**The Effects of Model Aromatic Lignin Compounds on Growth and Lipid
Accumulation of *Rhodococcus rhodochrous***

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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. Dry cell weight and lipids were analyzed daily. *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.

1.0 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 and 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 and 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 and 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-hydroxybenzoic acid also occurs (Palmqvist and Hahn-Hagerdal, 2000; Saadia and 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 and Ashfaq, 2010; Wells et al., 2015).

Microbial degradation of lignin is limited to white-rot and brown-rot fungi, which are able to mineralize lignin, and to some soil bacteria, which are able to depolymerize lignin or further break-down lignin fragments (Bugg et al., 2011; Gelbrich et al., 2008). Several species of soil bacteria capable of aromatic degradation are currently being investigated for lignin degrading abilities, and these are focused on identification of specific enzymes and catabolic pathways (Bugg et al., 2011). 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 grow in up to 7.5 g/L phenol as the sole carbon source (Rehfuss and 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).

Phenol and its derivatives can be serious health hazards if released into the environment and aerobic degradation of phenolic compounds by bacteria, such as *Rhodococcus* species, is an important method of remediation (Buchholz and Pawliszyn, 1993;

Michalowicz and Duda, 2007). The process of phenol degradation begins with the formation of catechol from the aromatic ring by phenol hydroxylase. *R. erythropolis* was genetically modified with phenol catabolism genes, including phenol hydroxylase and catechol 1,2-dioxygenase, suggesting that phenol degradation can be improved in *Rhodococcus* (Zídková et al., 2013). Catechol 1,2-dioxygenase, an *ortho*-cleaving enzyme, or catechol 2,3-dioxygenase, a *meta*-cleaving enzyme, are proteins involved in the Beta-ketoadipate pathway in which the bacterial degradation of catechol to central metabolic intermediates occurs (Guzik et al., 2011; Szokol et al., 2014; Zídková et al., 2013). The Beta-ketoadipate pathway converts catechol, derived from simple aromatics like phenol, or protocatechuate, derived from aromatic lignin monomers, to succinyl-CoA and Acetyl-CoA. Acetyl Co-A is necessary precursor for the fatty acid synthesis and lipid accumulation in *Rhodococcus* (Wynn and Ratledge, 2005). Phenol degraded via phenol hydroxylase and the Beta-ketoadipate pathway can generate acetyl-CoA for lipid accumulation by *Rhodococcus* species (Wells and Ragauskas, 2012) and this study aims to further elucidate this phenomenon using *R. rhodochrous*.

Another pathway involved in fatty acid synthesis in actinomycetes is the Beta-oxidation pathway, which has been shown to degrade hydrocarbons, including those with alkyl side chains, and is capable of producing membrane lipids and triglycerides (Alvarez, 2003). The Beta-oxidation pathway is active in normal growth conditions and in nutrient-restricting conditions, such as those present in the natural environments of actinomycetes, suggesting that the routine hydrocarbon degradation activity of actinomycetes can be used for growth or for fatty acids (Alvarez, 2003). The incorporation of hydrocarbons

into cellular lipids, causing uncommon fatty acids (in regard to the substrate) has been observed in several species of *Rhodococcus* (Alvarez et al., 1996; Alvarez and Steinbuchel, 2002) and is explained by the key role of the Beta-oxidation pathway in fatty acid synthesis in actinomycetes (Alvarez, 2003).

Oleaginous microbes, which are those that can accumulate over 20% of their dry cellular weight as lipids (Wynn and Ratledge, 2005), have been 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 and Ragauskas, 2012) and tolerate lignocellulosic hydrolysates during growth and lipid accumulation (Wang et al., 2014; Wells et al., 2015). *Rhodococcus rhodochrous*, which is known for its degradation capabilities, was recently identified 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*.

2.0 Materials and Methods

2.1 Culture and Experimental conditions

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

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, or 20g/L glucose and 1g/L phenol. Bacterial inocula (10% v/v) for phenol experiments were prepared using a starter culture of *R. rhodochrous* grown in glucose-supplemented medium for 48 hours. 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 v23 when necessary and significance was determined when $p < 0.05$.

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. A 1.25g/L concentration of VA or HBA was used based on previous literature (Kosa and Ragauskas, 2012) as well as previous observations (unpublished) of reduced growth and chemical solubility at higher concentrations.

Inocula (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.

BIOFLO 310 5L fermenters (New Brunswick Scientific, Edison, NJ) with a 3L working volume were used 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.

2.2 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).

2.21 Lipid Analysis

Lipids were extracted from the cell pellet using a modified Bligh and Dyer method (Bligh and 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.

2.22 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 and 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) using a modified method (Verette). 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% H₂O/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.

2.23 Protein Analysis

Culture protein lysates were prepared using a modified *Rhodococcus* protein extraction method (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 (0.1 µg/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 discovery 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 2).

3.0 Results and Discussion

3.1 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 was significantly less than the glucose control (Figure 1A and 1C). Phenol concentrations were below the level of detection by GC-FID at 48 hours in both the phenol and phenol with glucose treatments (Figure 1A), 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 significantly less 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 1A and 1C), 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 and 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). In this study, *R. rhodochrous* could tolerate and 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 suggests 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; Zídková et al., 2013). While the data shows 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.

3.2 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 1 B and 1D). *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 had a significantly greater yield of 60% lipid (Figure 1B). When *R. rhodochrous* was grown in just phenol, lipid content was significantly lower than both glucose control and glucose and phenol treatment and lipid content decreased to 0.03g/L (3% of CDW) by 120 hours (Figures 1B and 1D), suggesting that these lipids were consumed for growth after phenol exhaustion at 48 hours (Figure 1C and 1D). 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 and Hahn-Hagerdal, 2000). A previous study (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, the data indicated an increase in unknown FAMES at 72 hours (Table 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). Changes in FAME profiles have also been observed in *Pseudomonas* species during phenol contaminated soil studies between 40-60 days suggesting that FAME analysis may be a good tool to monitor phenol bioremediation efforts (Mrozik et al., 2010; Mrozik et al., 2008).

3.3 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 2). The mechanism of lipid synthesis and accumulation in yeasts is generally well understood and provides the mechanistic foundation for lipid accumulation in oleaginous microbes (Jin et al., 2015). Recent proteomic comparisons have gained valuable insight towards the

triacylglycerol (TAG) biosynthesis, storage, and degradation among oleaginous organisms, including *Rhodococcus* (Chen et al., 2014; Davila Costa et al., 2015).

When cultured in glucose, *R. rhodochrous* accumulated 25% of CDW as lipid within 24 hours (Figures 1B and D). 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 the protein abundance at 24 hours was compared 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 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 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 1A). Several proteins involved in aromatic degradation (Guzik et al., 2011; Mamma et al., 2004; Prieto et al., 1993;

Wells and Ragauskas, 2012; Zídková et al., 2013) were in high abundance in phenol cultures at 24 hours compared to 0 hour: multiple putative HpaB/PvcC/4-BUDH (phenol hydroxylase); catechol 1, 2-dioxygenases; and catechol 2, 3-dioxygenase (Table 2). 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 (phenol hydroxylase) 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 (phenol hydroxylase), 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*.

3.4 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 and Hahn-Hagerdal, 2000) and can be used as model compounds representing the major components of grass, hardwood, and softwood lignin (Kasi and 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 2A). Furthermore, percent lipid accumulation was much greater in the cultures supplemented with glucose than those without (Figure 2 B).

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 3D). 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 3A). 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 3B), implying that model aromatic lignin compounds do not inhibit the growth or lipid accumulation of *R. rhodochrous*.

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 euric acid at time 0 and an increase of unknowns, likely odd chain carbon fatty acids, at

24 hours (Figure 4). 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 3C) 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 and Ragauskas, 2012).

4.0 Conclusions

Rhodococcus rhodochrous is a versatile bacterium with a diverse metabolism capable of degradation and lipid accumulation. The prevalence and potential of the lignin component of lignocellulosic biomass for bioconversion to a product remains elusive. The purpose for using phenolic compounds in this study is twofold: phenol and its derivatives can be environment pollutants and phenol also serves as a basic model lignin compound as lignin is a polyaromatic structure. 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.

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CONFLICT OF INTEREST

The authors declare that all experiments documented in this manuscript comply with the current laws of the U.S.A.

The authors declare that they have no conflict of interest.

FIGURES

Figure 1. Phenol concentration (grey; g/L $\pm$ SD) and *R. rhodochrous* cell dry weight (black; g/L $\pm$ SD; **A**), percent lipid ($\pm$ SD; **B**), non-lipid biomass (g/L $\pm$ SD; **C**), and lipid (g/L $\pm$ SD; **D**) of *R. rhodochrous* shake flask cultures supplemented with 20g/L glucose ($\bullet$), 1g/L phenol ($\blacksquare$), or 1g/L phenol and 20g/L glucose ($\blacklozenge$).

Figure 2. *R. rhodochrous* cell dry weight (**A**; g/L $\pm$ SD) and percent lipid accumulation (**B**; $\pm$ SD) of shake flask cultures supplemented with VA, HBA, VA + HBA, with and without glucose. Data shown from the 72-hour time point.

Figure 3. *R. rhodochrous* cell dry weight (**A**; g/L), percent lipid accumulation (**B**) and pH (**C**) of 3L fermentations supplemented with glucose ($\bullet$) and VA($\blacksquare$), HBA($\blacklozenge$), or VA + HBA (-). Vanillic acid and 4-hydroxybenzoic acid concentrations (**D**; g/L) were analyzed using HPLC-ELSD. Fermentation samples of VA ($\blacksquare$), HBA ($\blacklozenge$) and VA + HBA (grey; $\blacksquare$, $\blacklozenge$) were taken every 2 hours and filtered before analysis.

Figure 4. Fatty acid methyl ester profile (% w/w) of *R. rhodochrous* grown in glucose (Glu), vanillic acid (VA), 4-hydroxybenzoic acid (HBA), or a mixture of VA and 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.

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Figure

D

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

Figure 2

Figure 2. *R. rhodochrous* cell dry weight (**A**; g/L $\pm$ SD) and percent lipid accumulation (**B**; $\pm$ SD) of shake flask cultures supplemented with VA, HBA, VA + HBA, with and without glucose. Data shown from the 72-hour time point.

Figure 3

A

B

C

D

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

Figure 4

Figure 4. Fatty acid methyl ester profile (% w/w) of *R. rhodochrous* grown in glucose (Glu), vanillic acid (VA), 4-hydroxybenzoic acid (HBA), or a mixture of VA and 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.

Table 1

Table 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). 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.

	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

Table 2

Table 2. Proteins of *R. rhodochrous* cultured in glucose, phenol, and glucose with phenol.

Glucose 24 hrs vs Glucose 0 hrs Fold change	Fisher's Exact Test (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
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
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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
-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	dihydroipoamide 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
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
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 Test (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]		gi 573528361	41 kDa
-25.0	< 0.00010	catechol 1,2-dioxygenase [Rhodococcus rhodochrous ATCC 21198]	Central metabolism 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
-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 phosphonmutase-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	0.0037	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
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 <=	Protein Name	Role	Accession Number	Molecular Weight

0.00579)

-33.3	< 0.00010	6-phosphogluconate dehydratase [Rhodococcus rhodochrous ATCC 21198]	central metabolism	gi 573526218	64 kDa
-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
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
-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