (Figure 3.1B), which coincides with the start of lipid accumulation (Figure 3.1D) that occurs as the cells enter into stationary phase due to depletion of nitrogen (Figure 3.1A).

Cell growth was observed in all treatments and the 40g/L glucose treatment achieved the greatest cell dry weight of almost 7g/L at 96 and 120 hours ($p<0.05$; Figure 3.1A). The 10g/L and 20 g/L glucose treatment achieved significantly greater lipid accumulation by 72 hours than the 40g/L treatment ($p<0.05$; Figure 3.1D). There was no significant difference in lipid accumulation between 10g/L and 20 g/L glucose treatments, but the 20 g/L glucose treatment achieved slightly greater percent lipid to cell dry weight yield of 47 % in 72 hours (Figure 3.1D) compared to the 10g/L treatment, suggesting that 20 g/L of glucose is better for lipid accumulation as non-lipid biomass did not increase after 48 hours (data not shown). These results show that *R. rhodochrous* was able to grow in various concentrations of glucose as indicated by an increase in cell dry weight (Figure 3.1A) but maximum lipid accumulation occurred in shake-flasks with 20

g/L of glucose (Figures 3.1A and 3.1D). Controlled pH conditions could help to promote glucose consumption and increase lipid accumulation.

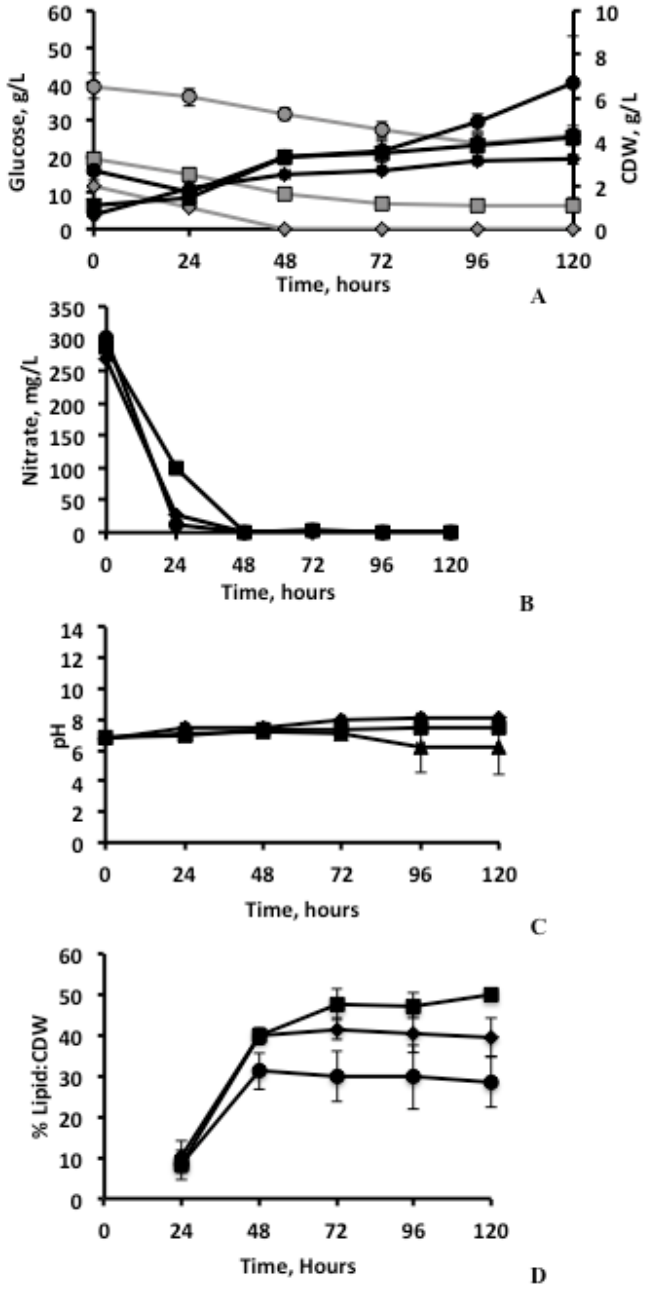

Figure 3.1 Effects of glucose on lipid growth and lipid accumulation of *R. rhodochrous*.

Glucose concentration (gray; g/L ±SD) and *R. rhodochrous* cell dry weight (black; g/L ±SD; A), nitrate concentration (mg/L ±SD; B), pH (±SD; C), and percent lipid (±SD; D) of cell dry weight accumulated by *R. rhodochrous* shake flask cultures supplemented with 10 (◆), 20 (■), or 40g/L (•) of glucose.

The initial pH for all experiments was 6.8 and this increased throughout the duration of the experiment (Figure 3.1C), unlike most fermentations of oleaginous yeasts in which the pH decreases with lipid accumulation and glucose consumption (Ratledge 1982, Brown, Hasan et al. 1990, Johnson, Singh et al. 1992). Uncontrolled pH could have caused the observed plateau of glucose consumption and lipid accumulation by *R. rhodochrous* (Figures 3.1A and D). This citrate-producing strain of *R. rhodochrous* has an optimal pH range of 6-9 (Kimura and Nakanishi 1975) and was in this range throughout the experiment (Figure 3.1C). If citrate is being produced during fermentation, not only is this citrate potentially driving lipid accumulation (Evans and Ratledge 1984), but an abundance could be acting as a buffer to maintain a neutral to alkaline pH. Furthermore, an abundance of citrate could also be used as a substrate, ultimately producing the alkaline by-products of sodium bicarbonate and ammonia.

3.4.2 Characterization of Lipid Accumulation

A New Brunswick BIOFLO 410 fermenter was used to investigate the effect of larger scale fermentation on growth, glucose consumption and lipid accumulation by *R. rhodochrous*. The inoculum size and larger volume of the fermenter could have contributed to the longer lag phase, although biomass reached 7g/L after one week of growth (Figure 3.2A). Similar total glucose consumption (decreased to less than 5g/L) and percent lipid accumulation (over 40 %) were observed in the fermenter trial (Figures 3.2A and B) as was observed in the shake flask experiments.

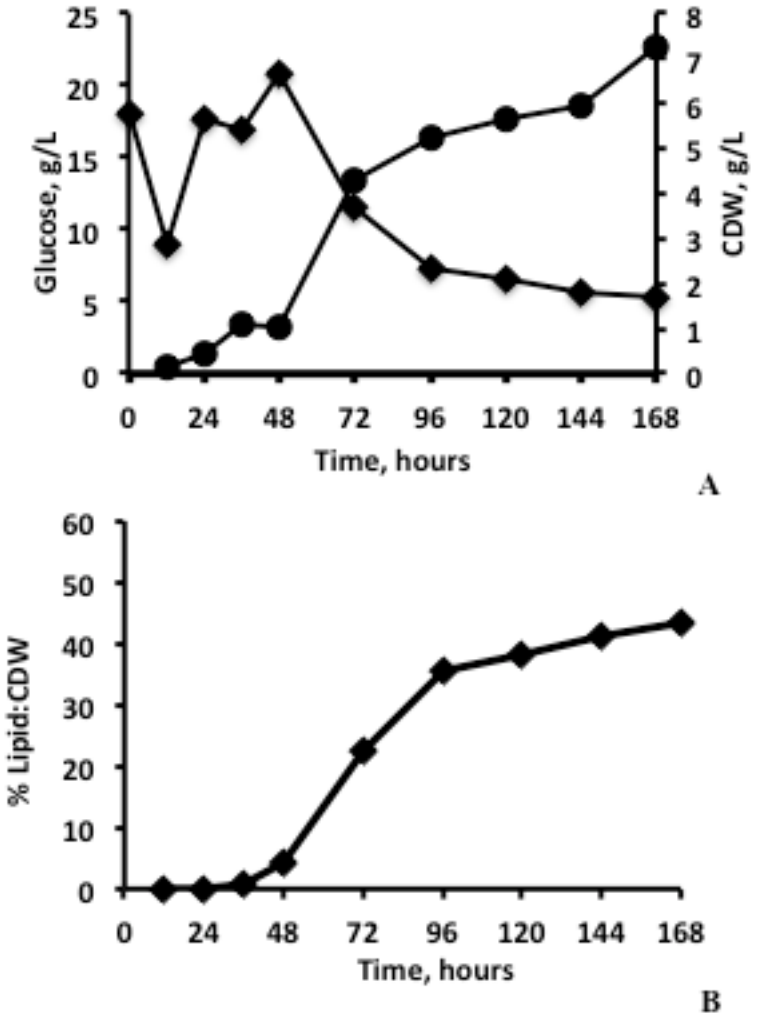

Figure 3.2 Growth and lipid accumulation by *R. rhodochrous* in fermenter cultivation. Glucose concentration (◆g/L ±SD) and *R. rhodochrous* cell dry weight (•; g/L; A) and percent lipid of cell dry weight accumulated (B) of fermenter experiment using 20 g/L of glucose-supplemented medium.

Growth kinetics of *R. rhodochrous* on glucose fermentation showed that the lipid accumulation increased rapidly after 48 hours of fermentation, which is attributed to the nitrogen depletion time. The cell growth also increased rapidly with a rate of biomass production of 0.0503 g/l-h. The rate of lipid accumulation (0.0206 g/L-h) increased after 48 hours of fermentation as interpreted from the slope of the graph for lipid accumulation

versus time. However, the lipid is produced during both growth phase (growth and accumulation) and stationary phase (accumulation). This led to a lipid yield $Y_{Lipid/Glu}$ of 0.3 g lipid/g of sugar consumed. In Table 3.1 some oleaginous microorganisms and their corresponding kinetic values considering the type of substrate and cultivation mode were presented (Papanikolaou, Fakas et al. 2008, Economou, Aggelis et al. 2011, Saenge, Cheirsilp et al. 2011). This suggests that the cultivation of *R. rhodochrous* in larger scale fermentations can also achieve oleaginous lipid yields.

Table 3.1 Comparison of parameters related to biomass and lipid production among different oleaginous species grown on various substrates.

Organism	Maximum CDW (g/L)	Biomass Productivity (g/L-h)	Lipid (%)	Lipid Productivity (g/L-h)	Lipid Yield (g/g)	Growth mode	Substrate	Reference
Rhodococcus rhodochrous	7.3	0.0503	43	0.0206	0.3	batch	glucose	This study
Mortierella isabellina	8.5	0.02	51.7	0.0125	NA	batch	glycerol	23
Rhodotorula glutinis	8.17	NA	52.91	0.058	0.067	batch	glycerol	28
Mortierella isabellina	14.2	NA	52	NA	0.242	batch	Sweet sorghum	7

Transmission electron microscope images further reveal intracytoplasmic lipid inclusion bodies from fermenter-cultivated cells (Figure 3.3), which resemble the electron-transparent inclusions shown in images of other oleaginous microbes (Alvarez, Mayer et al. 1996, Waltermann, Hinz et al. 2005, Waltermann and Steinbuchel 2005, Kalscheuer, Stoveken et al. 2006, Kalscheuer, Stoveken et al. 2007). A thin section sample at 120 hours shows intracellular lipid inclusion bodies that correspond to 40 % gravimetric lipid accumulation (Figure 3.3). This lipid accumulation occurred in stationary phase and is consistent with that of other oleaginous microbes (Waltermann and Steinbuchel 2005).

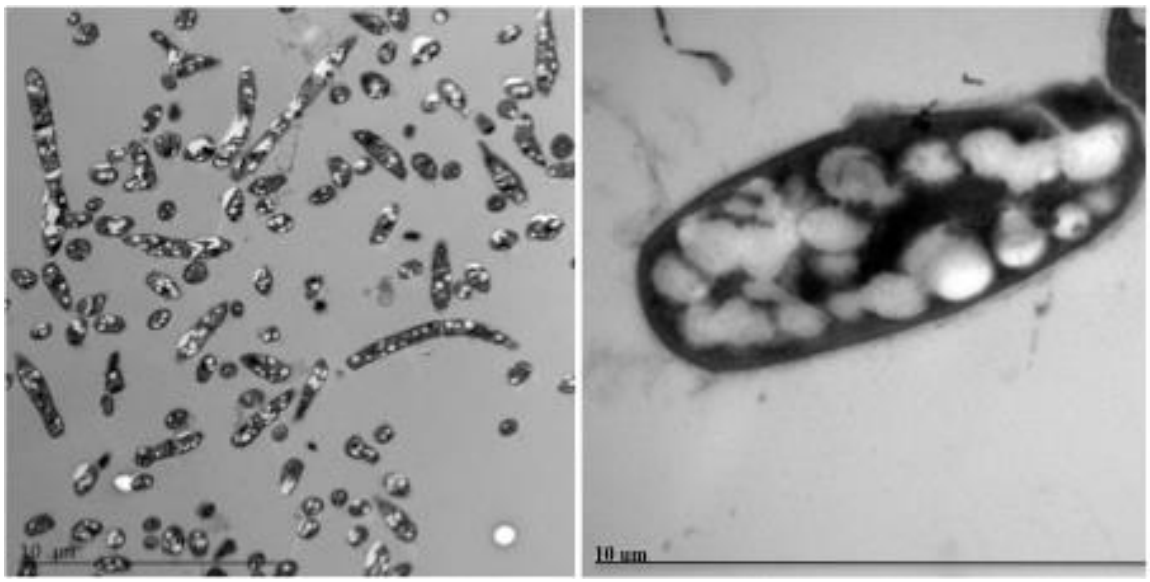

Figure 3.3 Transmission electron microscopy images of *R. rhodochrous*. Scale bar represents 10um.

Thin-layer chromatography analysis was used to investigate lipid classes present in *R. rhodochrous* extractables (Figure 3.4). The lipid extract from *R. rhodochrous* cultivated in the BIOFLO410 fermenter (Figure 3.4-lane RR) showed bands corresponding to triglycerides (Figure 3.4-lanes S1 and S3), which are esters of glycerol with three fatty acids and commonly found in animals and plants. However, some bacteria such as *Gordonia, Nocardia, Acinetobacter*, *Mycobacterium*, and *Rhodococcus* have been shown to accumulate lipids in the form of triglycerides (Alvarez and Steinbuchel 2002). Similarly, microbes can accumulate wax esters for use in times of inorganic nutrient starvation or stress. Wax esters have been identified in *Acinetobactor, Pseudomonas, Nocardia, Mycobacterium* and *Rhodococcus* species (Waltermann and Steinbuchel 2005) and were identified in the lipid extractable of *R. rhodochrous* (Figure 3.4-lanes S1 and S4). Both wax esters and triglycerides are valuable raw materials that

can be used in cosmetics, soaps, plastics, lubricants, and other industrial purposes.

Triglycerides and fatty acids could also be potential feedstocks for renewable fuels.

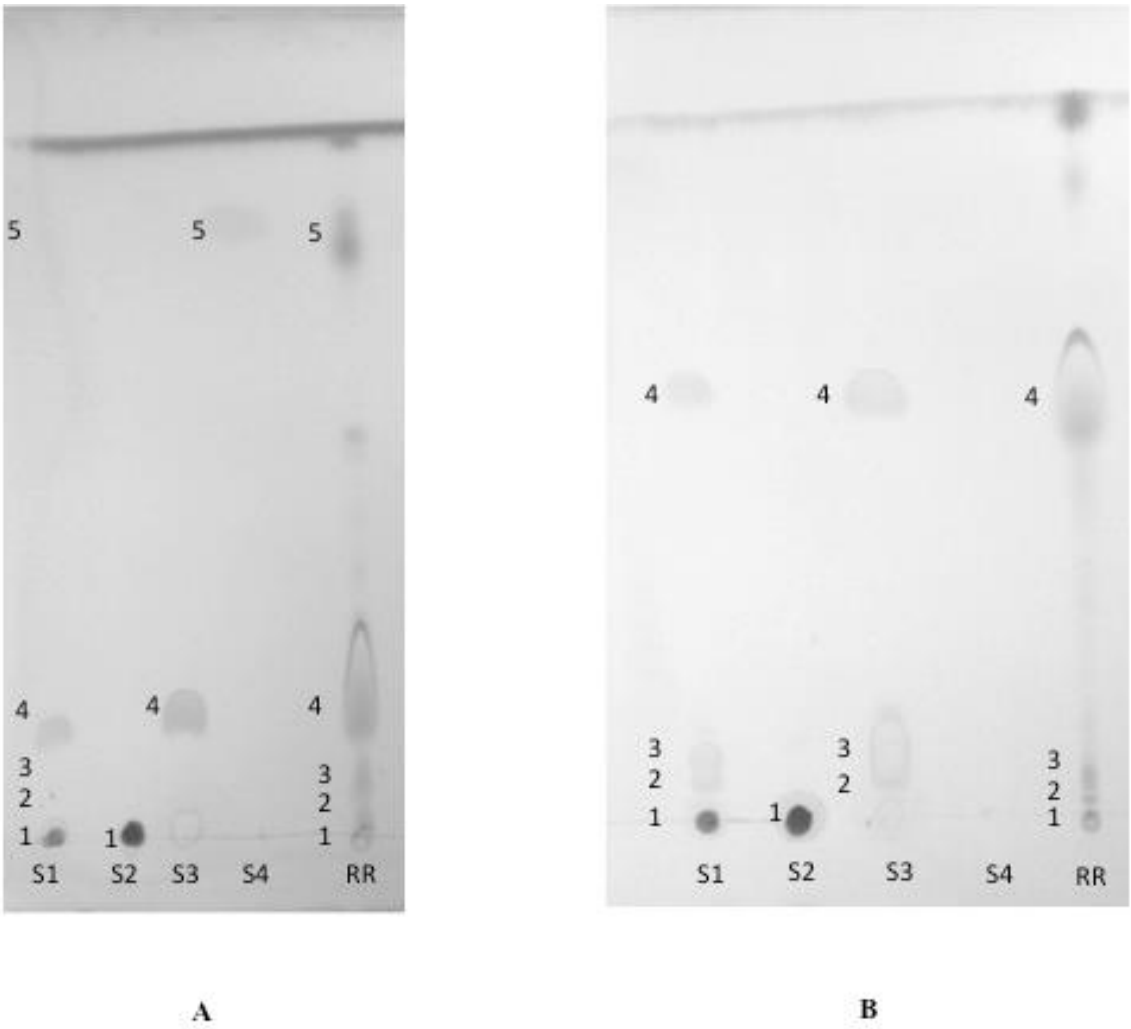

Figure 3.4 Thin layer chromatography of lipid extract from *R. rhodochrous* fermenter culture (RR).

(a) Lipid extract developed in hexane/diethyl ether (96/4). (b) Lipid extract developed in hexane/diethyl ether/acetic acid (85/15/2). S1 is a standard mixture of S2, S3, and S4. S2 is a (1) phospholipid standard mixture (S2). S3 is a mixture of (2) monoolein, (3) 1, 2-diolein, and (4) triolein. S4 is (5) palmityl palmitate

3.4.3 Fatty Acid Methyl Esters Analysis

Soybean oil is a common feedstock for biodiesel production and has a FAME profile dominant in palmitic, oleic, linoleic and linolenic fatty acids (Canakci and Sanli 2008) similar to some oleaginous yeast (Li, Du et al. 2008, Ratledge and Cohen 2008, Leiva-Candia, Pinzi et al. 2014). Palmitic and oleic methyl esters were the most prevalent of the FAMEs in both the shake flask experiments and in the fermenter trial (Figures 3.5 and 3.6), which could be advantageous for oxidative stability, cetane number and balancing cold flow of biodiesel (Bringe 2005). Palmitic and oleic methyl esters were also found to be dominant in the fatty acid composition of *R. opacus* PD630 after growth on various substrates (Alvarez, Mayer et al. 1996). The yields of total FAMEs from the lipid extracts of the shake flask experiments range from 19 % to 49 % (w/w of total lipid extract; data not shown). The percent yield of FAMEs of the cell dry weight was also highest after 72 hours of growth in 20 g/L of glucose.

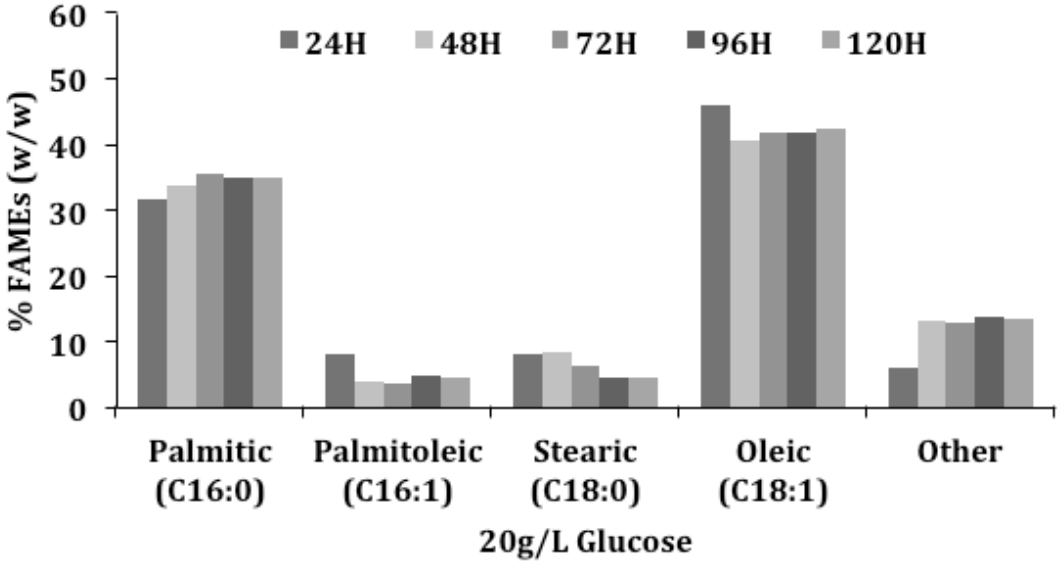

Figure 3.5 FAME profile over time of *R. rhodochrous* grown in shake flasks supplemented with 10 (top), 20 (middle), or 40g/L (bottom) of glucose.

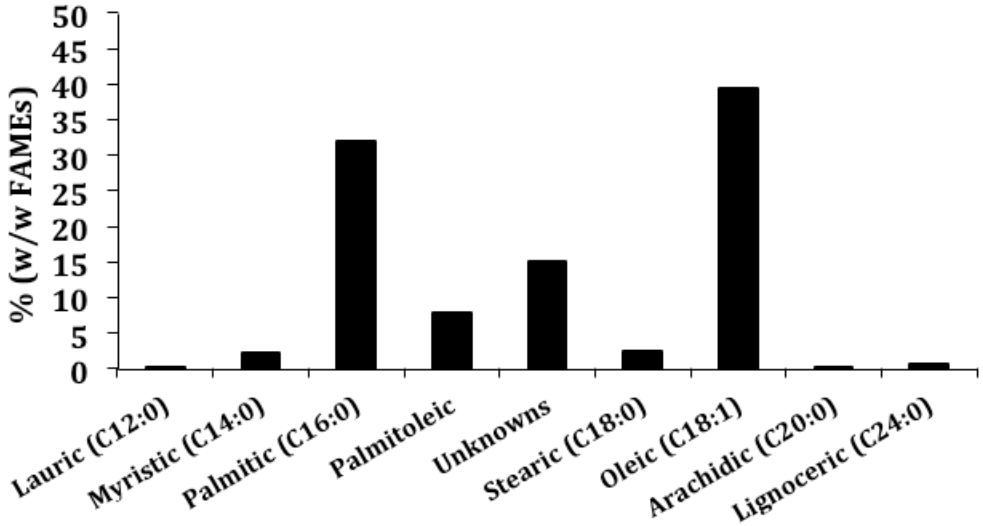

Figure 3.6 Percent FAMEs based on lipid extract of *R. rhodochrous* grown for 144 hours in 20 g/L in a BioFlo410 fermenter.

Other FAMEs were present in small amounts in the 10 g/L and 20 g/L glucose treatments accounting for the large total FAME yield in 20 g/L at 72 hours (Figure 3.5). FAMEs denoted as other consisted of unknown methyl esters (3-13% w/w) and small amounts (less than 3 % w/w) of Lauric, Myristic, Arachidic, and/or Erucic methyl esters. This FAME profile is similar to other oleaginous microbes although *R. rhodochrous* does not produce linoleic or linolenic acids as observed in oleaginous yeasts (Li, Du et al. 2008, Ratledge and Cohen 2008, Leiva-Candia, Pinzi et al. 2014). *Rhodococcus opacus* PD630, an oleaginous bacterium, has a FAME profile dominant with palmitic, oleic, and heptadecanoic methyl esters (Alvarez, Mayer et al. 1996). The percentage of unknown FAMEs of *R. rhodochrous* with retention times between those of palmitoleic and stearic methyl esters could be representative of heptadecanoic methyl esters that were not included in the standard FAME mix used in this study The effects of fermentation conditions and substrate could influence the fatty acid composition of microbial oils (Alvarez, Mayer et al. 1996, Mondala, Hernandez et al. 2012, Leiva-Candia, Pinzi et al. 2014). Further optimization of *R. rhodochrous* fermentation parameters must be investigated.

3.5 Conclusion

R. rhodochrous, a gram-positive bacterium belonging to the actinomycete group, was grown in varying concentrations of glucose in an effort to characterize this organism as oleaginous. All glucose concentrations promoted lipid accumulation and *R. rhodochrous* accumulated over 40 % lipid when grown in 20 g/L. A scaled-up fermentation achieved similar lipid yields and greater biomass (7g/L). TEM images show intracellular lipid bodies in samples collected during these fermentations. Triglycerides

and wax esters were found to be the major components of lipids accumulated by *R. rhodochrous*. Transesterification of extractable lipids yielded FAMEs with high percentages of palmitic and oleic methyl esters.

Microbial derived products, such as triglycerides, are potential feedstock alternatives for biofuels. Several bacteria have been identified as oleaginous, accumulating over 20 % of the cell dry weight as lipids, usually in the form of triglycerides. Here, we show lipid accumulation of 40 % in *R. rhodochrous* when grown in glucose-supplemented medium. However, as a well-known and documented bioremediator, the potential for *R. rhodochrous* to accumulate lipid when grown on more sustainable and cost-effective substrates is being explored.

3.6 Acknowledgements

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CHAPTER IV

LIPID ACCUMULATION BY *RHODOCOCCUS RHODOCHROUS* GROWN IN MODEL LIGNOCELLULOSIC SUGARS AND INHIBITORS

4.1 Abstract

Lignocellulosic biomass requires pretreatment to release pentose and hexose sugars for microbial fermentation to commercially valuable bioproducts. During pretreatment, inhibitory compounds such as acetic acid and furfural are also released, which can affect microbial growth and yields. *Rhodococcus rhodochrous* is a red-pigmented, gram-positive, soil bacterium commonly used for industrial production of acrylamide and for bioremediation of hydrocarbons and aromatic compounds. Despite the widespread use of *R. rhodochrous* in industry, the understanding of its ability for lipid accumulation is limited. Here, the lipid accumulation capabilities of *R. rhodochrous* were analyzed in an effort to offer a unique approach to addressing the need for alternative fuels. Growth was analyzed using glucose or xylose as a substrate with limited nitrogen conditions to promote lipid accumulation. Glucose experiments were supplemented with acetic acid and furfural to investigate any inhibitory effects of these compounds. Cells from all treatments were harvested daily for 5 days and lipids were extracted from total cell dry weight using a modified Bligh and Dyer method. *R. rhodochrous* accumulated 52% of cell dry weight as lipid after 5 days of growth in 20g/L of glucose. Minimal growth was observed when *R. rhodochrous* was grown in xylose. In the presence of 3g/L

of acetic acid (sodium acetate) and glucose, 50% lipid accumulation by cell dry weight was observed in *R. rhodochrous*. Similar results were observed with the addition of 0.3 ml/L furfural suggesting that *R. rhodochrous* is not inhibited by the addition of furfural or acetic acid in the media. Future work is needed to determine the fate of these inhibitors in the media and the concentrations of inhibitors that *R. rhodochrous* can tolerate during cultivation and lipid accumulation.

4.2 Introduction

Lignocellulosic biomass is the most abundant and renewable organic material in the world (Claassen et al., 1999). Examples of lignocellulosic biomass include wood, grass, forestry waste and agricultural residues (Palmqvist & Hahn-Hagerdal, 2000b). The advantages of lignocellulosic residues over food crops are that lignocellulosics do not compete with food economics and do not require the environmental resources of food crops (Hill et al., 2006). The lignocellulosic complex is composed of cellulose, hemicellulose, and lignin. Cellulose and hemicellulose account for 70% of dry cellulosic material, which makes this biomass an ideal feedstock for biofuels (Claassen et al., 1999; Franden et al., 2009; Palmqvist & Hahn-Hagerdal, 2000a; Pandey & Kim, 2011). However, pretreatment of the biomass is often necessary to disrupt the lignocellulosic complex to release sugars for microbial fermentation. Acid hydrolysis is a common pretreatment that releases hexose and pentose sugars (Mosier et al., 2005), but in the process, also forms chemical byproducts that can inhibit microbial growth and fermentation of sugars (Palmqvist & Hahn-Hagerdal, 2000b). Hemicellulose is more easily hydrolyzed than cellulose resulting in the release of glucose, xylose and other pentose sugars, and acetic acid (Palmqvist & Hahn-Hagerdal, 2000b). At high

temperatures and pressure, xylose is further degraded to furfural (Dunlop, 1948). Cellulose is hydrolyzed to glucose, which can form hydroxymethylfurfural, while the hydrolysis of lignin produces phenolic compounds (Palmqvist & Hahn-Hagerdal, 2000b).

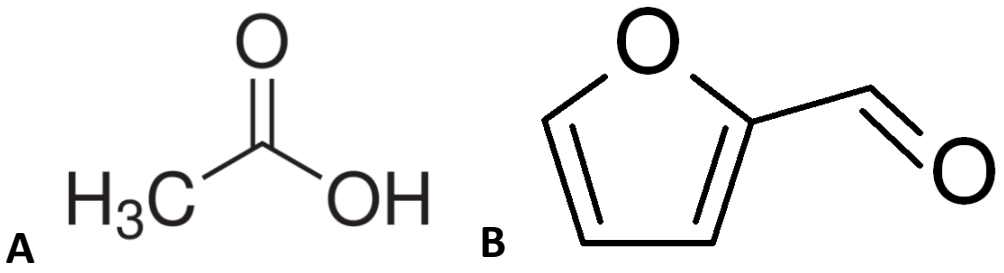

Figure 4.1 Chemical structures of acetic acid and furfural.

Furfural and acetic acid have been shown to significantly impact the yield of microbial fermentations, which is more widely documented for ethanol-producing microbes (Figure 4.1). As concentrations of acetic acid, formic acid, and levulinic acid increased, yeast ethanol yield decreased (Larsson et al., 1998). Acetate and furfural decreased the growth rates of *Zymomonas mobilis*, an ethanol-producing bacterium (Franden et al., 2009). Even if inhibitors do not affect ethanol yields, the growth rates and ethanol production rates can be inhibited by furans and aromatic compounds present in hydrolysates (Klinke et al., 2004). Thus, the challenge of using lignocellulosic biomass for microbial conversion to a product lies in the ability of an organism to utilize pentose and hexose sugars while surviving in the presence of chemical inhibitors.

Oleaginous microbes are often able to utilize a range of carbon sources (Alvarez & Steinbuchel, 2002) and can accumulate over 20% of their biomass as intracellular

lipids when nutrients are limited, but carbon is in excess (Wynn & Ratledge, 2005). These lipids can be extracted and used for biodiesel or other commercial application. Few oleaginous microbes can use lignocellulosic hydrolysates for lipid accumulation (Huang et al., 2013); however, certain yeast species have been shown to utilize both glucose and xylose to achieve lipid accumulation (Evans & Ratledge, 1984; Huang et al., 2009; Mondala et al., 2012). Even fewer oleaginous microbes have been shown to thrive when lignocellulosic inhibitors are present (Huang et al., 2013). The hydrolysate must first be detoxified to remove or reduce the concentration of inhibitors (Palmqvist & Hahn-Hagerdal, 2000a; Palmqvist & Hahn-Hagerdal, 2000b) or the organisms must be modified to increase their tolerance (Liu, 2011).

The effects of lignocellulosic sugars and inhibitors on the growth and productivity of oleaginous microbes, specifically bacteria, have only recently been studied. We aim to use a pigmented, gram-positive Actinomycete, *Rhodococcus rhodochrous*, recently characterized as oleaginous to research the use of lignocellulosic biomass as a substrate for oleaginous bacteria. This study investigated the use of xylose as a sole carbon source or when combined with glucose and the influence of the lignocellulosic inhibitors acetic acid and furfural on the growth and lipid accumulation of *Rhodococcus rhodochrous*.

4.3 Methods

4.3.1 Culture Conditions

Rhodococcus rhodochrous (American Type Culture Collection #21198) glycerol stocks, stored at -80°C were used to inoculate minimal salts media (Sorkhoh et al., 1990) supplemented with 20g/L glucose or nutrient agar plates. A 1:10 inoculum was used for all experiments (unless otherwise specified) from a starter culture of *R. rhodochrous*

grown for 48 hours in MSM supplemented with 20g/L glucose. All shake-flask experiments were conducted in triplicate and incubated in baffled flasks at 30°C in a shaking incubator at 125 rpm (New Brunswick Scientific Model I26, Edison, New Jersey).

4.3.2 Inhibitor Growth Conditions

Minimal salts media (MSM; Sorkhoh 1990) was supplemented with xylose (20g/L), glucose and xylose (10g/L each), or glucose and xylose (20g/L, 10g/L). MSM with 20g/L glucose was also supplemented with either sterile-filtered acetic acid (3g/L) or furfural (0.3mL/L). The pH was adjusted to 7.0 for all media before inoculation using sterile sodium hydroxide. Inoculums were prepared by transferring lawns of *R. rhodochrous* grown for 5 days on nutrient agar to phosphate buffer saline or an aliquot of sterile medium (Shields-Menard et al., 2015). All shake-flask experiments were conducted in triplicates and the experimental mean and standard deviation were calculated for all gravimetric and sugar analyses. Repeated measures ANOVA were performed using the Statistical Package for the Social Sciences (SPSS) software when necessary and significance was determined when $p<0.05$.

R. rhodochrous was also grown in 4 BIOFLO 310 5L fermenters (New Brunswick Scientific, Edison, NJ) with a 3L working volume to further evaluate the effects of acetic acid or furfural combined with glucose, in comparison to glucose only controls. The pH of all cultures was adjusted to 7 using NaOH and monitored (but not adjusted) using the fermenters' pH probes. Temperature was maintained at 30 °C and fermentation parameters were set as described in Chapter 5. One sample (50mL) from

each fermenter (treatment) was collected every 12 hours for the first 48 hours and then daily until 168 hours.

4.3.3 Analyses

Samples were analyzed for percent gravimetric lipid content (% lipid:CDW), cell dry weight (CDW), and sugar concentration (g/L). Samples were collected and stored as previously described (Shields-Menard et al., 2015). Glucose and xylose concentrations were measured from the supernatant using a YSI 2900 Biochemistry Analyzer (YSI Incorporated Life Sciences, Yellow Springs, Ohio).

Lipids were extracted from a freeze-dried pellet using a modified Bligh and Dyer (Bligh & Dyer, 1959) method and analyzed as previously described (Revellame et al., 2012; Shields-Menard et al., 2015). The extracted lipids were then transesterified and fatty acid methyl esters (FAMEs) were analyzed using an Agilent 6890N gas chromatograph with a flame-ionization detector (GC-FID; Agilent Technologies Inc., Wilmington, Delaware) with a Zebron ZB-FFAP column (30 m x 0.25mm, film thickness 0.25 μm), using an oven temperature of 50-250°C with a rate of 10°C per minute. The carrier gas, helium, had a flow rate of 1.5 mL per minute and the detector temperature was 260°C. A standard solution of known concentrations of C9-C24 FAMEs (Sigma) and an internal standard was used to calibrate the instrument. Mean and standard deviations of triplicate injections of triplicate samples are reported for shake flask experiments.

4.4 Results and Discussion

4.4.1 Glucose and Xylose Affect Growth and Lipid Accumulation

While glucose is the primary sugar of pretreated lignocellulosic biomass, the amount of available xylose is still an abundant component (Palmqvist & Hahn-Hagerdal, 2000a). *R. rhodochrous* was cultured in media supplemented with glucose, xylose, and a mixture of glucose and xylose to investigate cell growth, lipid accumulation, and sugar consumption. *R. rhodochrous* grew in glucose and the mixture containing glucose and xylose as observed by a significant increase in CDW ($p<0.05$; Figure 4.2). However, *R. rhodochrous* was not able to grow in MSM supplemented with xylose (Figure 4.1). Glucose was consumed to less than 5 g/L within 72 hours, but xylose was not consumed in either the xylose or mixed sugars treatment (Figure 4.3). Relatively few bacteria and yeasts are able to use xylose as a sole carbon source and several research efforts have genetically modified microbes in an effort to efficiently use total sugars from lignocellulosic biomass (Alper & Stephanopoulos, 2009; Fernandes & Murray, 2010; Kim et al., 2010). The well-known lipid accumulating bacterium, *Rhodococcus opacus* was recently engineered, with the addition of the xylose isomerase (*xyl*A) gene and xylulokinase (*xyl*B) gene, to grow on varying concentrations of xylose as the sole carbon source (Kurosawa et al., 2014; Xiong et al., 2012). In both cases, the genetically modified *R. opacus* PD630 strain accumulated over 45% lipid when grown on xylose suggesting that the metabolic engineering approach could prove useful for other *Rhodococcus* species and oleaginous microbes (Kurosawa et al., 2014; Xiong et al., 2012).

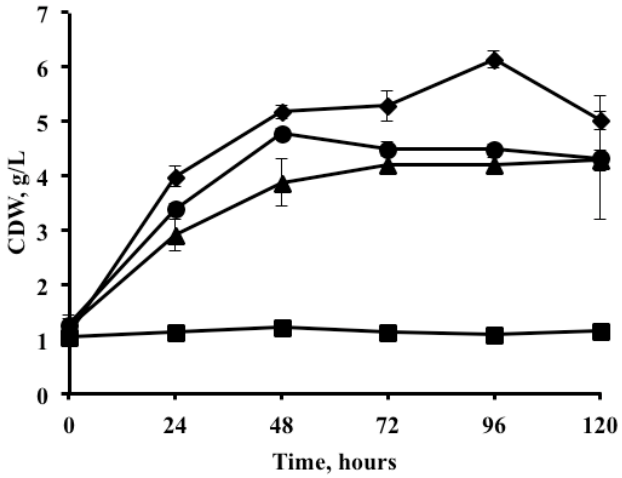

Figure 4.2 *R. rhodochrous* cell dry of shake flask cultures supplemented with glucose, xylose, or a mixture of glucose and xylose.

R. rhodochrous cell dry weight (g/L ±SD) of shake flask cultures supplemented with 20g/L glucose (◆), 20g/L xylose (■), or 10g/L xylose mixed with either 10g/L glucose (GX 1; ▲) or 20g/L glucose (GX 2; •).

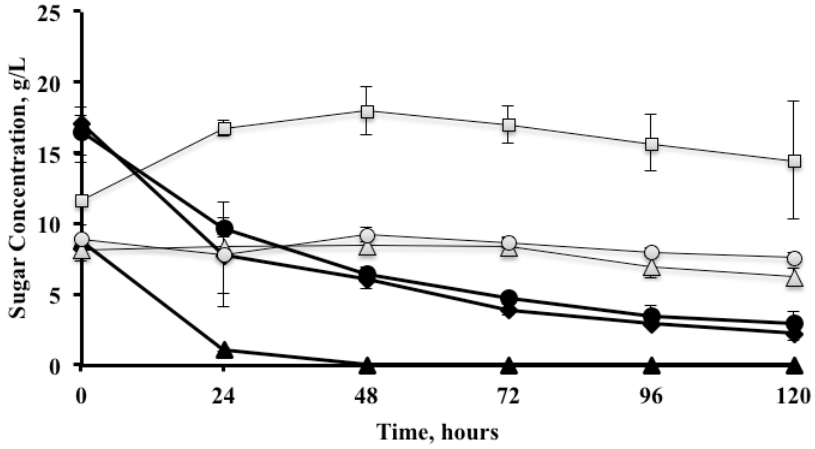

Figure 4.3 Glucose or xylose concentration of *R. rhodochrous* shake flask cultures.

Glucose (black fill) or xylose concentration (no fill; g/L ±SD) of *R. rhodochrous* shake flask cultures supplemented with 20g/L glucose (◆), 20g/L xylose (□), or 10g/L xylose mixed with either 10g/L glucose (GX 1; ▲) or 20g/L glucose (GX 2; •).

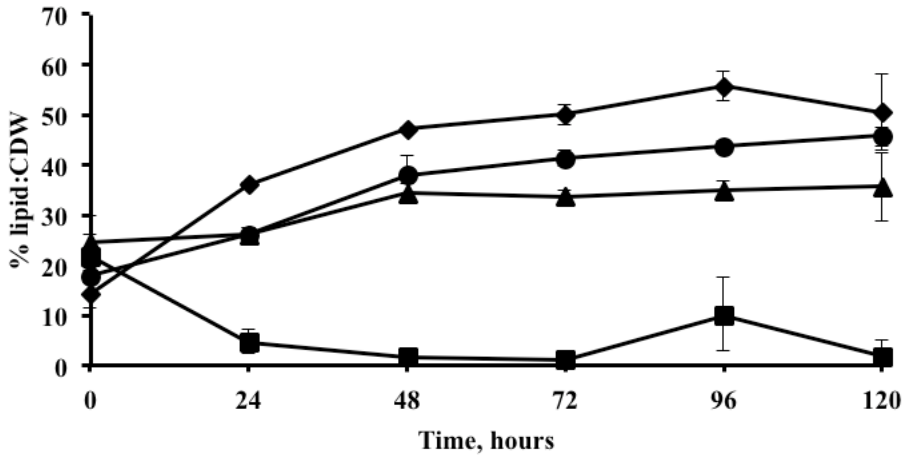

Figure 4.4 Percent lipid accumulation by *R. rhodochrous* grown in glucose or xylose supplemented shake flask cultures.

Percent lipid (±SD) of cell dry weight accumulated by *R. rhodochrous* shake flask cultures supplemented with 20g/L glucose (◆), 20g/L xylose (■), or 10g/L xylose mixed with either 10g/L glucose (GX 1; ▲) or 20g/L glucose (GX 2; •).

Table 4.1 Fatty acid methyl ester profile (% w/w) of *R. rhodochrous* grown in glucose, xylose, or a mixture of glucose and xylose.

Treatment Group	Time	Lauric (C12:0)	Myristic (C14:0)	Palmitic (C16:0)	Palmitoleic (C16:1)	Unknown	Stearic (C18:0)	Oleic (C18:1)	Arachidic (C20:0)	Erucic (C22:1)	Lignoceric (C24:0)
	0	0.00	2.27	39.45	7.03	7.82	6.29	37.14	0.00	0.00	0.00
Glucose	24	0.10	2.42	37.49	8.31	7.55	6.81	36.00	0.19	1.15	1.15
Xylose	24	0.00	0.00	38.86	9.27	0.00	1.46	38.18	0.00	12.23	12.23
GX1	24	0.00	2.79	37.39	9.57	7.56	5.87	35.30	0.00	1.52	1.52
GX2	24	0.00	2.59	36.21	9.27	8.17	6.62	35.82	0.19	1.13	1.13
Glucose	48	0.18	2.68	36.32	9.61	9.85	5.07	34.61	0.18	1.49	1.49
GX 1	48	0.22	3.28	37.31	11.61	9.87	3.73	32.65	0.04	1.29	1.29
GX 2	48	0.20	3.21	35.55	11.29	11.10	4.06	33.24	0.12	1.23	1.23
Glucose	72	0.18	2.80	35.64	10.79	10.26	4.25	34.80	0.15	1.13	1.13
GX 1	72	0.24	3.50	36.13	12.79	10.98	3.18	31.80	0.09	1.30	1.30
GX 2	72	0.22	3.20	34.99	12.86	11.55	3.23	32.68	0.12	1.16	1.16

FAMEs (% w/w) of *R. rhodochrous* grown in 20g/L glucose, 20g/L xylose, or a mixture of 10g/L xylose with 10g/L glucose (GX 1) or 20g/L glucose (GX 2). Percentages are an average of triplicate injections. Standard deviation is less than 3 %.

R. rhodochrous achieved over 50% lipid accumulation when grown in glucose and the mixtures of glucose and xylose (Figure 4.4), but lipid accumulation was not observed when xylose was used as the sole carbon source (Figure 4.4). The nitrogen concentration was unchanged in the media (data not shown), suggesting that nitrogen was not used by *R. rhodochrous* to increase biomass and therefore was not limited for lipid accumulation to occur in the xylose only treatment, which further supports the indication that xylose does not promote growth or lipid accumulation. In the treatments with both glucose and xylose, lipid accumulation was significantly reduced ($p< 0.05$; Figure 4.4), suggesting that xylose could have a slight inhibitory effect on lipid accumulation. Similarly, this inhibitory effect of xylose, albeit small, was also observed in experiments culturing the genetically modified *R. opacus* PD630 in a mixture of glucose and xylose (Kurosawa et al., 2014). FAME data (Table 4.1) shows a prevalence of palmitic and oleic methyl esters for all treatments throughout the experiment. When *R. rhodochrous* was grown in just xylose-supplemented media, there was a shift of FAMEs from 0 to 24 hours: the percentage of stearic acid decreased (from 6% to 1%) while erucic and lignoceric increased (from 1% to 12%). As there was little lipid recovery from lipid extraction, membrane lipids may account for the majority of lipids extraction as well as the noticeable shift in FAMEs. Since there was no lipid recovered after 24 hours, FAME analysis was not possible.

4.4.2 The Effect of Acetic Acid and Furfural on Growth and Lipid Accumulation

R. rhodochrous cell dry weight increased across all inhibitor treatments (Figure 4.5) with no significant difference ($p<0.05$) among treatments or the control indicating

that acetic acid and furfural were not toxic and did not impede cell growth. Glucose was depleted by 72 hours and a similar trend of consumption was observed in the glucose control and flasks supplemented with inhibitors (Figure 4.6). Other oleaginous microbes have shown increased growth rates when cultivated in glucose media supplemented with acetic acid and furfural (Huang et al., 2009; Zhang et al., 2011). However, inhibitor concentration has been shown to be an important factor that affects microbial growth (Mondala et al., 2012; Wang et al., 2014).

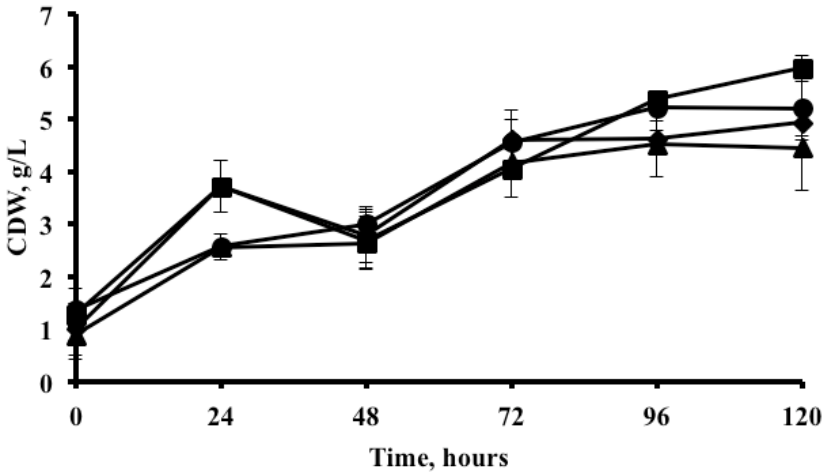

Figure 4.5 *R. rhodochrous* cell dry weight of shake flask cultures with glucose supplemented with acetic acid, furfural, or a mix of acetic acid and furfural.

R. rhodochrous cell dry weight (g/L ±SD) of shake flask cultures with 20g/L glucose (◆) and supplemented with 3g/L acetic acid (■), 0.3mL/L furfural(▲), or a mix of 3g/L acetic acid and 0.3mL/L furfural (•).

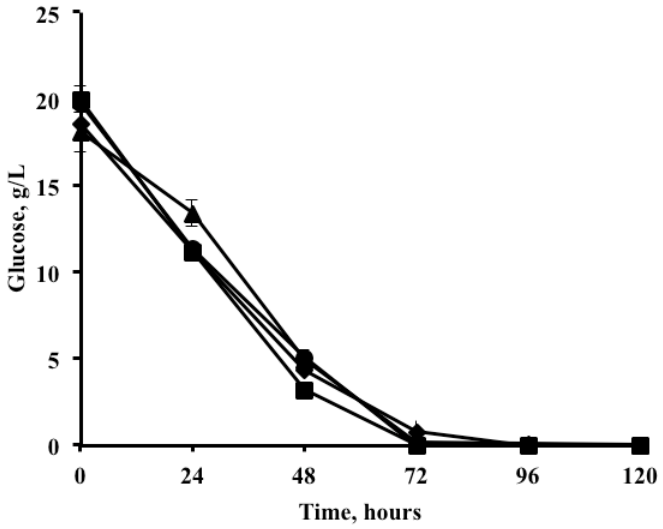

Figure 4.6 Glucose concentrations of *R. rhodochrous* shake flask cultures supplemented with acetic acid, furfural, or a mix of acetic acid and furfural (•).

Glucose concentrations (g/L ±SD) of *R. rhodochrous* shake flask cultures supplemented with no inhibitors (20g/L glucose control; ◆) or supplemented with 20g/L glucose and 3g/L acetic acid (■), 0.3mL/L furfural (▲), or a mix of 3g/L acetic acid and 0.3mL/L furfural (•).

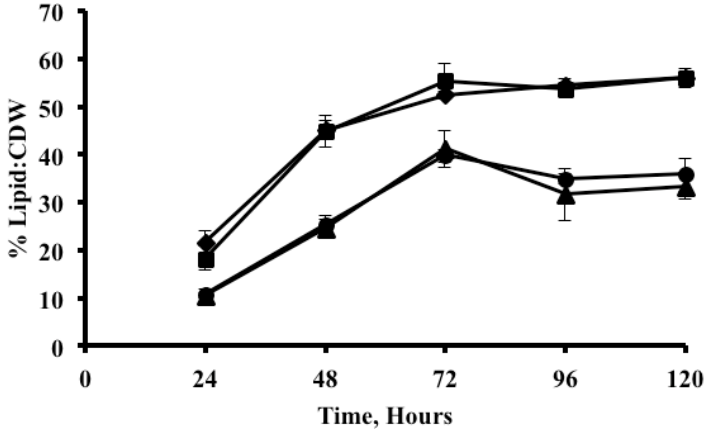

Figure 4.7 Percent lipid accumulation by *R. rhodochrous* grown in inhibitor-supplemented shake flask cultures.

Percent lipid (±SD) of cell dry weight accumulated by *R. rhodochrous* shake flask cultures with 20g/L glucose (◆) and supplemented with 3g/L acetic acid (■), 0.3mL/L furfural (▲), or a mix of 3g/L acetic acid and 0.3mL/L furfural (•).

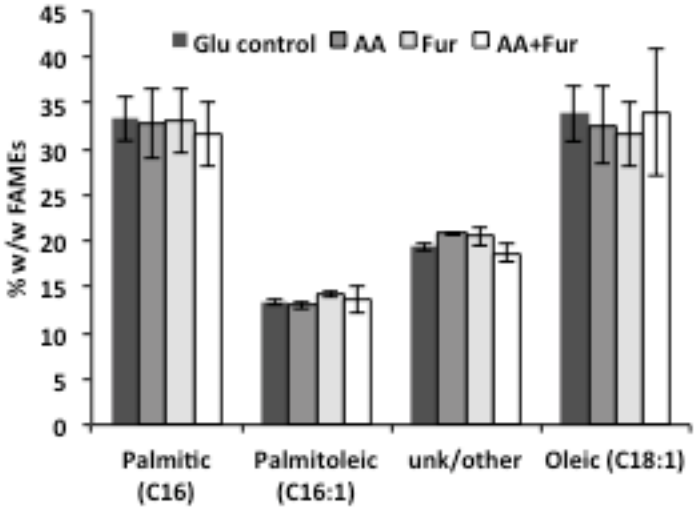

Figure 4.8 FAME profile of *R. rhodochrous* cultures at 72H grown in model lignocellulosic inhibitors, acetic acid and furfural.

R. rhodochrous cultures were grown in 20g/L glucose and supplemented with 3g/L acetic acid, 0.3mL/L furfural, or a mix of 3g/L acetic acid and 0.3mL/L furfural. Error bars denote standard deviation.

R. rhodochrous achieved over 50% lipid accumulation in the glucose control and acetic acid (sodium acetate) supplemented media, which was significantly greater than 40% lipid accumulation in inhibitor treatments containing furfural ($p<0.05$; Figure 4.7), suggesting that the addition of furfural reduces lipid accumulation. In this experiment lipid accumulation was more sensitive to furfural than cell growth, which is the opposite of what occurs during ethanol fermentation (Palmqvist & Hahn-Hagerdal, 2000a). The reduction of furfural to furfuryl alcohol could inactivate cell replication or cause a metabolic shift towards furfural reduction rather than other metabolic processes (Palmqvist & Hahn-Hagerdal, 2000a). However, other oleaginous microbes, including *R. opacus*, have been shown to increase lipid yields when grown in the presence of lignocellulosic inhibitors (Wang et al., 2014; Zhang et al., 2011).

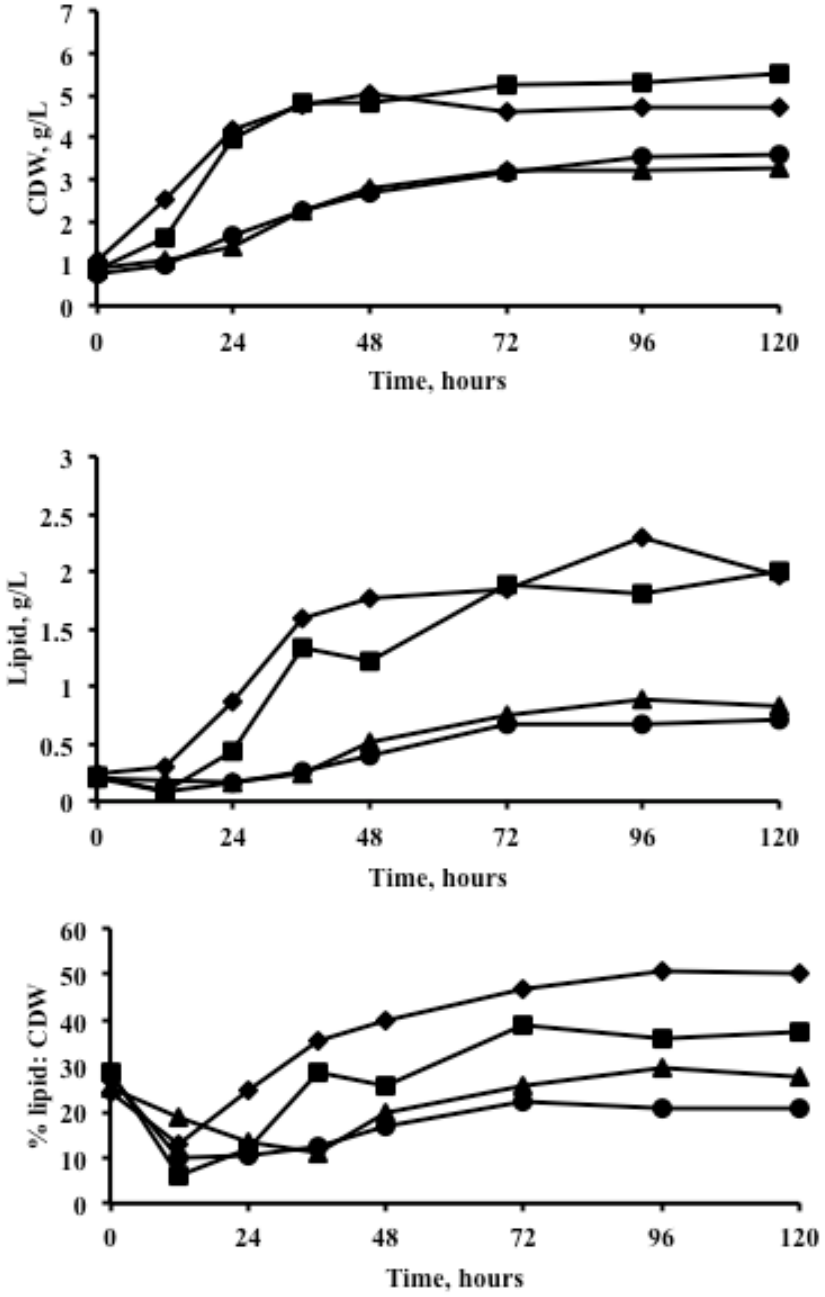

Figure 4.9 *R. rhodochrous* growth and lipid accumulation in the presence of inhibitors in fermenter cultivation.

R. rhodochrous cell dry weight (g/L ±SD; top), lipid (g/L ±SD; middle), and percent lipid (±SD) of cell dry weight accumulated by *R. rhodochrous* cultivated in BIOFLO 310 Fermenters with 20g/L glucose (◆) and supplemented with 3g/L acetic acid (■), 0.3mL/L furfural (▲), or a mix of 3g/L acetic acid and 0.3mL/L furfural (•).

Despite a 40% lipid accumulation in furfural supplemented *R. rhodochrous*, detoxification of hydrolysate may increase lipid yields as observed in a study of *Trichosporon fermentans* grown in rice straw hydrolysate (Huang et al., 2009). Gravimetric lipid analysis showed the largest lipid accumulation for all treatments at 72H (Figure 4.7) and palmitic and oleic methyl esters were shown to be the most prevalent of the FAMEs in all treatments (Figure 4.8), which is consistent with our previous FAME profile characterization of *R. rhodochrous*. Furthermore, this experiment was repeated in larger scale fermenters (3L total volume) with the same treatments of acetic acid and furfural (Figure 4.9). The fermenter results complimented the shake-flask data, indicating that acetic acid and furfural reduce lipid accumulation by *R. rhodochrous*, but are not inhibitory. Future studies would aim to determine the fate of these inhibitors in the media during cultivation of *R. rhodochrous*.

4.5 Conclusions

R. rhodochrous is an oleaginous microbe that can accumulate over 50% of its biomass as lipid when grown in glucose and has a FAME profile consistent with other *Rhodococcus* species (Alvarez and Steinbuchel 2002). Xylose significantly reduced lipid accumulation when mixed with glucose, although over 30% lipid accumulation occurred in both mixtures compared to less than 5% in xylose alone. Furfural also reduced lipid accumulation, but was not inhibitory. These data suggest that lignocellulosic biomass or hydrolysate could be a possible substrate for *R. rhodochrous* lipid accumulation. Future work aims to determine the fate of furfural using analytical methods and to investigate the effects of lignin degradation compounds on lipid accumulation.

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CHAPTER V

THE EFFECTS OF MODEL AROMATIC LIGNIN COMPONENTS ON GROWTH AND LIPID ACCUMULATION OF *RHODOCOCCUS RHODOCHROUS*

5.1 Abstract

Lignocellulosic biomass is one of the most abundant and renewable organic materials in the world. The lignocellulosic complex is composed of cellulose, hemicellulose, and lignin, which can be pretreated to release sugars that can be utilized for microbial production of valued metabolites. Oleaginous microbes can accumulate over 20% of their cell dry weight as lipids, which are stored as intracellular energy reserves. The characterization of oleaginous bacteria creates opportunities for the development of alternative feedstocks and technologies. *Rhodococcus rhodochrous* is a bacterium recently determined to be oleaginous when grown in glucose-supplemented media. The purpose of this study was to evaluate model lignin phenolic compounds as substrates for lipid accumulation. Lipid accumulation in *R. rhodochrous* was evaluated using phenol, 4-hydroxybenzoic acid (HBA) and vanillic acid (VA) as model lignin compounds with and without glucose as a co-substrate. Cell dry weight increased in all treatments, indicating that growth was not impaired in these conditions. However, alterations were observed in the amount of lipids produced. *R. rhodochrous* accumulated over 40% of its cell dry weight as lipids when grown in glucose with HBA and VA, but less than 20% when grown in HBA and VA alone. When grown in phenol and glucose,

R. rhodochrous accumulated 35% of its dry weight at lipids, but did not accumulate lipids when grown in phenol alone. These data indicate that *R. rhodochrous* may have the capability to tolerate and utilize lignin-like aromatic compounds for lipid accumulation. Future research will aim to optimize culture conditions of lignocellulosic compounds for growth and lipid accumulation by *R. rhodochrous*.

5.2 Introduction

Lignocellulosic biomass (i.e. agricultural residues, wood, grasses, and energy crops) is one of the most abundant organic materials in the world, gaining interest from sustainable energy researchers to be used for high-value products. Lignocellulosic material is comprised of cellulose, hemicellulose, and lignin, which are chemically bonded and intricately intertwined. Several pretreatment methods have been developed to depolymerize lignocellulose and release the glucose and pentose sugars, mainly xylose, from the cellulose and hemicellulose, respectively (Hu & Ragauskas, 2012; Mosier et al., 2005). As a result of lignocellulosic pretreatment, inhibitors such as acids, furans, and phenolic compounds form in lignocellulosic hydrolysate, which can affect fermentation productivity (Palmqvist & Hahn-Hagerdal, 2000). While cellulose and hemicellulose can be used to produce various bioproducts, lignin is a complex polyaromatic structure that is recalcitrant to most bacteria and therefore limited in its use as a renewable and valuable resource.

Lignin accounts for 10-25% of lignocellulosic material and the global paper industry alone produces almost 50 million tons of lignin per year (Gosselink et al., 2004). Only 2% of lignin is used commercially, while the remainder is usually burned for energy. However, bioconversion of lignin offers exceptional potential for the microbial

production of alternative fuels or bioproducts (Kosa & Ragauskas, 2012; Zakzeski et al., 2010). During the pretreatment of lignocellulosic material to release sugars for fermentation purposes, degradation of lignin to lignin oligomers and phenolic derivatives, such as vanillic acid, trans-p-coumaric acid and 4-hyroxybenzoic acid also occurs (Figure 5.1; Palmqvist & Hahn-Hagerdal, 2000; Saadia & Ashfaq, 2010). Degraded lignin fractions are often discarded due to lack of use and are ultimately found in industrial wastewaters (Lamichhane Upadhyaya et al., 2013; Saadia & Ashfaq, 2010; Wells et al., 2015).

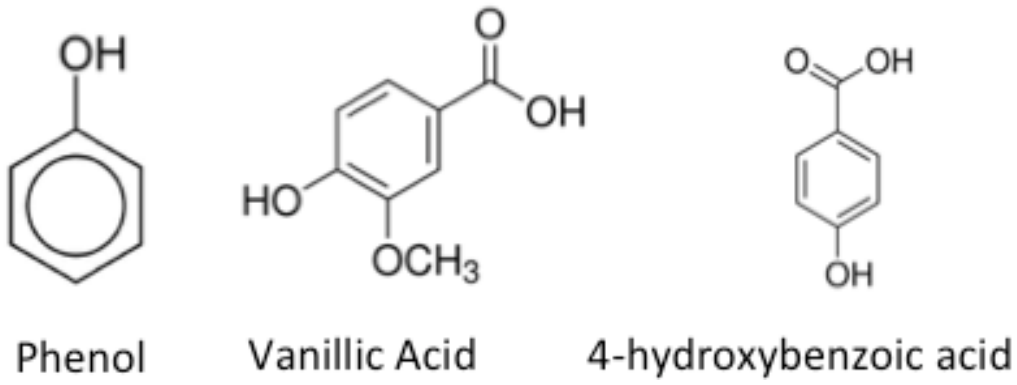

Figure 5.1 Chemical structures of phenol, vanillic acid, and 4-hydroxybenzoic acid.

Microbial degradation of lignin is limited to white-rot and brown-rot fungi, which are able to mineralize lignin, and some soil bacteria, which are able to depolymerize lignin or further break-down lignin fragments (Bugg et al., 2011). Several species of soil bacteria capable of aromatic degradation are currently being investigated for lignin degrading abilities, based on identification of specific enzymes and catabolic pathways (Bugg et al., 2011).

Phenol and its derivatives can be serious health hazards if released into the environment (Buchholz & Pawliszyn, 1993; Michalowicz & Duda, 2007). Biodegradation of phenolic contaminants by microorganisms has been analyzed extensively. For instance, several *Rhodococcus* species have been shown to degrade phenol and other aromatic hydrocarbons (Larkin et al., 2005; Martinkova et al., 2009). *Rhodococcus phenolicus* sp. nov. was isolated from a wastewater bioprocessor and was shown to grown in media with up to 7.5 g/L phenol as the sole carbon source (Rehfuss & Urban, 2005). *Rhodococcus opacus* GM-14 was also shown to use up to 1.2 g/L of phenol and its halogenated derivatives as a sole carbon source (Zaitsev et al., 1995).

Oleaginous microbes, which are those that can accumulate over 20% of their dry cellular weight as lipids (Wynn & Ratledge, 2005), have also recently begun to be studied for their biodegradative properties. For instance, the well-characterized oleaginous bacterium *R. opacus* (Alvarez et al., 1996) was recently found to convert model lignin compounds to triglycerides (Kosa & Ragauskas, 2012) and tolerate lignocellulosic hydrolysates during growth and lipid accumulation (Wang et al., 2014; Wells et al., 2015). We recently identified *Rhodococcus rhodochrous*, which is known for its degradation capabilities, as an oleaginous bacterium when grown in glucose (Shields-Menard et al., 2015). In an effort to better understand the mechanisms of aromatic degradation as it promotes lipid accumulation, the purpose of this study was to evaluate the effects of model lignin aromatic compounds, specifically phenol, vanillic acid (VA), and 4-hydroxybenzoic acid (HBA) on growth and lipid accumulation of *R. rhodochrous*.

5.3 Materials and Methods

5.3.1 Culture and Experimental conditions

R. rhodochrous (ATCC #21198) glycerol stocks were stored at -80ºC and used to inoculate nutrient agar plates (Fisher Scientific, Pittsburg, Pennsylvania). Lawns of *R. rhodochrous* were harvested to inoculate experimental starters.

5.3.1.1 Shake-Flask Conditions

Shake-flask experiments were used to evaluate the growth and lipid accumulation by *R. rhodochrous* when grown in media supplemented with model lignin compounds. For phenol experiments, a minimal salts medium (Sorkhoh et al., 1990) was supplemented with 20g/L glucose, 1g/L phenol (Sigma), or 20g/L glucose and 1g/L phenol. Bacterial inoculums (10% v/v) for phenol experiments were prepared using a starter culture of *R. rhodochrous* grown in glucose-supplemented medium for 48 hours.

For HBA and VA shake-flask experiments, Sorkhoh media (Sorkhoh et al., 1990) was supplemented with or without glucose (20g/L) and with 1.25g/L VA, 1.25g/L HBA, or both 1.25g/L HBA and 1.25g/L VA. HBA and VA (purchased from Sigma) were added to the media, mixed thoroughly until dissolved, and then filter sterilized. We used a 1.25g/L concentration of VA or HBA based on previous literature (Kosa & Ragauskas, 2012) as well as our own observations (unpublished) of reduced growth and chemical solubility at higher concentrations. Inoculums (10% v/v) were prepared for each treatment using the respective 48-hour starter cultures of *R. rhodochrous* grown in glucose and VA, glucose and HBA, or glucose, HBA, and VA. The pH was adjusted with NaOH to 6.8 prior to inoculation. All cultures were incubated at 30°C in a shaking incubator at 125 rpm (New Brunswick Scientific Model I26, Edison, New Jersey). All

shake-flask experiments were conducted in triplicates and the experimental mean and standard deviation were calculated for all gravimetric and sugar analyses.

5.3.1.2 Fermenter Conditions

To better control pH and mixing, we used BIOFLO 310 5L fermenters (New Brunswick Scientific, Edison, NJ) with a 3L working volume to further evaluate the effects on growth and lipid accumulation of VA or HBA with glucose, in comparison to glucose only controls. The pH of all cultures was adjusted to 7 using NaOH and monitored (but not controlled) using the fermenters' pH probes. Temperature was maintained at 30°C and fermentation parameters were set as previously described (Shields-Menard et al., 2015). Samples were collected as described below every 12 hours until 48 hours and then daily until 168 hours.

5.3.2 Analyses

Culture samples (50mL) were collected daily from all experiments. Samples were centrifuged at 2825 x g for 20 minutes and the supernatant was transferred to another vial for pH (Accumet), glucose, HBA, VA, and phenol analysis. The pellets were stored at -20°C and then freeze-dried (Freezone). Cell dry weight (CDW) was determined by subtracting the initial mass of the sample tube from final mass of the tube containing the freeze-dried cell pellet. Glucose concentration was measured for all samples in triplicate using a YSI 2900 Biochemistry Analyzer (YSI Incorporated Life Sciences, Yellow Springs, OH).

5.3.3 Lipid Analysis

Lipids were extracted from the cell pellet using a modified Bligh and Dyer method (Bligh & Dyer, 1959), with minor modifications as described previously (Revellame et al., 2012; Shields-Menard et al., 2015). Solvent was evaporated using a TurboVap LV (Caliper Life Sciences, Hopkinton, MA, USA) at 50°C with a 15 psi stream of nitrogen gas for 50 minutes before the extracted lipids were weighed to determine the gravimetric lipid accumulation. The extracted lipids were then transesterified into fatty acid methyl esters (FAMEs) and analyzed by three injections of each sample (Revellame et al., 2012). An Agilent 6890N gas chromatograph with a flame-ionization detector (GC-FID; Agilent Technologies Inc., Wilmington, DE, USA) and a Zebron ZB-FFAP column (30 m x 0.25 mm, film thickness 0.25 μm) was calibrated using a standard solution of C9-C24 FAMEs in known concentrations (Sigma Aldrich, St. Louis, MO, USA) with 1, 3-dichlorobenzene as an internal standard. Helium at a flow rate of 1.5 mL per minute was used as the carrier gas. The detector temperature was 260°C and the oven temperature was 50-250°C with a rate of 10°C/minute.

5.3.4 Phenol, HBA, and VA Analysis

An Agilent 6890N GC-FID (Agilent Technologies Inc., Wilmington, DE, USA) equipped with a solid-phase microextraction fiber (SPME; Sigma Aldrich, St. Louis, MO, USA) was used to determine phenol concentration as it avoids loss of analytes compared to other methods (Buchholz & Pawliszyn, 1993). The SPME was set for a vial penetration of 25mm and an extraction time of 30 seconds followed by desorbing to the injector for 30 seconds. Injection penetration was 50mm. The injector was maintained at 280°C and split in a ratio of 10:1 and the detector was set at 300°C. The oven

temperature was programmed to 80°C for 2 minutes with an increase of 10°C/minute to 150°C for 9 minutes and an increase of 20°C/minute to 300°C for 17 minutes. A Phenomenex ZB-5 5% phenyl capillary column (30 m x 250 μm x 0.25 μm) was calibrated using known concentrations of phenol.

To monitor HBA and VA concentrations, samples were collected every two hours from BIOFLO 310 fermenters. Samples were filtered using a 0.45 μm Teflon filter. Analysis was performed on an Agilent 1260 Infinity Liquid Chromatography (LC) system coupled to Agilent 380 evaporative light scattering detector (Santa Clara, CA). The chromatography separation was achieved with a Poroshell 120 EC-C18 column (3.0 x 150 mm, 2.7 μm) preceded by a Poroshell 120 EC-C18 guard column (2.1 x 5 mm, 2.7 μm), both from Agilent (Santa Clara, CA, USA). The mobile phase consisted of: A (99.5% H_2O/0.5% formic acid v/v) and B (99.5% acetonitrile/0.5% formic acid v/v). Formic acid (>99.5%) and liquid chromatography grade acetonitrile and water were obtained from Fisher Scientific (Bridgewater, NJ, USA). Flow rate was set at 0.6 mL/minute with an injection volume of 10 μL. The gradient was initially held at 5% B for 2 minutes. There was a linear increase from 2 to 9 minutes (12% B), from 9 to 11 minutes (15% B), from 11 to 14 minutes (50% B), and a linear decrease from 14 to 15 minutes (5% B). The equilibration was achieved 4 minutes after returning to the initial conditions. Identification and quantification were carried out using the evaporative light scattering detector (ELSD). ELSD parameters included nebulization with nitrogen at 28°C and a flow rate of 2.75 standard L per minute (SLM). The evaporator temperature was 30°C.

5.3.5 Protein Analysis

Culture protein lysates were prepared using a modified method by Patrauchan et al. (Patrauchan et al., 2005). Briefly, cell pellets were disrupted by bead-beating in 10:1 v/v cell pellet to lysis buffer (8 M urea, 4% CHAPS, 30 mM Tris pH 8.5, and protease inhibitor cocktail (Sigma P2714)). Zirconia/silica beads (0.5 g of 0.1 mm beads) were used for bead-beating for 5 cycles of 1 minute and cooled on ice between cycles. Debris was removed by centrifugation at 34,180 x *g* for 30 minutes at 10°C, discarding the pellet, and centrifuging the supernatant at 16,100 x *g* at 10°C for 10 minutes. Protein concentration was quantified using a Fluka Protein Quantification Kit and equal amounts (100ug) of protein from each sample was precipitated using methanol and chloroform (4:1). Protein pellets were resuspended in 100 mM ammonium bicarbonate and 5% acetonitrile for trypsin digestion. Dithiothreitol (DTT) (50 mM) was added for 10 minutes and proteins were incubated at 65°C followed by the addition of 100 mM Iodoacetamide (IAA) for 30 minutes in a dark incubator at 30°C. Trypsin (0.1µg/µl; Pierce Trypsin Protease, Mass Spectrometry- grade, Thermo Scientific) was added and proteins were digested overnight at 37°C. Glacial acetic acid was used to stop the digestion and detergents and salts were removed by using a Pierce Detergent Removal Spin Column (Thermo Scientific) and a macrotrap (Michrome Bioresources), both by using manufacturer's instructions. The samples were dried by vacuum centrifuge at room temperature. Desalted samples were resuspended in 0.1% formic acid with 5% acetonitrile for analysis.

Peptide mass spectrometry was accomplished using a Dionex UltiMate 3000 (Thermo Scientific) high performance liquid chromatography machine (HPLC) coupled

with an LTQ-OrbiTrap Velos (Thermo Scientific) tandem mass spectrometer. The Dionex UltiMate 3000 was configured for reversed phase chromatography using an Acclaim PepMap RSLC column (Thermo Scientific) with a flow rate of 300 nL/minute. Peptides were separated for mass spectrometry analysis using an acetonitrile gradient starting at 2% ACN, 0.1% FA and reaching 50% ACN, 0.1% FA in 120 minutes, followed by a 15 minutes wash of 95% ACN, 0.1% FA. Column equilibration was handled automatically using the Dionex UltiMate 3000. The eluate from the HPLC was fed directly to the LTQ-OrbiTrap Velos for nanospray ionization followed by MS/MS analysis of detected peptides. The LTQ-OrbiTrap Velos was configured to perform 1 ms scan followed by 18 MS/MS scans of the 18 most intense peaks repeatedly over the 135 min duration of each HPLC run.

Tandem mass spectra (MS/MS) were extracted and the charge state was deconvoluted and deisotoped by Proteome Discoverer (Proteome Discoverer ver. 1.4.0.288, Thermo Fisher Scientific, CA, USA). The analysis was performed by using Sequest (XCorr) (Thermo Fisher Scientific, CA, USA) and X! Tandem (version CYCLONE 2010.12.01.1). The NCBI database for *Rhodococcus rhodochrous* ATCC (version from March 2015, 6034 entries) was used for the proteomic comparisons, using the *in silico* trypsin digestion, with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 1.5 Da. Oxidation of methionine and carbamidomethylation of cysteine were chosen as variable modifications in both searches. Additionally, Glu->pyro-Glu of the N-terminus, ammonia-loss of the N-terminus, and gln->pyro-Glu of the N-terminus were specified in X! Tandem as variable modifications. Scaffold (version 4.4.5, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and

protein identifications. Peptide identifications were accepted if they achieved a false delivery rate (FDR) less than 0.1%. Peptide probabilities from X! Tandem were assigned by the Peptide Prophet algorithm (Keller et al., 2002) with Scaffold delta-mass correction. Peptide probabilities from Sequest (XCorr Only) were assigned by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Fold change was calculated using weighted spectral counts and a minimum value of 0.5 counts was used for the proteins with a spectral count of 0 in order to calculate the fold change; minimum 1.5 fold change was required for differential expression analysis. The statistical analysis was performed using Fisher's test with Hochberg-Benjamini correction (see p-values in Table 5.2).

5.4 Results and Discussion

5.4.1 Phenol reduces, but does not inhibit growth and lipid accumulation of *R. rhodochrous*

Phenol was used to investigate the effects of a common aromatic compound present in lignocellulosic hydrolysates on the growth and lipid accumulation of *R. rhodochrous*. Cell dry weight of *R. rhodochrous* increased to 4g/L after 120 hours of growth in glucose and phenol supplemented media, although overall growth in the mixture of glucose and phenol occurred at a slower rate than the glucose control (Figure 5.2A and 5.2C). Phenol concentrations were below the level of detection by GC-FID at

48 hours in both the phenol and phenol with glucose treatments (Figure 5.2A), but the phenol concentration in the abiotic control remained close to 1g/L, suggesting that *R. rhodochrous* utilized phenol as a carbon source for growth. The increase in cell dry weight of *R. rhodochrous* grown in phenol as the sole carbon source is minimal compared to that of the glucose control and combination of glucose and phenol; however, when comparing the cell dry weight at 120 hours to time 0, there is a noticeable increase (Figure 5.2A and 5.2C), further suggesting that *R. rhodochrous* can tolerate and utilize 1g/L of phenol as a carbon source. Phenol, a common ingredient in cleaning supplies, is generally considered toxic to most microbes and has been shown to have inhibitory effects on growth and yields of fermentative organisms (Klinke et al., 2004; Palmqvist & Hahn-Hagerdal, 2000). *Rhodococcus* species have been well described for their metabolic capabilities in degrading a plethora of compounds (Larkin et al., 2005; Martinkova et al., 2009). A new species of *Rhodococcus*, *Rhodococcus phenolicus*, was shown to degrade phenol as a sole carbon source and grow in phenol concentrations up to 7.5g/L (Rehfuss & Urban, 2005).

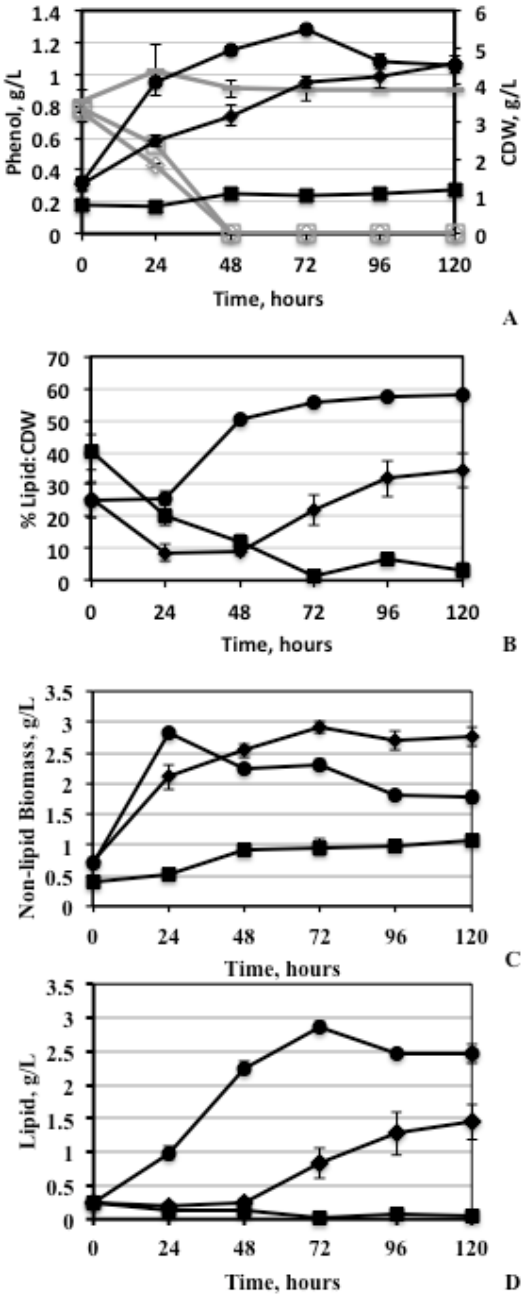

Figure 5.2 Growth and lipid accumulation of *R. rhodochrous* grown in phenol-supplemented media.

Phenol concentration (gray; g/L ±SD) and *R. rhodochrous* cell dry weight (black; g/L ±SD; **A**), percent lipid (±SD; **B**), non-lipid biomass (g/L ±SD; **C**), and lipid (g/L ±SD; **D**) of *R. rhodochrous* shake flask cultures supplemented with 20g/L glucose (•), 1g/L phenol (■), or 1g/L phenol and 20g/L glucose (◆).

In this study, *R. rhodochrous* could use 1g/L phenol as a sole carbon source for growth, but achieved greater growth when the media was supplemented with glucose. Similarly, *Pseudomonas putida* overcame the inhibitory effect of phenol when cultivated with glucose as an added growth substrate (Mamma et al., 2004). A recent study further suggested that a key enzyme of the phenol degradation pathway, phenol hydroxylase was repressed by succinate, but not by the addition of glucose (Szokol et al., 2014). While our data show the degradation of phenol and growth of *R. rhodochrous* using both glucose and phenol, the slower growth rate might indicate the presence of another catabolite repressing an enzyme involved in phenol degradation.

5.4.2 Lipid analysis and FAME profiles of *R. rhodochrous* grown in phenol

Lipid accumulation by *R. rhodochrous* grown in a mixture of glucose and phenol was evident within 48 hours, though glucose only controls began accumulating within the first 24 hours (Figure 5.2B and 5.2D). *R. rhodochrous* accumulated over 30% of cell dry weight as lipids when grown in phenol with glucose, which is still considered oleaginous (over 20%) even though the glucose control yielded 60% lipid (Figure 5.2B). When *R. rhodochrous* was grown in just phenol, lipid content decreased to 0.03g/L (3% of CDW) by 120 hours (Figures 5.2B and 5.2D), suggesting that these lipids were consumed for growth after phenol exhaustion at 48 hours (Figure 5.2C and 5.2D). Lipid accumulation by *R. rhodochrous* grown in phenol as a sole carbon source was likely limited due to the lack of available carbon (1g/L phenol) in the media. Further studies should aim to increase the amount of phenol available to *R. rhodochrous* to promote lipid accumulation without having toxic effects.

Low molecular weight phenolic compounds have been shown to be more toxic than larger phenolics, inhibiting microbial growth and fermentation by causing membrane instability (Klinke et al., 2004; Palmqvist & Hahn-Hagerdal, 2000). A study by Tsitko et al. (1999) investigated the effects of aromatic compounds on the fatty acid composition of *R. opacus* and found that cells grown on aromatic substrates had increased amounts of odd-chain fatty acids. Similarly, our data indicated an increase in unknown FAMEs at 72 hours (Table 5.1), which corresponded to an unidentifiable peak with a retention time between that of C16:0 and C18:1. If these unknown FAMEs are indeed odd-chain saturated fatty acids, then an increase in saturated fatty acids suggests that *R. rhodochrous* may be reacting to the presence of membrane-active compounds and has a protection mechanism against cell disruption by aromatic compounds (Tsitko et al., 1999).

5.4.3 Proteomic analysis of *R. rhodochrous* grown in phenol

Almost 500 proteins from each treatment were identified in the proteomic analysis, several of which were related to aromatic degradation, central metabolism, fatty acid metabolism, nitrogen metabolism, and other important cellular functions (Table 5.2). The mechanism of lipid synthesis and accumulation in yeasts is generally well understood and provides the mechanistic foundation for lipid accumulation in all oleaginous microbes (Jin et al., 2015). Recent proteomic comparisons have gained valuable insight towards the triacylglyercol (TAG) biosynthesis, storage, and degradation among oleaginous organisms, including *Rhodococcus* (Chen et al., 2014; Davila Costa et al., 2015).

R. rhodochrous was recently characterized as oleaginous (Shields-Menard et al., 2015). When cultured in glucose, this bacterium accumulated 25% of CDW as lipid within 24 hours (Figures 5.2B and 5.2D). As lipid accumulation is beginning, there is also a large increase in non-lipid biomass (growth) between 0 and 24 hours as nitrogen is not yet limited (previous data). When we compared the protein abundance at 24 hours to that at 0 hours, there was a 25-fold increase in biotin carboxylase (oxaloacetate decarboxylase), an enzyme subunit of acetyl-CoA carboxylase (ACC) responsible for shunting the flux of carbon to lipids by catalyzing the conversion of acetyl-CoA to malonyl-CoA for fatty acid synthesis (Davila Costa et al., 2015; Magdouli et al., 2014). The switch from cell growth (oxidative catabolism) to lipid accumulation (reductive anabolism) was also evident by the increased expression of acyltransferase, citrate synthase and 3-isopropylmalate dehydrogenase and the decrease in abundance of adenylate kinase (Table 5.2). Malic enzyme, which is critical in replenishing NADPH during fatty acid synthesis in nitrogen starvation, had a 16.7-fold reduction within 24 hours (Table 5.2). The abundance of proteins involved in both lipid synthesis and cell growth suggest that *R. rhodochrous* is likely entering limited nitrogen conditions, slowing cell growth, and beginning lipid accumulation.

Phenol was undetected in the media by 48 hours, suggesting that *R. rhodochrous* was able to degrade and use phenol as a carbon source (Figure 5.2A). Several proteins involved in aromatic degradation (Prieto et al., 1993; Wells & Ragauskas, 2012) were in high abundance in phenol cultures at 24 hours compared to 0 hour: multiple putative HpaB/PvcC/4-BUDH; catechol 1, 2-dioxygenases; and catechol 2, 3-dioxygenase (Table 5.2).

Table 5.1 Fatty acid methyl ester profile (% w/w) of *R. rhodochrous* grown in glucose (Glu), phenol (Phe) or a mixture of glucose and phenol (GP).

	Myristic (C14:0)	Palmitic (C16:0)	Palmitoleic (C16:1)	Unknown	Stearic (C18:0)	Oleic (C18:1)	Erucic (C22:1)
Glu 0H	2.44	39.42	9.22	8.70	4.30	32.96	2.96
Phe 0H	2.48	39.61	9.48	8.61	4.37	32.51	2.95
GP 0H	2.53	39.32	9.69	8.61	4.20	32.77	2.88
Glu 24H	2.46	34.26	10.00	7.40	5.91	37.13	2.01
Phe 24H	2.75	36.18	9.12	8.02	4.72	36.53	2.37
GP 24H	3.26	38.60	13.22	7.28	4.12	29.88	3.60
Glu 48H	2.46	33.08	10.13	9.29	4.27	37.48	2.29
Phe 48H	2.90	37.31	10.10	7.41	3.73	32.86	4.98
GP 48H	3.15	38.73	8.73	13.48	4.05	28.41	2.38
Glu 72H	2.85	34.70	10.47	12.81	3.50	35.15	0.00
Phe 72H	0.92	49.89	7.62	4.04	1.42	36.10	0.00
GP 72H	3.45	37.14	15.05	14.19	3.86	25.83	0.00

Percentages are an average of triplicate injections of all experimental treatment replicates. Standard deviation is less than 11 %. FAMEs less than 1% are not shown.

Table 5.2 Proteins of *R. rhodochrous* cultured in glucose, phenol, and glucose with phenol.

Glucose 24 hrs vs Glucose 0 hrs Fold change	Fisher's ExactTest (P-Value): *(p <= 0.00610)	Protein Name	Role	Accession Number	Molecular Weight
-50.0	< 0.00010	Adenylate kinase [Rhodococcus rhodochrous ATCC 21198]	energy balance	gi|573523828	19 kDa
-33.3	< 0.00010	ABC-type branched-chain amino acid transport systems periplasmic component-like protein [Rhodococcus rhodochrous ATCC 21198]	transporter	gi|573527736	45 kDa
-16.7	< 0.00010	malic protein NAD-binding protein [Rhodococcus rhodochrous ATCC 21198]	fatty acid synthesis	gi|573526831	41 kDa
-14.3	< 0.00010	Succinyl-CoA ligase (ADP-forming) subunit beta [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573528361	41 kDa
-10.0	0.0023	transketolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525360	74 kDa
-5.0	< 0.00010	6-phosphogluconate dehydratase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526218	64 kDa
-3.3	< 0.00010	Enolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525840	45 kDa
-3.3	< 0.00010	Phosphoglycerate kinase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525373	42 kDa
-2.0	0.005	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526276	61 kDa
-2.0	0.0027	nitrogen regulatory protein P-II [Rhodococcus rhodochrous ATCC 21198]	nitrogen metabolism	gi|573526092	12 kDa
-2.0	< 0.00010	glyceraldehyde-3-phosphate dehydrogenase, type I [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525374	36 kDa

Table 5.2 (Continued)

2.5	< 0.00010	dihydrolipoamide dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573523617	57 kDa
3.2	0.0028	Citrate (Si)-synthase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573528494	40 kDa
4.3	< 0.00010	pyruvate carboxylase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573529586	121 kDa
5.2	< 0.00010	Acyl transferase [Rhodococcus rhodochrous ATCC 21198]	fatty acid synthesis	gi|573527113	323 kDa
5.3	< 0.00010	Biotin carboxylase, Propionyl-CoA carboxylase [Rhodococcus rhodochrous ATCC 21198]	fatty acid synthesis	gi|573528207	199 kDa
5.5	0.00047	3-isopropylmalate dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573529634	35 kDa
15.0	0.00056	isocitrate lyase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573523598	47 kDa
25.0	< 0.00010	Biotin carboxylase, Oxaloacetate decarboxylase [Rhodococcus rhodochrous ATCC 21198]	fatty acid synthesis	gi|573525143	64 kDa

Glucose 24 hrs vs Glucose+phenol 24 hrs Fold Change	**Fisher's Exact Tes (P-Value) *(p <= 0.01128)**	**Protein Name**	**Role**	**Accession Number**	**Molecular Weight**
-33.3	< 0.00010	Succinyl-CoA ligase (ADP-forming) subunit beta [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573528361	41 kDa
-25.0	< 0.00010	malic protein NAD-binding protein [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573526831	41 kDa
-12.5	< 0.00010	catechol 1,2-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573524225	32 kDa

Table 5.2 (Continued)

-11.1	< 0.00010	glutamate synthase, NADH/NADPH, small subunit [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526911	52 kDa
-10.0	0.0016	glucokinase, ROK family [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526222	33 kDa
-10.0	0.0022	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573523521	27 kDa
-10.0	0.00012	UTP--glucose-1-phosphate uridylyltransferase [Rhodococcus rhodochrous ATCC 21198]	nitrogen metabolism	gi|573528320	32 kDa
-5.0	0.0077	transketolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525360	74 kDa
-3.3	0.007	Transaldolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525361	41 kDa
-2.0	< 0.00010	Phosphoglycerate kinase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525373	42 kDa
-1.7	< 0.00010	Enolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525840	45 kDa
1.6	0.0043	pyruvate carboxylase [Rhodococcus rhodochrous ATCC 21198]	Anaplerotic pathway	gi|573529586	121 kDa
1.7	0.00034	aconitate hydratase 1 [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573527327	101 kDa
2.1	0.0018	3-isopropylmalate dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	citrate/malate cyle	gi|573529634	35 kDa
2.3	< 0.00010	fructose-1,6-bisphosphatase, class II [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526483	36 kDa
2.3	< 0.00010	Triosephosphate isomerase, bacterial/eukaryotic [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525372	27 kDa
2.3	0.00074	Biotin carboxylase, Oxaloacetate decarboxylase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism/ ACC	gi|573525143	64 kDa
2.5	< 0.00010	dihydrolipoamide dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573523617	57 kDa
2.6	0.0046	6-phosphogluconate dehydrogenase, decarboxylating [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573524711	52 kDa

Table 5.2 (Continued)

2.9	< 0.00010	cysteine synthase A [Rhodococcus rhodochrous ATCC 21198]	Amino acid and protein metabolism	gi|573523949	33 kDa
3.4	< 0.00010	2-oxo-acid dehydrogenase E1 subunit, homodimeric type [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525512	105 kDa
3.4	< 0.00010	Acyl transferase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573527113	323 kDa
3.6	< 0.00010	urea ABC transporter, urea binding protein [Rhodococcus rhodochrous ATCC 21198]	Nitrogen metabolism	gi|573523602	44 kDa
4.2	0.00046	Acetyl-coenzyme A synthetase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573529490	70 kDa
4.7	0.00048	IMP dehydrogenase family protein [Rhodococcus rhodochrous ATCC 21198]	citrate/malate cycle	gi|573524713	50 kDa
5.0	< 0.00010	Biotin carboxylase, Propionyl-CoA carboxylase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573528207	199 kDa
5.5	< 0.00010	succinate dehydrogenase, flavoprotein subunit [Rhodococcus rhodochrous ATCC 21198]	Amino acid and protein metabolism	gi|573525085	64 kDa
5.6	< 0.00010	succinate dehydrogenase and fumarate reductase iron-sulfur protein [Rhodococcus rhodochrous ATCC 21198]	Amino acid and protein metabolism	gi|573525084	29 kDa
5.7	0.00089	3-oxoacyl-(acyl-carrier-protein) reductase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573527700	46 kDa
7.2	< 0.00010	Beta-ketoacyl-acyl-carrier-protein synthase III [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573528205	71 kDa
7.9	< 0.00010	Catalase-peroxidase [Rhodococcus rhodochrous ATCC 21198]	antioxidant	gi|573526986	80 kDa

Table 5.2 (Continued)

8.2	< 0.00010	acetyl-CoA acetyltransferase [Rhodococcus rhodochrous ATCC 21198]	B-oxidation	gi|573527699	46 kDa

Glucose 24 hrs vs Phenol 24 hrs Fold Change	**Fisher's Exact Tes (P-Value) *(p <= 0.01037)**	**Protein Name**	**Role**	**Accession Number**	**Molecular Weight**
-50.0	< 0.00010	malic protein NAD-binding protein [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573526831	41 kDa
-50.0	< 0.00010	putative HpaB/PvcC/4-BUDH, partial [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573528977	36 kDa
-33.3	< 0.00010	Catechol 2,3-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	phenol metabolism	gi|573526750	41 kDa
-33.3	< 0.00010	Succinyl-CoA ligase (ADP-forming) subunit beta [Rhodococcus rhodochrous ATCC 21198]	Central metabolism	gi|573528361	41 kDa
-25.0	< 0.00010	catechol 1,2-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	phenol metabolism	gi|573523683	31 kDa
-20.0	< 0.00010	Adenylate kinase [Rhodococcus rhodochrous ATCC 21198]	energy balance	gi|573523828	19 kDa
-20.0	< 0.00010	putative HpaB/PvcC/4-BUDH, partial [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573524229	11 kDa
-20.0	< 0.00010	catechol 1,2-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	phenol metabolism	gi|573524225	32 kDa
-12.5	< 0.00010	Acetaldehyde dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526749	31 kDa
-11.1	< 0.00010	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573523521	27 kDa
-11.1	< 0.00010	putative HpaB/PvcC/4-BUDH, partial [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573525281	17 kDa

Table 5.2 (Continued)

-10.0	0.00033	glutamate synthase, NADH/NADPH, small subunit [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526911	52 kDa
-10.0	0.0072	PEP phosphonomutase-like protein [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573524216	26 kDa
-10.0	0.0016	Alcohol dehydrogenase, zinc-type, actinomycete [Rhodococcus rhodochrous ATCC 21198]	energy balance	gi|573527429	39 kDa
-10.0	0.01	Phosphoenolpyruvate carboxykinase (GTP) [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573523953	67 kDa
-10.0	0.0016	acetyl-CoA acetyltransferase [Rhodococcus rhodochrous ATCC 21198]	B oxidation	gi|573524197	42 kDa
-2.0	< 0.00010	Phosphoglycerate kinase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525373	42 kDa
-1.7	0.002	Enolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525840	45 kDa
1.5	0.0075	aconitate hydratase 1 [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573527327	101 kDa
1.6	0.0037	Triosephosphate isomerase, bacterial/eukaryotic [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525372	27 kDa
1.6	0.00	Triosephosphate isomerase, bacterial/eukaryotic [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525372	27 kDa
2.3	0.0015	3-isopropylmalate dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	malate cycle	gi|573529634	35 kDa
2.5	0.00092	Biotin carboxylase, Oxaloacetate decarboxylase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573525143	64 kDa
2.7	0.0027	succinate dehydrogenase, flavoprotein subunit [Rhodococcus rhodochrous ATCC 21198]	Amino acid and protein metabolism	gi|573525085	64 kDa
2.8	0.0053	6-phosphogluconate dehydrogenase, decarboxylating [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573524711	52 kDa
2.8	< 0.00010	D-3-phosphoglycerate dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573529635	55 kDa

Table 5.2 (Continued)

2.9	< 0.00010	fructose-1,6-bisphosphatase, class II [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526483	36 kDa
3.1	< 0.00010	dihydrolipoamide dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	Acetyl-CoA synthesis	gi|573523617	57 kDa
3.1	< 0.00010	pyruvate carboxylase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573529586	121 kDa
3.3	0.00065	IMP dehydrogenase family protein [Rhodococcus rhodochrous ATCC 21198]	citrate/malate cycle	gi|573524713	50 kDa
4.2	0.00076	Glucose-6-phosphate isomerase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573528367	58 kDa
4.5	0.0042	IMP dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	citrate/malate	gi|573524120	52 kDa
4.5	0.0027	acetyl-CoA acetyltransferase [Rhodococcus rhodochrous ATCC 21198]	B oxidation	gi|573527699	46 kDa
5.5	< 0.00010	Phosphoserine aminotransferase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573528495	40 kDa
7.4	0.00063	6-phosphogluconate dehydratase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526218	64 kDa
9.0	< 0.00010	Beta-ketoacyl-acyl-carrier-protein synthase III [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573528205	71 kDa
44.0	< 0.00010	Acyl transferase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573527113	323 kDa
78.0	< 0.00010	Biotin carboxylase, Propionyl-CoA carboxylase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573528207	199 kDa

Phenol 24 hrs vs Phenol 0 hrs Fold Change	**Fisher's Exact Tes (P-Value) *(p <= 0.00579)**	**Protein Name**	**Role**	**Accession Number**	**Molecular Weight**
-33.3	< 0.00010	6-phosphogluconate dehydratase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526218	64 kDa

Table 5.2 (Continued)

-20.0	< 0.00010	ABC-type branched-chain amino acid transport systems periplasmic component-like protein [Rhodococcus rhodochrous ATCC 21198]	transporter	gi|573527736	45 kDa
-14.3	< 0.00010	Biotin carboxylase, Propionyl-CoA carboxylase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573528207	199 kDa
-10.0	< 0.00010	Acyl transferase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573527113	323 kDa
-2.5	0.00018	aspartate-semialdehyde dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	amino acid metabolism	gi|573524856	36 kDa
-2.5	0.0043	urea ABC transporter, urea binding protein [Rhodococcus rhodochrous ATCC 21198]	nitrogen metabolism	gi|573523602	44 kDa
-2.5	< 0.00010	glyceraldehyde-3-phosphate dehydrogenase, type I [Rhodococcus rhodochrous ATCC 21198]	Central metabolism	gi|573525374	36 kDa
-2.0	0.0015	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526276	61 kDa
-2.0	0.0013	D-3-phosphoglycerate dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	amino acid metabolism	gi|573529635	55 kDa
-2.0	0.00036	Adenylate kinase [Rhodococcus rhodochrous ATCC 21198]	energy balance	gi|573523828	19 kDa
-2.0	< 0.00010	Enolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525840	45 kDa
-1.4	0.0035	Phosphoglycerate kinase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525373	42 kDa
2.7	0.00013	malic protein NAD-binding protein [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573526831	41 kDa
9.9	0.0046	Biotin carboxylase, Oxaloacetate decarboxylase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism/ACC	gi|573525143	64 kDa
11.0	0.0027	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573523521	27 kDa
11.0	0.0027	putative HpaB/PvcC/4-BUDH, partial [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573525281	17 kDa

Table 5.2 (Continued)

12.0	0.00093	Transaldolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525361	41 kDa
13.0	0.0016	Acetaldehyde dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526749	31 kDa
15.0	0.00054	isocitrate lyase [Rhodococcus rhodochrous ATCC 21198]	Dioxylate/dicarboxylate metabolism	gi|573523598	47 kDa
16.0	< 0.00010	Betaine-aldehyde dehydrogenase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526747	52 kDa
19.0	< 0.00010	putative HpaB/PvcC/4-BUDH, partial [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573524229	11 kDa
20.0	< 0.00010	catechol 1,2-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573524225	32 kDa
23.0	< 0.00010	catechol 1,2-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573523683	31 kDa
31.0	< 0.00010	Catechol 2,3-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573526750	41 kDa
54.0	< 0.00010	putative HpaB/PvcC/4-BUDH, partial [Rhodococcus rhodochrous ATCC 21198]	phenol degradation	gi|573528977	36 kDa

Phenol+glucose 24 hrs vs 0 hrs Fold Change	**Fisher's Exact Tes (P-Value) *(p <= 0.00610)**	**Protein Name**	**Role**	**Accession Number**	**Molecular Weight**
-33.3	< 0.00010	ABC-type branched-chain amino acid transport systems periplasmic component-like protein [Rhodococcus rhodochrous ATCC 21198]	Transporter	gi|573527736	45 kDa
-10.0	< 0.00010	ABC-type transporter, periplasmic subunit family 3 [Rhodococcus rhodochrous ATCC 21198]	transporter	gi|573527959	29 kDa

Table 5.2 (Continued)

-5.0	< 0.00010	Adenylate kinase [Rhodococcus rhodochrous ATCC 21198]	energy balance	gi|573523828	19 kDa
-5.0	< 0.00010	urea ABC transporter, urea binding protein [Rhodococcus rhodochrous ATCC 21198]	Nitrogen metabolism	gi|573523602	44 kDa
-3.3	< 0.00010	6-phosphogluconate dehydratase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526218	64 kDa
-2.5	< 0.00010	glyceraldehyde-3-phosphate dehydrogenase, type I [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525374	36 kDa
-2.0	< 0.00010	glutamine synthetase, type I [Rhodococcus rhodochrous ATCC 21198]	nitrogen metabolism	gi|573526282	54 kDa
-2.0	0.0011	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526276	61 kDa
-1.7	< 0.00010	Enolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525840	45 kDa
2.1	0.0026	Succinyl-CoA ligase (ADP-forming) subunit beta [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573528361	41 kDa
4.9	0.0028	2-oxoglutarate dehydrogenase, E1 subunit [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573526837	138 kDa
10.0	0.0045	UTP--glucose-1-phosphate uridylyltransferase [Rhodococcus rhodochrous ATCC 21198]	Nitrogen metabolism	gi|573528320	32 kDa
11.0	0.003	Biotin carboxylase, Oxaloacetate decarboxylase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism/acc	gi|573525143	64 kDa
11.0	0.003	isocitrate lyase [Rhodococcus rhodochrous ATCC 21198]	glyoxylate/dicarboxylate metabolism	gi|573523598	47 kDa
12.0	0.0013	catechol 1,2-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	Phenol degradation	gi|573524225	32 kDa
16.0	< 0.00010	Transaldolase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi|573525361	41 kDa
16.0	< 0.00010	3-hydroxybutyryl-CoA epimerase [Rhodococcus rhodochrous ATCC 21198]	fatty acid metabolism	gi|573526670	75 kDa
22.0	< 0.00010	putative HpaB/PvcC/4-BUDH, partial [Rhodococcus rhodochrous ATCC 21198]	Phenol degradation	gi|573528977	36 kDa

Proteins involved in central metabolism and fatty acid synthesis, such as 6-phosphogluconate dehydratase, an amino acid transport system, propionyl-CoA carboxylase and acyltransferase, were less abundant at 24 hours than 0 hour, which corresponds to the decrease of lipid accumulation at 24 hours. TadA (2,3-bisphosphoglycerate-dependent phosphoglycerate mutase) is involved in the structural formation of lipid bodies (Ding et al., 2012; MacEachran et al., 2010) and was found to be increased by 11-fold at 24 hours. Biotin carboxylase and malic enzyme were also over 2-fold higher at 24 hours than 0 hour. The presence of proteins involved in fatty acid synthesis may indicate that greater lipid accumulation is possible using phenol as a substrate if phenol can be used in larger concentrations.

The primary metabolic activities of *R. rhodochrous* grown in glucose or in phenol are evident when the abundance of proteins in each are compared at 24 hours. Catechol 2,3-dioxygenase, catechol 1, 2-dioxygenase, and multiple putative HpaB/PvcC/4-BUDH are significantly less abundant, which shows the difference in activity of phenol degradation. In comparison, propionyl-CoA carboxylase and acyltransferase were 78- and 44-fold higher in *R. rhodochrous* grown in glucose at 24 hours than in phenol at 24 hours, further supporting the increased lipid accumulation observed in glucose grown cells at 24 hours.

When *R. rhodochrous* was grown in a combination of glucose and phenol, there was a slower increase in lipid accumulation. The proteomic analysis of *R. rhodochrous* grown in glucose and phenol indicated an increase at 24 hours in proteins associated with phenol degradation, such as catechol 1, 2-dioxygenase and a putative HpaB/PvcC/4-BUDH enzyme, as well as those proteins associated with lipid synthesis, 3-

hydroxybutyryl-CoA epimerase and biotin carboxylase. Proteins associated with nitrogen scavenging, ABC-type branched-chain amino acid transport systems periplasmic component-like protein and a urea ABC transporter, had the highest negative fold change compared to that at 0 hours. Similarly, when the proteins identified in glucose and phenol at 24 hours were compared to those in glucose at 24 hours, phenol degradation proteins and lipid synthesis proteins were more prevalent, further supporting that the metabolic diversity of *R. rhodochrous* is capable of concurrent aromatic degradation and lipid accumulation. Further research is needed to identify the specific roles and regulatory components of *R. rhodochrous*.

5.4.4 VA and HBA do not inhibit growth or lipid accumulation when *R. rhodochrous* is grown in the presence of glucose

VA and HBA are major aromatic components in untreated hydrolysate (Palmqvist & Hahn-Hagerdal, 2000) and can be used as model compounds representing the major components of grass, hardwood, and softwood lignin (Kasi & Ragauskas, 2010; Pu et al., 2011). *R. rhodochrous* was cultured in shake flasks with HBA or VA as a sole carbon source and supplemented with glucose to investigate the effects of these model aromatic lignin compounds on growth and lipid accumulation. After 72 hours of cultivation, growth was observed to be greater when *R. rhodochrous* was supplemented with glucose than those with HBA and VA as a sole carbon source (Figure 5.3A). Furthermore, percent lipid accumulation was much greater in the cultures supplemented with glucose than those without (Figure 5.3B).

The utilization of HBA and VA by *R. rhodochrous* was also analyzed using BIOFLO 310 fermenters; concentrations of HBA and VA were measured every 2 hours

for 24 hours. The data indicated that HBA and VA were undetected by 18 hours and 20 hours, respectively (Figure 5.4D). This suggests that the low CDW and lipid accumulation analyzed at 72 hours was due to an exhaustion of carbon. However, when *R. rhodochrous* was cultured with glucose in the presence of VA and HBA, CDW increased to over 5g/L, only slightly less than the glucose control (Figure 5.4A). Lipid accumulation of over 40% was also similar between cells grown in the presence of VA and HBA and that of the glucose control (Figure 5.4B), implying that model aromatic lignin compounds do not inhibit the growth or lipid accumulation of *R. rhodochrous*.

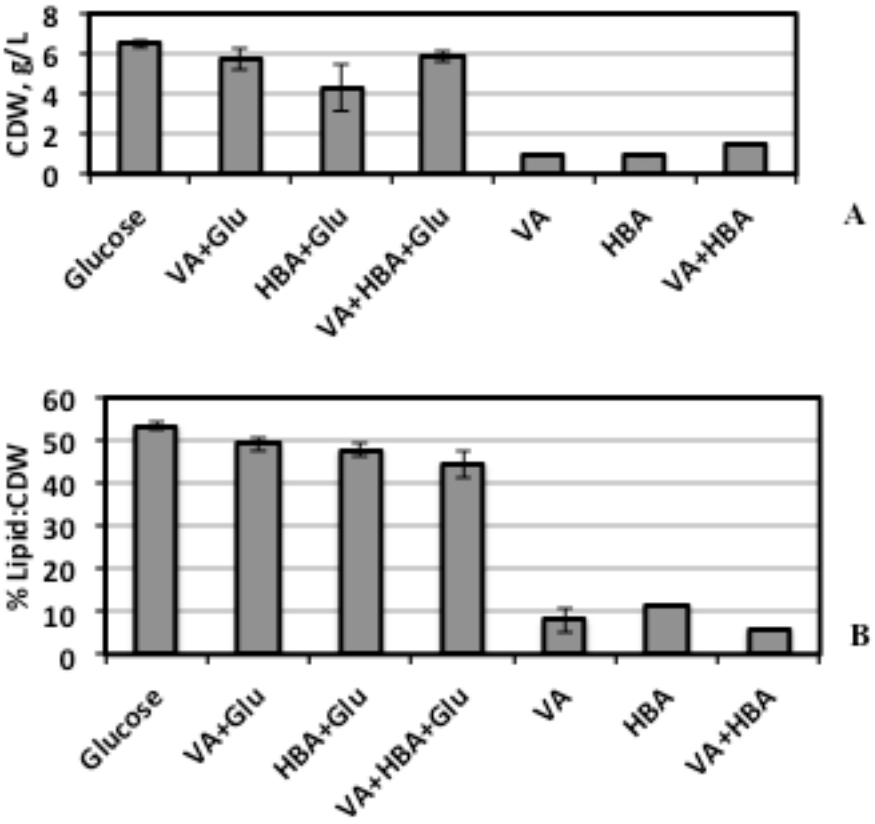

Figure 5.3 *R. rhodochrous* cell dry weight and percent lipid accumulation of shake-flask cultures supplemented with model lignin compounds.

R. rhodochrous cell dry weight (**A**; g/L ±SD) and percent lipid accumulation (**B**; ±SD) of shake flask cultures supplemented with 1.25g/L VA, 1.25g/L HBA, 1.25g/L VA + 1.25g/L HBA, with and without 20g/L glucose. Data shown from the 72-hour time point.

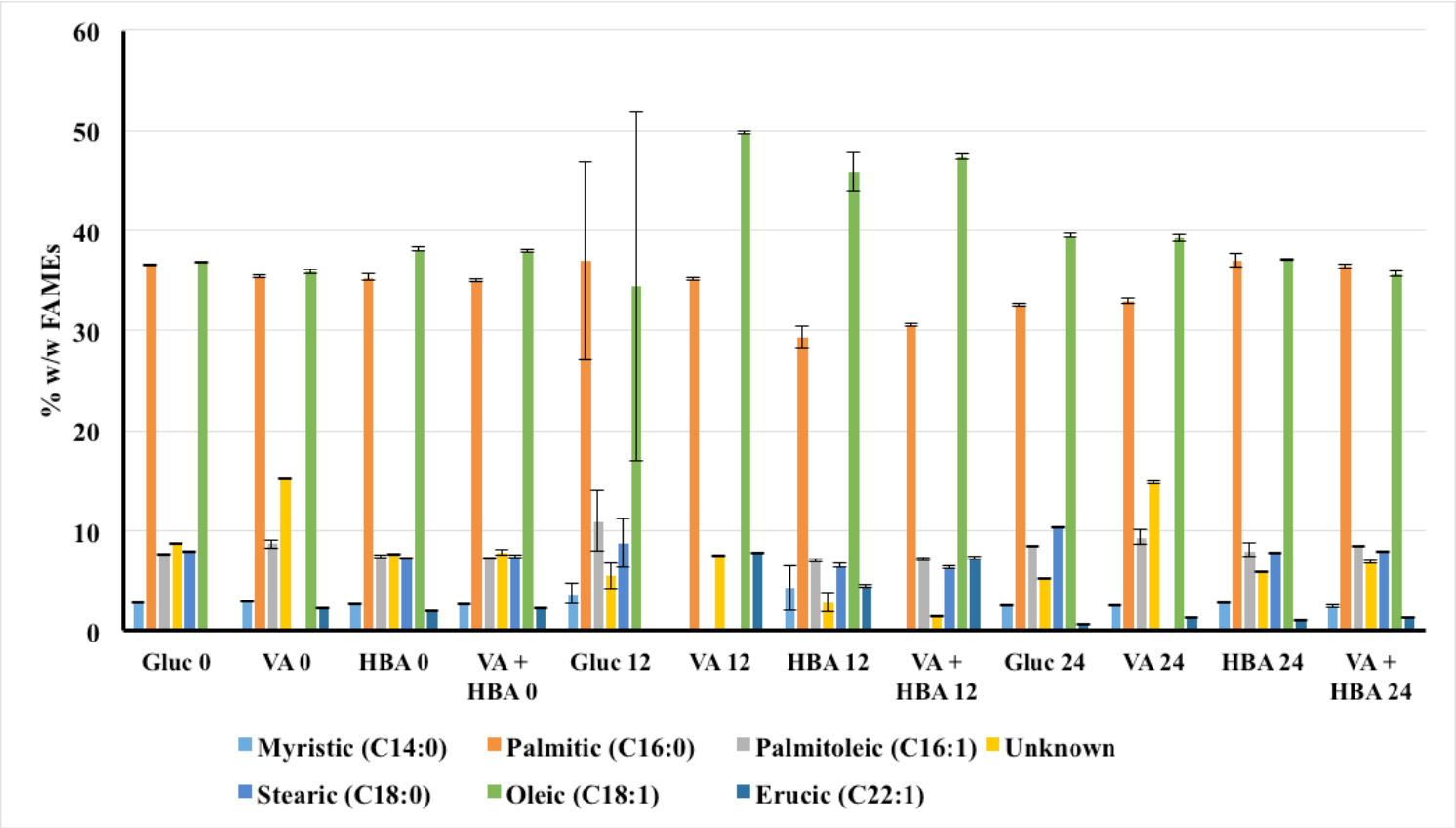

Figure 5.4 Fatty acid methyl ester profile (% w/w) of *R. rhodochrous* grown in model lignin compounds.

FAME profile of *R. rhodochrous* grown in shake flasks supplemented with 20g/L glucose (Glu), 1.25g/L vanillic acid (VA), 1.25g/L 4-hydroxybenzoic acid (HBA), or a mixture of 1/25g/L VA and 1/25g/L HBA (VA + HBA) at 0, 12, and 24 hours. Percentages are an average of triplicate injections of all experimental treatment replicates. Standard deviation is shown by error bars. FAMEs less than 1% are not shown.

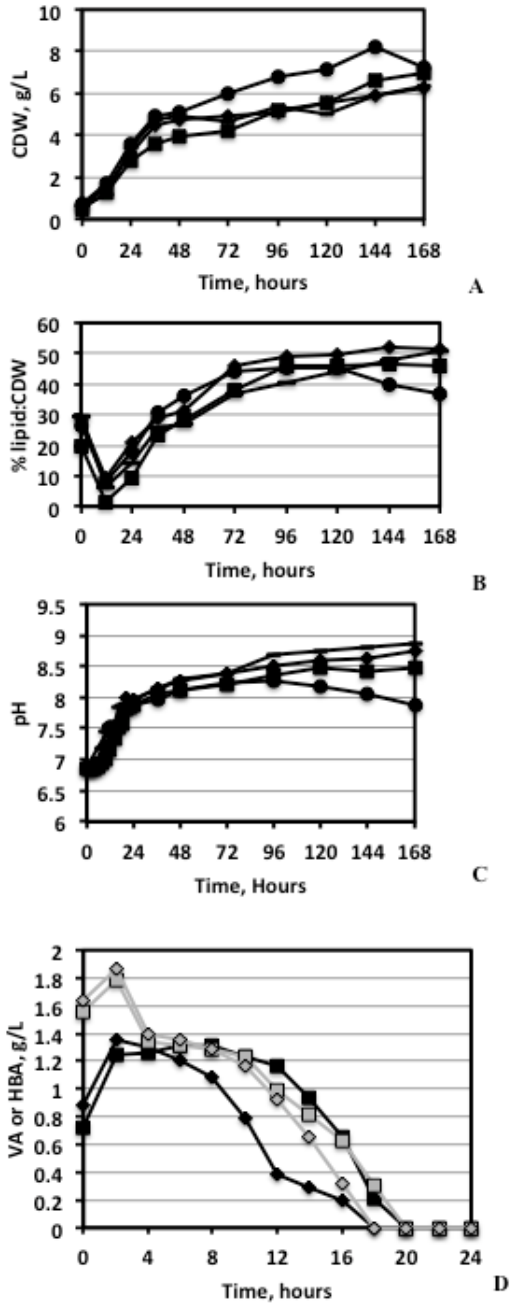

Figure 5.5 *R. rhodochrous* cell dry weight, percent lipid accumulation and pH of 3L fermentations supplemented with glucose and VA, HBA, or VA and HBA.

R. rhodochrous cell dry weight (**A**; g/L), percent lipid accumulation (**B**) and pH (**C**) of 3L fermentations supplemented with 20g/L glucose (•) and 1.25g/L VA(■), 1.25g/L HBA(◆), or 1/25g/L VA + 1.25g/L HBA (-). Vanillic acid and 4-hydroxybenzoic acid concentrations (**D**; g/L) were analyzed using HPLC-ELSD. Fermentation samples of VA (■), HBA (◆) and VA + HBA (gray; ■, ◆) were taken every 2 hours and filtered before analysis.

Since HBA and VA were undetected by 20 hours of cultivation, it was expected that lipid accumulation and cell growth would be comparable between treatments, assuming inhibitory effects were not present from 0-20 hours. The FAME analysis showed slight differences between the glucose control and the model lignin compound treatments in erucic acid at time 0 and an increase of unknowns, likely odd chain carbon fatty acids, at 24 hours (Figure 5.5). The slight differences in FAMEs may indicate a fatty acid response to aromatic compounds as observed when *R. rhodochrous* was cultivated in phenol. Furthermore, increasing pH (Figure 5.4C) could positively impact membrane transport, influencing the cell growth and lipid accumulation as proposed in previous work using the model oleaginous bacterium, *R. opacus* PD630 (Kosa & Ragauskas, 2012).

5.5 Conclusions

The purpose for using phenolic compounds in this study is twofold: phenol and its derivatives can be serious health hazards if released into the environment (Buchholz & Pawliszyn, 1993; Michalowicz & Duda, 2007) and phenol also serves as a basic model lignin compound as lignin is a polyaromatic structure. *R. rhodochrous* was recently characterized as oleaginous (Shields-Menard et al., 2015) and, this work is the first glimpse into the proteins associated with *R. rhodochrous* lipid accumulation. Furthermore, proteomic analysis provides insight into the concurrent aromatic substrate degradation and lipid accumulation by *R. rhodochrous*. When cultivated in model aromatic lignin compounds (phenol, HBA, VA) and supplemented with glucose, *R. rhodochrous* growth and lipid accumulation increased. Furthermore, phenol, HBA, and VA did not have any considerable negative effects on *R. rhodochrous* and were

undetected by 24 hours. Further work is necessary to fully elucidate the impact of lignin compounds on *R. rhodochrous*, so that growth and lipid yields can be improved without the use of glucose as a substrate.

5.6 Acknowledgements

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CHAPTER VI

EVALUATION OF PROPANE AS A CARBON SOURCE FOR GROWTH AND LIPID ACCUMULATION BY *RHODOCOCCUS RHODOCHROUS*

6.1 Abstract

Hydrocarbon gasses are an inexpensive and abundant substrate for microorganisms. In this study, the capability of the oleaginous bacterium *Rhodococcus rhodochrous* to utilize propane as a carbon course for growth and lipid accumulation was analyzed. Growth was observed when propane was used a sole carbon source, but was sufficiently less than growth observed in the presence of glucose. Reduced lipid accumulation occurred when *R. rhodochrous* was grown on propane as the sole carbon source (6% cell dry weight) in comparison to glucose controls (60% cell dry weight). Analysis of the fatty acid methyl esters profiles indicated a prevalence of palmitic, stearic, oleic and unknown methyl esters in both propane and glucose treatments. This work gives us a better insight towards understanding the limited potential of propane as a sole carbon source for growth and lipid accumulation by *R. rhodochrous*.

6.2 Introduction

Hydrocarbon gasses are abundant, readily available, and offer potential as an affordable, alternative carbon source for microorganisms. Natural seeps of hydrocarbon gasses are found in oil or natural gas deposits (Reed and Kaplan 1977) and consist primarily of methane (70-99%), with smaller amounts of ethane (1-10%) and propane (1-

2%; Hunt 1996). Organisms that can grow on short-chain gaseous hydrocarbons as a carbon source have been used previously for petroleum exploration and single cell protein (Perry 1980, Shennan 2006). The advantages of using propane as a potential source of single cell protein or other high value metabolic intermediates are the cost, availability, and prevalence of microbes able to use propane (Perry 1980). Propane has low solubility (although more than other gaseous alkanes) in water, which would allow unused substrate to be easily recycled back into the fermentation system; however, as oxygen is required for microbial growth, the combination of propane and oxygen poses a safety challenge (Perry 1980).

Several microorganisms belonging to the actinomycete group, such as *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus,* have been shown to utilize *n*-alkanes (Shennan 2006). Tanaka et al. (1973) showed the utilization of ethane, n-butane, and propane as sole carbon sources for the growth of *Nocardia paraffinica* (ATCC 21198, now *Rhodococcus rhodochrous*). Recently, *R. rhodochrous* was found to be oleaginous, being able to accumulate over 50% of cell dry weight as lipids when grown on glucose as a sole carbon source (Shields-Menard, Amirsadeghi et al. 2015). Despite the well-documented ability of *R. rhodochrous* to grow on gaseous hydrocarbons, the ability for *R. rhodochrous* to accumulate lipids on alternative and waste carbon sources, such as gaseous hydrocarbons, is not well understood.

Natural gas is often a waste byproduct of oil refining and has potential as a low-cost, plentiful substrate for microbial conversion to food or commercially relevant chemicals. This study aimed to gain insight in the lipid accumulation capabilities of *R. rhodochrous* when grown on propane as a sole carbon source. *R. rhodochrous* was grown

on agar and broth in prescription bottles flushed with a gas mixture of propane, oxygen and carbon dioxide. Growth was observed in both broth and agar cultures of *R. rhodochrous* grown solely on propane; however, lipid accumulation was minimal. In this report, we show detailed methodology intended to gain a preliminary understanding of the utilization of propane to yield valued products by an oleaginous bacterium.

6.3 Methods

6.3.1 Culture Conditions

Rhodococcus rhodochrous (American Type Culture Collection #21198) glycerol stocks, stored at -80°C were used to inoculate a starter culture. Experiments were conducted first, to observe the growth of *R. rhodochrous* on propane and to identify potential conditions for lipid accumulation and second, to determine propane utilization and analyze the associated lipids accumulated in the presence of propane.

6.3.2 Experimental Set-up

To determine if *R. rhodochrous* could use propane as the sole carbon source, three prescription bottles (10.16 cm x 6.35 cm) containing 30mL of minimal salts medium (Sorkhoh, Ghannoum et al. 1990) were inoculated with 1mL of *R. rhodochrous* stock culture. The prescription bottles were sealed with rubber stoppers and flushed for approximately 1 minute with a gas mixture of 65% propane, 30% oxygen, and 5% carbon dioxide (Figures 6.1, 6.2; French 2000). The gas mixtures were sterilized using a sterile glass syringe packed with glass wool. The starter cultures were incubated at 30°C in a shaking incubator at 125 rpm (New Brunswick Scientific Model I26, Edison, New Jersey) and observed daily for increased turbidity. After four days the bottles were

flushed again for one minute with the gas mixture to ensure a source of carbon and oxygen. Seven days after the initial inoculation, cells were collected in a tube, centrifuged for 15 minutes at 2825 x *g*, resuspended in fresh media, and flushed again with the gas mixture. Starter cultures were flushed with the gas mixture and resuspended in fresh media once more before the start of the experiment.

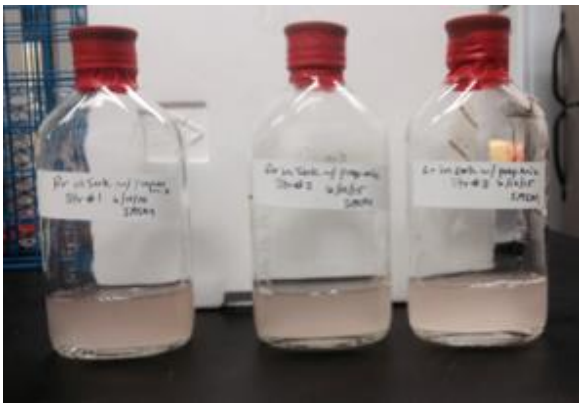

Figure 6.1 *R. rhodochrous* starter cultures were grown in Sorkhoh media with propane as the sole carbon source.

Prescription bottles were incubated at 30°C with 125rpm agitation. Photo shows cultures immediately after inoculation with freezer stocks.

Figure 6.2 *R. rhodochrous* cultures were flushed with a gas mixture of approximately 65% propane, 30% oxygen, and 5% carbon dioxide for 1 minute.

A sterile needle was used to vent the headspace as the gas mixture flowed through a glass wool stuffed glass syringe.

Minimal salts agar, made by adding 10g/L agar to minimal salts media (Sorkhoh, Ghannoum et al. 1990), was autoclaved and 30mL of liquid agar was transferred to sterile prescription bottles and placed flat side down to cool (Figure 6.3). To promote lipid accumulation, 11 bottles had agar containing limited nitrogen (0.45g/L $NaNO_3$) and another 11 bottles had agar containing normal nitrogen (0.9g/L $NaNO_3$) concentrations. Each bottle was inoculated with 2 mL of starter culture to coat the surface of the agar and flushed for 1 minute with the gas mixture. The bottles were flushed again after 4 days of growth. Seven days post initial inoculation, the cells were harvested by washing the agar surfaces with phosphate buffer solution (PBS). The washings from all 11 prescription bottles of either limited nitrogen or normal nitrogen concentrations were combined so that enough dry cell mass was available for lipid extractions as previously described

(Shields-Menard et al. 2015). Headspace samples were not measured during this experiment.

6.3.3 Experimental Set-up for Lipid analysis

The second study aimed to determine utilization of propane and to analyze the associated lipid accumulation. To ensure a starter culture with a high cell density, we transferred a frozen stock to glucose-supplemented medium and incubated at 30°C for 72 hours in a shaking incubator at 125 rpm (New Brunswick Scientific Model I26, Edison, New Jersey). The starter culture was resuspended in fresh media and distributed evenly (30mL per bottle) into prescription bottles, which were then sealed with rubber stoppers and flushed for 1 minute with the gas mixture as previously described. The cells were resuspended with fresh media 4 days later and flushed again with the gas mixture. Seven days post initial inoculation, the starter culture was used to inoculate a series of prescription bottles: 20% (v/v) inoculum in Sorkhoh broth and 2 mL to coat the surface of Sorkhoh agar. We used 3 prescription bottles for each control and 4 for the experimental treatment containing Sorkhoh broth media: glucose supplemented (20g/L), no propane, with *R. rhodochrous* (Biotic control); propane without *R. rhodochrous* (Abiotic control); and Propane with *R. rhodochrous*. We used 3 prescription bottles for each control and 4 for each experimental treatment containing Sorkhoh agar media: glucose-supplemented, no propane, with *R. rhodochrous* (biotic control); propane without *R. rhodochrous* (abiotic control); *R. rhodochrous* with no propane; and propane with *R. rhodochrous*. All cultures were incubated for 72 hours at 30°C in a benchtop incubator (bottles with agar; Fisher Scientific Isotemp Incubator Model 637D, Pittsburgh, PA) or a

shaking incubator at 125rpm (bottles with broth; New Brunswick Scientific Model I26, Edison, New Jersey). Cells from each treatment were combined into one sample and freeze dried for lipid analysis.

6.3.3.1 Growth analysis

A random bottle of each treatment of broth containing bottles was analyzed using 1.5 mL of broth taken using a sterile 22-gauge needle and syringe. Optical density was measured daily using a GENSYS 20 spectrophotometer at 595nm (Thermo Scientific, model 4001/4).

6.3.3.2 Lipid analysis

Lipids were extracted from a freeze-dried pellet using modified Bligh and Dyer (Bligh and Dyer 1959) method as previously described (Revellame, Hernandez et al. 2012). The extracted lipids were then transesterified and fatty acid methyl esters (FAMEs) were analyzed using an Agilent 6890N gas chromatograph with a flame-ionization detector (GC-FID; Agilent Technologies Inc., Wilmington, Delaware) with a Zebron ZB-FFAP column (30 m x 0.25mm, film thickness 0.25 um). The method used an oven temperature of 50-250°C with a rate of 10°C per minute. The carrier gas, helium, had a flow rate of 1.5 mL per minute and the detector temperature was 260°C. A standard solution of known concentrations of C9-C24 FAMEs (Sigma) and an internal standard was used to calibrate the instrument. Mean and standard deviations of triplicate injections of each are reported.

6.4 Results and Discussion

Prescription bottles with agar surfaces were used to evaluate the growth of *R. rhodochrous* when a gas mixture of propane was used as the sole carbon source. After 4 days growth of *R. rhodochrous* was observed on the agar surfaces (Figure 6.3). The bottles were then flushed with more gas to promote lipid accumulation and then cells were harvested on day 7 (Figure 6.3). Lipid accumulation by oleaginous microbes occurs in culture conditions of excess carbon and limited nitrogen (Wynn and Ratledge 2005). We used the normal concentration of nitrogen, which has previously shown over 50% lipid accumulation (Shields-Menard, Amirsadeghi et al. 2015), as a control and used a limited concentration of nitrogen to determine if lipid accumulation might be stimulated faster. Nitrogen depletion has been previously shown to occur after 24 hours of cultivation, followed by lipid accumulation using the excess glucose in the medium (Shields-Menard, Amirsadeghi et al. 2015). To ensure propane utilization for lipid accumulation and to ensure an excess of propane was still available, we limited the nitrogen for some prescription bottles so that lipid accumulation would ideally occur without a lag time. While growth was observed visually (Figure 6.3), the data are inconclusive if there was an actual difference in cell dry weight or lipid yield in regard to nitrogen concentration (Figure 6.4). Furthermore, nitrogen concentration was not determined in this study and bioavailability of nitrogen could be a limitation when *R. rhodochrous* is grown on agar surfaces. Nevertheless, a total of 145 mg of cell dry weight was achieved when *R. rhodochrous* was grown on propane as the sole carbon source (Figure 6.4).

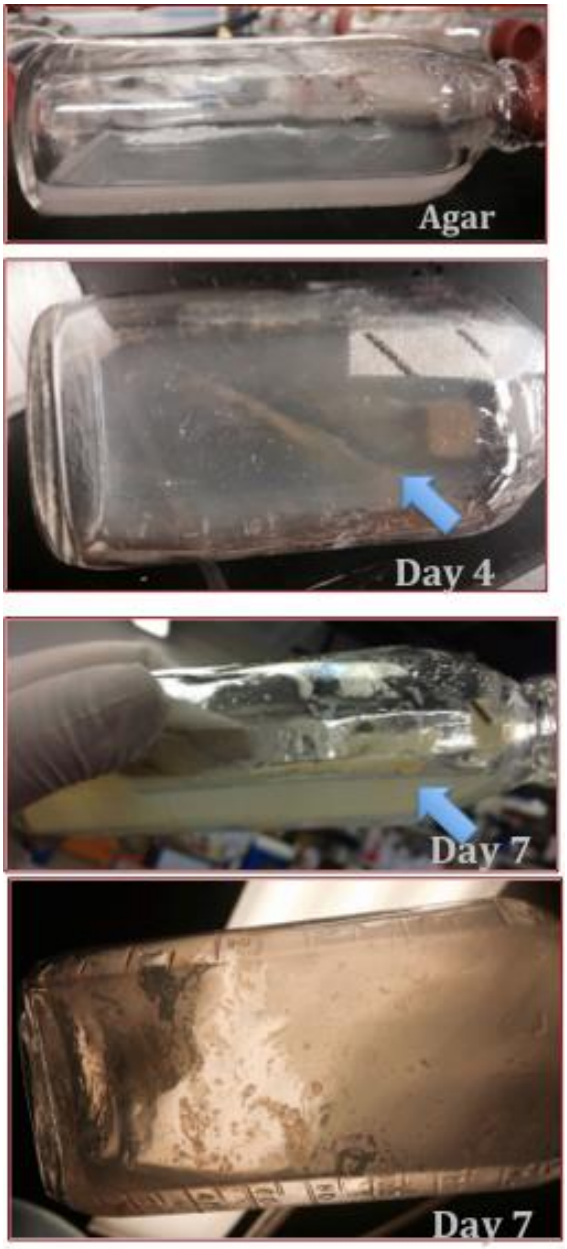

Figure 6.3 *R. rhodochrous* was grown on agar surfaces in prescription bottles flushed with a propane gas mixture as the sole carbon source.

Growth is observed by day 4 and bottles were again flushed with the gas mixture. More growth was observed at day 7 and cells were harvested for analysis.

Tanaka et al. (1973) cultured a closely related *Nocardia* strain on propane as a sole carbon source and yielded 10mg/mL, which would be considerably higher than what

we have previously determined when *R. rhodochrous* was grown in glucose-supplemented media (5-7g/L) suggesting that propane can be used as a sole carbon source for growth and might have potential to yield a larger biomass than other substrates.

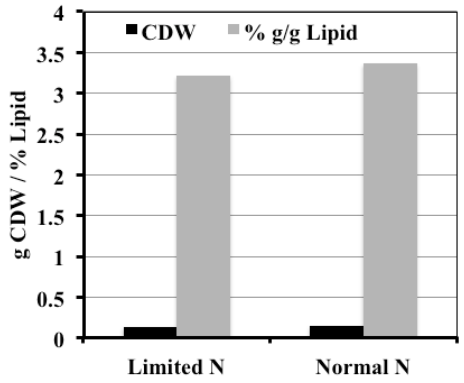

Figure 6.4 *R. rhodochrous* total cell dry weight (g; CDW) and percent (g/g) lipid of CDW of prescription bottle cultures grown on propane as a sole carbon source.

Cells from each treatment were pooled together for one analysis of CDW and lipid extraction. Agar was made using Sorkhoh media with normal nitrogen concentrations (0.9g/L) or limited nitrogen conditions (0.45g/L).

This study also aimed to investigate any associated lipid accumulation when *R. rhodochrous* was grown on propane as a sole carbon source. *R. rhodochrous* was again grown in prescription bottles with no carbon source (agar alone) or with glucose or propane as the sole carbon source. Broth cultures of similar conditions were also conducted to analyze any changes in absorbance. *R. rhodochrous* grown in glucose showed a similar increase in absorbance to previous experiments and when grown in propane, *R. rhodochrous* showed a trend of increasing absorbance (Figure 6.5), suggesting growth could occur when propane was used a sole carbon source, though

considerably less than when grown on glucose. When grown on propane and in broth, *R. rhodochrous* achieved less than 1g/L of CDW, considerably lower than results reported by Tanaka (Tanaka, Kimura et al. 1973), compared to over 6g/L when grown on glucose (Table 6.1). Figure 6.6 shows the observable differences in cell mass when grown on glucose-supplemented agar versus propane. After three days of cultivation, cells were harvested for analyses (Figure 6.6). Interestingly, aside from the glucose controls, lipid yield was 16% w/w when *R. rhodochrous* was grown on just agar without any supplementary carbon source (Table 6.1). Less than 7% of lipid to cell dry weight was achieved when *R. rhodochrous* was grown on propane as a sole carbon source (Table 6.1).

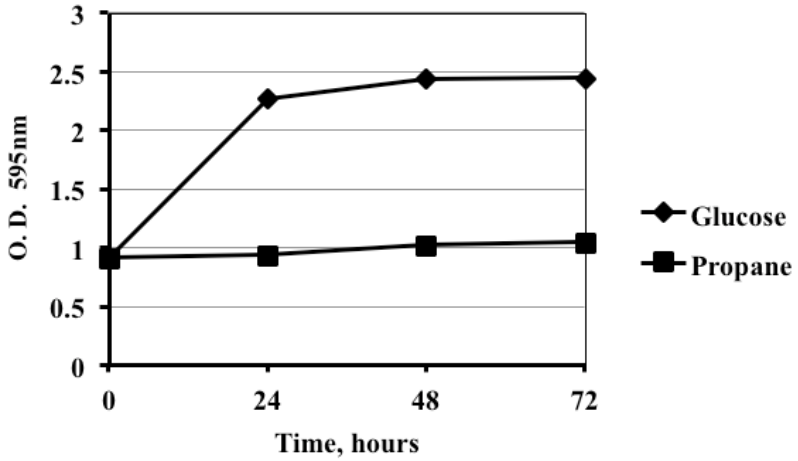

Figure 6.5 Absorbance (595nm) of *R. rhodochrous* prescription bottle broth cultures grown on propane or 20g/L glucose as a sole carbon source.

One sample was measured daily from random prescription bottles of each treatment.

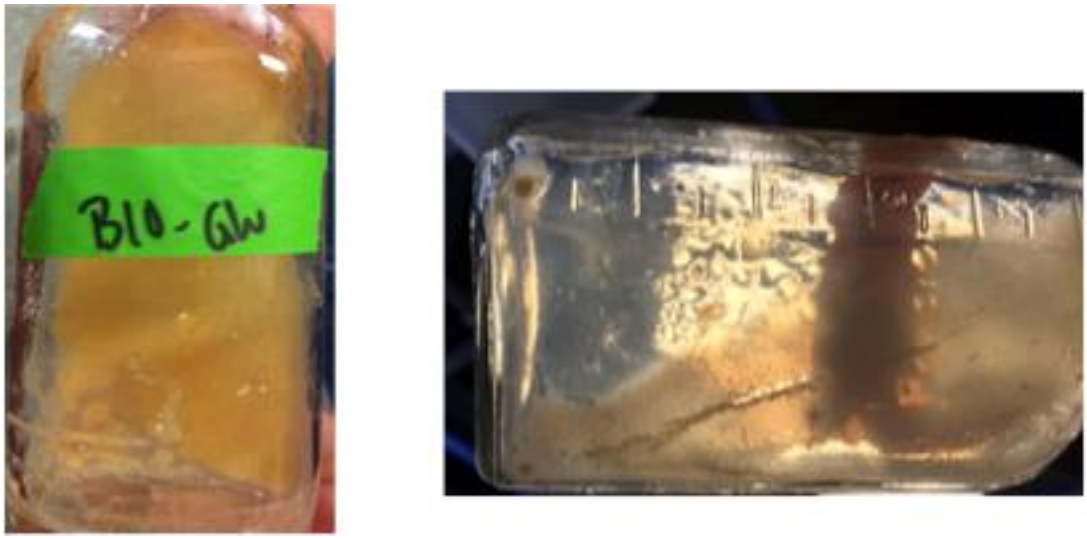

Figure 6.6 *R. rhodochrous* was grown on agar surfaces in prescription bottles supplemented with glucose or flushed with a propane gas mixture as the sole carbon source.

Growth was observed by 72 hours post-inoculation and cells were harvested for analysis.

Table 6.1 *R. rhodochrous* cell dry weight (g/L; CDW) and percent (g/g) lipid of CDW of prescription bottle agar and broth cultures grown on glucose or propane as a sole carbon source.

	CDW g/L	**% g/g Lipid**
Broth-Glucose	6.2179	57.0434
Broth-Propane	0.738	6.5597
Agar-Glucose	73.9167	50.1141
Agar-Propane	7.5375	3.78788
Agar	7.65	16.4384

Cells from each treatment were pooled together for one analysis of CDW and lipid extraction.

Propane utilization by microbes requires a monooxygenase to incorporate one atom of oxygen into the hydrocarbon (Shennan 2006). *R. rhodochrous* has been shown to possess several monooxygenases capable of assimilating propane and other alkanes

(Babu and Brown 1984, MacMichael and Brown 1987, French 2000). However in this study, there is no conclusive reduction in propane amounts in culture bottles. Furthermore, lipid yield was low when *R. rhodochrous* was grown on propane as a sole carbon source (Table 6.1). While rubber stoppers were used to ensure propane remained in the bottles, treatments without propane as a sole carbon source, such as the agar control, did not have stoppers, which may have influenced oxygen concentrations. If oxygen was limited in the presence of propane, then propane utilization by *R. rhodochrous* would have also been limited. Further work is necessary to determine best practices for ensuring gas mixing and availability.

FAME analysis revealed the prevalence of stearic acid methyl ester in cultures with propane as the sole carbon source and a large amount of unknowns when *R. rhodochrous* was grown on agar with propane (Figure 6.7). The slight increase in lipid yield of *R. rhodochrous* grown on agar versus broth with propane as the sole carbon source might be indicative of *R. rhodochrous* utilizing propane for lipid accumulation, which has been shown in other organisms to promote the accumulation of odd-chain fatty acids (Perry 1980), which are identified as "unknowns" by our procedures. When *R. rhodochrous* was grown on agar with no carbon source, we observed only palmitic and erucic acid methyl esters. This may be a result of losing a small amount of lipid extractables during processing or it might indicate that neutral lipids were not prevalent and the lipid extraction primarily pulled some of the membrane lipids, which show a prevalence of saturated fatty acids. Further work is necessary to achieve higher lipid yields that would provide sufficient samples for profiling the lipid classes present during growth on hydrocarbon gasses.

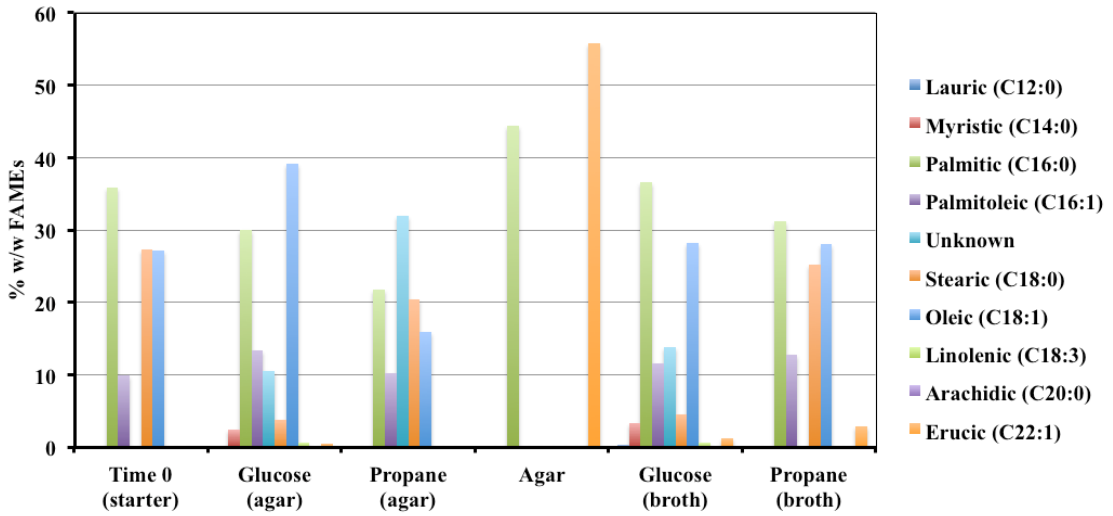

Figure 6.7 FAME profile of *R. rhodochrous* grown on propane as a sole carbon source.

FAME profile of R. rhodochrous starter culture (time 0 inoculation) and at 72 hours after growth in prescription bottles with glucose or propane as a sole carbon source. R. rhodochrous was also grown on agar (Agar) in a prescription bottle without any supplementation of carbon sources.

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CHAPTER VII

CONCLUSIONS

The increasing global demand for liquid energy requires investigation and development of alternative fuels while also mitigating rising environmental concerns. The volatile oil market, energy security, and greenhouse gas emissions have led the United States to implement renewable fuel policies with a research focus on novel substrates that can be repurposed as fuel. Oleaginous microbes can accumulate lipids intracellullarly when grown on various carbon substrates and are relevant towards the advancement of biodiesel feedstocks as well as the displacement of other petroleum-based products. *Rhodococcus rhodochrous* belongs to a well-described oleaginous group and has been studied for its degradation capabilities. This dissertation aimed to investigate the ability of *R. rhodochrous* to accumulate lipids when grown on waste or alternative carbon sources.

Bacteria belonging to the actinomycete group have been shown to accumulate lipids when grown in various substrates. The central hypothesis of this dissertation was that *R. rhodochrous* accumulates lipids when grown in glucose-supplemented media. In chapter II of this dissertation, we reported the genome sequence of *R. rhodochrous* ATCC 21198, which provided insight into a diverse metabolism with predicted genes involved in degradation and lipid accumulation. In chapter III it was reported that *R. rhodochrous* is an oleaginous bacterium, accumulating over 50% of cell mass as lipid

when grown using glucose as a carbon source. The lipid profile revealed the presence of phospholipids, mono-, di-, and triglycerides, and wax esters and the fatty acid methyl ester profile showed a prevalence of palmitic and oleic methyl esters, consistent with the characteristics of other oleaginous *Rhodococcus* species.

Lignocellulosic biomass, composed of lignin, cellulose, and hemicellulose, is an abundant and renewable resource and offers great potential as a substrate for microbes. After pretreatment of lignocellulosic biomass, the hydrolysate contains sugars (glucose and xylose) and inhibitory compounds such as acetic acid, furfural, and phenolics. In chapter IV, *R. rhodochrous* was studied using xylose as a carbon source and the effects of acetic acid and furfural on growth and lipid yield. Acetic acid and furfural were not inhibitory, although lipid yields were slightly reduced. *R. rhodochrous* was unable to consume xylose for growth or lipid accumulation. In chapter V, phenol was consumed by *R. rhodochrous*, but lipid accumulation was not observed, suggesting that perhaps more carbon was necessary to promote lipid accumulation. When *R. rhodochrous* was grown in phenol supplemented with glucose, there was delayed lipid accumulation compared to the glucose control. Proteomic analyses identified an abundance of phenol degradation enzymes present in cultures containing phenol and glucose with phenol as well as an abundance of enzymes involved in lipid synthesis and the central metabolism of *R. rhodochrous.* Model phenolic lignin compounds, VA and HBA, were consumed after 20 hours of cultivation and did not inhibit or affect growth or lipid accumulation by *R. rhodochrous*.

Previous work at Mississippi State University used *R. rhodochrous* to degrade vinyl chloride by first cultivating *R. rhodochrous* on hydrocarbon gasses, such as

propane. The researchers involved in this study observed a waxy sheen of the *R. rhodochrous* colonies when grown on propane. In chapter VI further examine this interaction of *R. rhodochrous* with propane to determine if these conditions favor lipid accumulation. This was a preliminary study to determine best practices for future experimental design and to observe growth and any associated lipid accumulation by *R. rhodochrous*. While this study did not observe as much growth or lipid accumulation by *R. rhodochrous* grown on propane compared to glucose, this study was able to identify and trouble-shoot limitations associated with hydrocarbon gas cultivation.

Collectively, the data support the ability of *R. rhodochrous* to tolerate and use unconventional carbon sources for growth and lipid accumulation. This study provided evidence of the oleaginicity of *R. rhodochrous* when grown on glucose and model lignocellulosic components. We further showed the growth of *R. rhodochrous* on propane, but were unable to achieve lipid accumulation. Future work should aim to optimize conditions for maximum lipid yield and investigate other bioproducts, such as carotenoids, that may be of commercial value.