Evaluating the compatibility of solvents used in reductive lignin depolymerization with microbial funneling using *Novosphingobium aromaticivorans*

by

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Abstract

The industrial revolution has resulted in the intensive use and dependence of fossil fuels and their products around the world. Fossil fuel emissions and the accumulation of carbon dioxide in the atmosphere are causing global climate change that has negative implications for the environment and our planet. Researchers and industries worldwide are developing technologies to make fuels and chemicals from lignocellulosic biomass to reduce fossil fuel dependance. Lignocellulosic biomass, a carbon-neutral and renewable feedstock, is composed of carbohydrate and aromatic polymers that can be used to produce clean fuels and value-added products, respectively. The conversion of carbohydrate components from lignocellulosic biomass into biofuels has been developed in a number of methods and processes, but the viability of these processes is limited by the need to produce large volumes of low-value biofuels. Hence, lignocellulosic biorefineries may be able to improve their viability and long-term sustainability if value-added products are made from lignin fractions. Despite the lack of economic viability at large scale to date, process pipelines have been proposed. One pipeline developed at the Great Lakes Bioenergy Research Center (GLBRC) includes the catalytic depolymerization of lignin into aromatic monomers using hydrogenolysis, followed by the biological transformation of the diverse set of aromatic monomers produced from the lignin into a single product using engineered strains of *Novosphingobium aromaticivorans*. Technoeconomic analyses of this pipeline have shown that using methanol as a solvent during hydrogenolysis leads to a process that requires costly reactors that are resistant to high pressure. It is therefore desirable to search for alternative solvents that allow the catalytic reactions to take place at lower pressures. The alternative solvents ethanol and ethylene glycol were investigated in this project, which focused on the biological process downstream of lignin depolymerization. To evaluate the effect of the solvent on the downstream microbial funneling step, the products of hydrogenolysis reactions performed with methanol, ethanol, or ethylene glycol (provided by Dr. Canan Sener) were used to evaluate the production of 2-pyrone-4,6-dicarboxylic acid (PDC) by an engineered strain of *N. aromaticivorans*. The PDC yield, per gram of biomass entering the hydrogenolysis process, was highest with ethanol, followed by ethylene glycol and methanol. In addition, the tolerance of *N. aromaticivorans* to the different solvents was also investigated. No inhibition to microbial growth was observed with ethylene glycol concentrations as high as 20% (by volume), whereas inhibition to microbial growth was observed at 2% with methanol and 1% with ethanol. These thresholds are pertinent to the use of *N. aromaticivorans* to produce value-added products with different lignocellulosic biomass streams.

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INTRODUCTION

Since the industrial revolution, the world has seen an intense use and dependence of fossil fuels and its products. The atmospheric accumulation of carbon dioxide derived from fossil fuel use is now causing global climate change with negative implications on the planet and its environment (Lindsey, 2023). These implications have strengthened the need for the creation of technology that could deliver sustainable biofuels and bioproducts, now and forward into the future. Lignocellulosic biomass, a carbon-neutral and renewable feedstock, is composed of sugar and aromatic polymers that can be used to produce clean fuels and value-added products, respectively (Cai et al., 2017; Gall et al., 2017; Ralph et al., 2019). While many methods and processes have been developed to convert the sugar components from lignocellulosic biomass into biofuels, the economic viability of these processes is limited by the need to produce large volumes of low-value biofuels. It is thus thought that the viability and long-term sustainability of lignocellulosic biorefineries could be improved if value-added products are made from aromatics in the lignin fraction of the biomass (Banu et al., 2019; Perez et al., 2022; Weng et al., 2021).

Although economic viability has not yet been achieved at large scale, process pipelines have been proposed. Figure 1 is an example of a strategy (Perez et al., 2022) that has been developed at the Great Lakes Bioenergy Research Center (GLBRC) to produce 2-pyrone-4,6-dicarboxylic acid (PDC), a chemical precursor for the manufacturing of polymers and adhesives (Hishida et al., 2009; Shikinaka et al., 2018). Starting from purified lignin obtained by the gamma-valerolactone (GVL) biomass deconstruction process (Luterbacher et al., 2014), the strategy includes the catalytic depolymerization of lignin into aromatic monomers using hydrogenolysis, followed by the biological transformation of the diverse set of aromatic monomers produced from the lignin into PDC by an engineered strain of the bacterium *Novosphingobium aromaticivorans* DSM12444 (Perez et al., 2022).

The hydrogenolysis process can be implemented on lignin that has already been separated from the rest of the plant biomass, as shown in Figure 1, or it can be implemented directly on the lignocellulosic biomass in a process that achieves biomass deconstruction (fractionation) and lignin depolymerization in a single process. When performed directly on the lignocellulosic biomass, the process is called reductive catalytic fractionation (RCF). In both, RCF and hydrogenolysis from lignin, methanol is typically used as a solvent that dissolves the lignin. After lignin is dissolved, a catalytic reaction (typically using a palladium catalyst) breaks interunit bonds in lignin, releasing mixtures of monoaromatic compounds, dimers, and small oligomers into the methanol solution. The type and ratio of aromatic monomers dissolved in the methanol solution depends on the type of biomass used as the feedstock and on the catalytic conditions used (Perez et al., 2022).

It has been shown in recent technoeconomic analyses (Bartling et al., 2021) of the RCF process that the use of methanol as a solvent requires pressurized reactors with a very high capital cost, and therefore, searching for alternative solvents that allow the catalytic reactions to occur at lower pressure is desirable (Facas et al., 2023; Jang et al., 2023; Ren et al., 2021). With methanol, the reactions take place at a starting pressure of 30 bar inside the reactor and have an average finishing pressure of 59 bar. With ethanol and ethylene glycol, two potential alternative solvents, reactions begin at 30 bar and 10 bar, respectively, and finish at 54 bar and 20 bar, respectively (Canan Sener, personal communication).

In an industrial pipeline, any solvent used during biomass deconstruction lignin depolymerization impact the downstream biological process because of its potential inhibitory effect on microbial growth. Although most of the solvent used upstream of biological funneling needs to be recovered and reused, this recovery is typically not 100% efficient, and therefore, the microorganisms will be exposed to some concentration of solvent that comes along with the delivery of the depolymerized aromatic mixtures. When testing PDC production from the products of RCF or lignin hydrogenolysis with *N. aromaticivorans*, our laboratory has limited the testing to a maximum methanol concentration of 2% in culture media (Perez et al., 2022), which is known to be tolerated by *N. aromaticivorans* strains.

This project investigated the downstream implication of using alternative solvents during the catalytical depolymerization of lignin. In one set of experiments, we used a novel instrument, Resipher, to evaluate the tolerance of *N. aromaticivorans* to different concentrations of solvents. Resipher tracks oxygen levels in cultures growing on 96-well plates, so changes in oxygen were used as a reporter of respiration when cells were grown in the presence of different solvents and aromatic monomers. In a second set of experiments, we performed batch experiments to measure PDC production by *N. aromaticivorans* when different solvents were used during the depolymerization of lignin in the hydrogenolysis step. Overall, *N. aromaticivorans* was shown to have the highest tolerance to ethylene glycol and the lowest tolerance to ethanol.

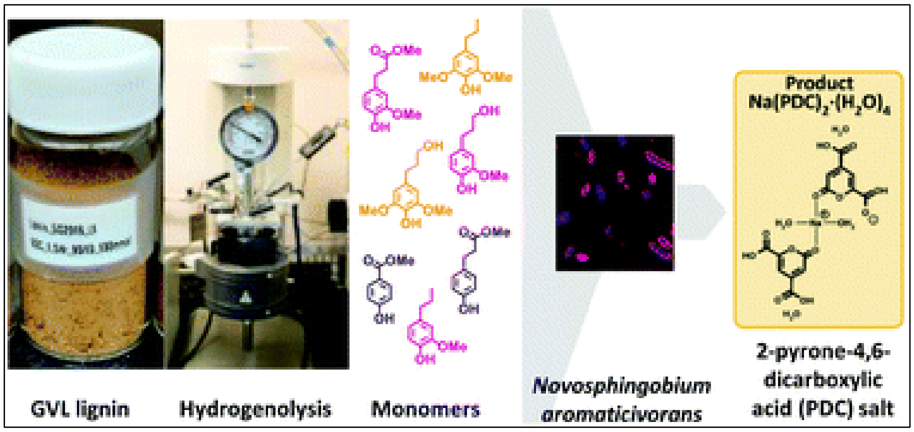

Figure 1. Example of the biomass-to-PDC pipeline studied at the Great Lakes Bioenergy Research Center. Figure from Perez et al. (2022).

BACKGROUND

Lignocellulosic Biomass

Lignocellulosic biomass is a promising feedstock for the creation and production of biofuels, chemicals, and materials. Lignocellulosic biomass consists of the carbohydrate-based polymers cellulose and hemicellulose, and the aromatics-based polymer lignin. The carbohydrates in cellulose and hemicellulose, after polymer hydrolysis, can be converted into biofuels such as ethanol and isobutanol via fermentation (Cai et al., 2017; Zheng et al., 2014). Lignin is a heterogeneous aromatic polymer that holds significance in the development of plant cell walls. Its presence in plants contributes to their structural integrity and support. Moreover, because lignin is an abundant renewable source of aromatic compounds, it is a promising and sustainable polymer that offers a plethora of opportunities for biotechnology (Deng et al., 2023; Ralph et al., 2019).

The lignin polymer is composed of syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) aromatic units connected by different C-C and C-O bonds (Figure 2). The different types of bioenergy crops contain lignin that varies by molecular weight, chemical composition, and inter-unit bonds (Perez et al., 2019; Ralph et al., 2019). The differing ratios of S, G, and H units in the lignin of different plants lead to differing monomer compositions when lignin is depolymerized (Abu-Omar et al., 2021; Constant et al., 2016; Ralph et al., 2019).

The differences in lignin composition can be seen in lignocellulosic crops, such as switchgrass, sorghum, poplar, miscanthus, and more (Jung et al., 2015; Perez et al., 2022; Samuel et al., 2011). Along with the varying composition of lignin within each species of plant, lignin depolymerization processes also play a role in the chemical composition of the monomeric aromatic compounds that would need to be processed in the microbial funneling stage into chemicals.

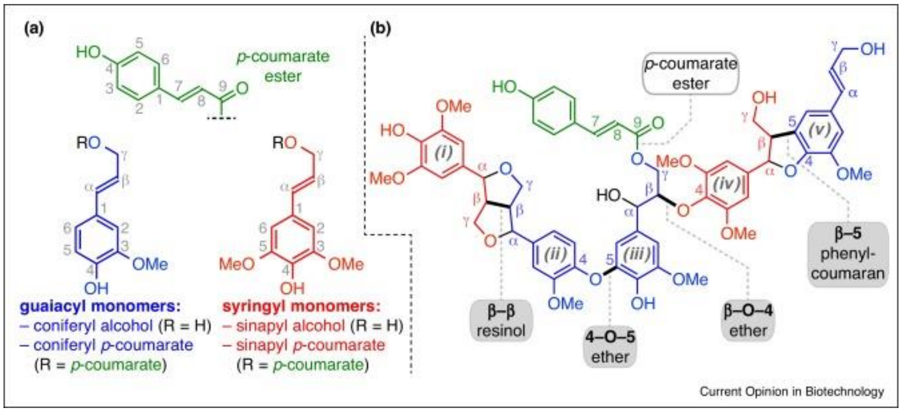

Figure 2. The structural components of lignin aromatic structures. **(a)** biosynthetic precursors, coniferyl (blue) and sinapyl (red) alcohols, listed with their related p-coumarate (green) conjugated esters, coniferyl *p*-coumarate and sinapyl *p*-coumarate are aromatic monomers that arise from the separation of **(b)** lignin oligomer fragment containing β-O-4, β-β, β-5, 4-0-5 bonds. Figure from Gall et al. (2017).

Reductive Catalytic Fractionation

Reductive catalytic fractionation (RCF) is one way to deconstruct lignocellulosic biomass for subsequent microbial processing. The major variables in the RCF process (Figure 3) are the type of lignocellulosic biomass, the solvent used to dissolve the lignin, and a redox-active catalyst. Changes in each of these variables may result in a different suite of aromatic monomers, and varying yields of monoaromatics from the lignin polymer (Renders et al., 2019; Schutyser et al., 2018). The RCF process employs a two-step chemical reaction, involving solvolysis to fractionate the different components of the biomass and solubilize the lignin, and hydrogenolysis to depolymerize the lignin. This process preserves the cellulose and hemicellulose as a solid fraction (Kuznetsov et al., 2023). Lignin extraction occurs under solvolysis in conditions such as methanol at 250 °C (Kumaniaev et al., 2017; Van den Bosch et al., 2017), to produce intermediate oxo aromatic fragments (Anderson et al., 2018). The solvolysis process is then followed by stabilization and depolymerization of these fragments through a redox reaction (Anderson et al., 2017). This reaction occurs at a slightly lower temperature, ~190 °C, the catalyst performs hydrogenolysis to cleave aryl-ether β-O-4 bonds in the lignin, releasing monoaromatics, dimers, barweight products of depolymerization is obtained (Brienza et al., 2023).

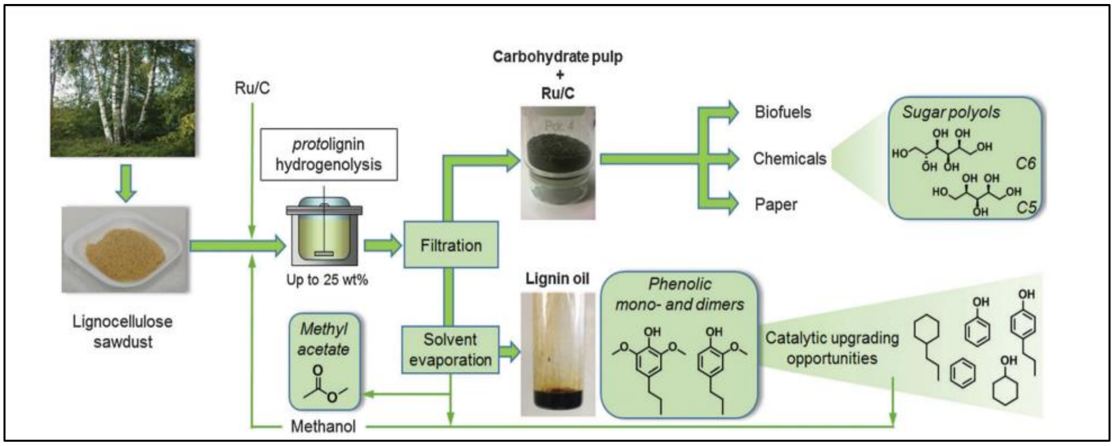

Figure 3. Figure illustrating one integrated biorefinery procedure. In the presence of catalyst Ru on carbon (Ru/C) in methanol, lignocellulose sawdust is subjected to a hydrogenolysis reaction under H2 pressure at a high temperature. The lignocellulose substrate is separated into two fractions: a depolymerized lignin fraction that is dissolved in the methanol solution, and a solid carbohydrate pulp that contains cellulose, hemicellulose, and s solid Ru/C catalyst. Lignin oil is produced during solvent recovery from the liquid phase, and the carbohydrate pulp is easily separated by mechanical filtering. The methoxyphenol mono- and dimers that make up lignin oil can be further refined to yield a wide range of downstream value-added products. Figure from Van den Bosch et al. (2015).

Degradation of Aromatic Compounds by *N. aromaticivorans*

We are using the alpha-proteobacterium *N. aromaticivorans* DSM12444 as a microbial chassis for converting biomass aromatics into valuable chemicals. *N. aromaticivorans* employs a central metabolic pathway and several peripheral pathways to degrade the S, G, and H type monomers that result from lignin depolymerization (Cecil et al., 2018; Perez et al., 2019). Figure 4 illustrates the central metabolic pathway of *N. aromaticivorans* DSM12444 (Perez et al., 2021).

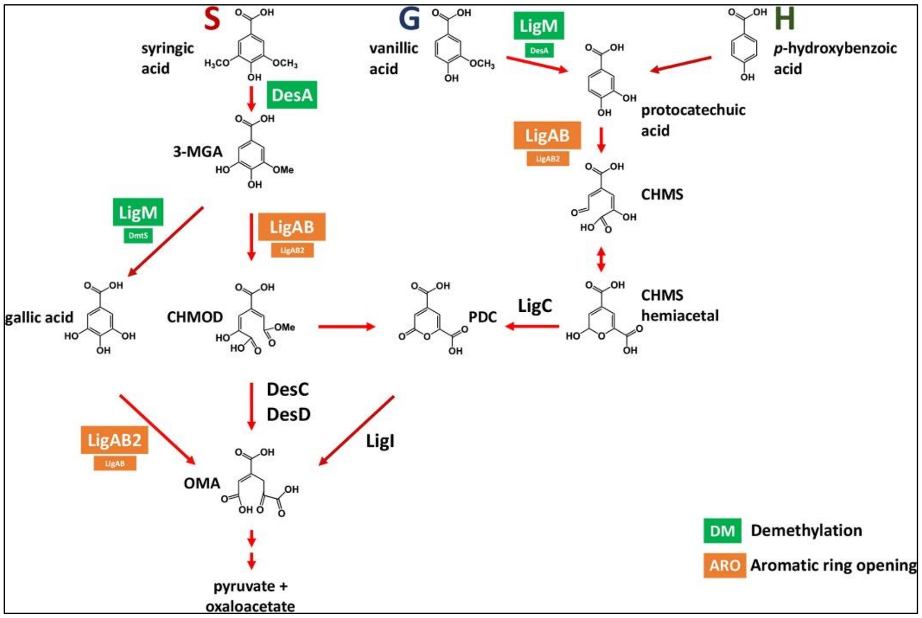

Figure 4. Central pathway for metabolism of S, G, and H phenolic subunits by *N. aromaticivorans* DSM12444. Abbreviations: 3-MGA, 3-methylgallate; CHMOD, 4-carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate; CHMS, 4-carboxy-2-hydroxy-cis,cis-muconate-6-semialdehyde; PDC, 2-pyrone-4,6-dicarboxylic acid; OMA, 4-oxalomesaconate. Figure from Perez et al. (2021).

Engineered *N. aromaticivorans* Strain that Produces PDC

The *N. aromaticivorans* metabolic pathway for degradation of aromatic compounds (Figure 5) was engineered to result in the accumulation of PDC (Perez et al., 2019). Three gene mutations were made to generate this strain. One of the genes removed (*ligI*) encodes an enzyme that hydrolyzes PDC to produce 4-oxalomesaconate (OMA) (Figure 4). The removal of this gene allowed for the accumulation of PDC from G and H aromatics. For S type aromatics, the removal of the genes encoding the enzyme catalyzing the step from 4-carboxy-2-hydroxy-6-methoxy-6-oxohexa-2,4-dienoate (CHMOD) to OMA (*desCD*) resulted in the accumulation of PDC from S type monomers (Perez et al., 2019).

By removing these genes, the engineered *N. aromaticivorans* strain converts most of the phenolic monomers derived from lignin into PDC. The engineered strain, termed *N. aromaticivorans* strain PDC (Umana et al., 2022), converts H aromatics to PDC with a ~73-84% yield, G aromatics with a ~76-100% yield, and S aromatics with a ~22-90% yield (Perez et al., 2019).

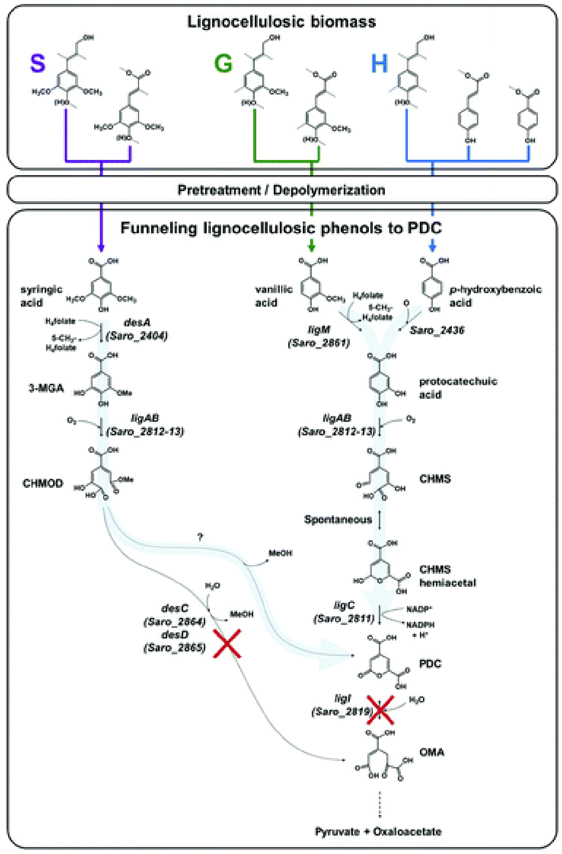

Figure 5. Metabolic pathway of *N. aromaticivorans* strain PDC that results in PDC accumulation with gene deletions shown in red. Figure from Perez et al. (2019).

Using Resipher to Measure Respiration Rates

Resipher Description. The Resipher (Figure 6), by Lucid Scientific (Atlanta, Georgia) is designed to measure and continuously track changes in oxygen concentrations within media containing metabolically active cells (https://lucidsci.com/) in a 96-well plate format (Figure 7). The Resipher instrument sits on top of a 96-well plate, and, using micro probes that move up and down in the media contained in the wells of a plate, dissolved oxygen levels are recorded at varying heights within the media. Data is automatically uploaded from the Resipher device to the cloud; there is no need to interrupt the metabolic activity to attain data. Thus, Resipher allows an investigator to use media oxygen concentrations as a reporter of cellular respiration.

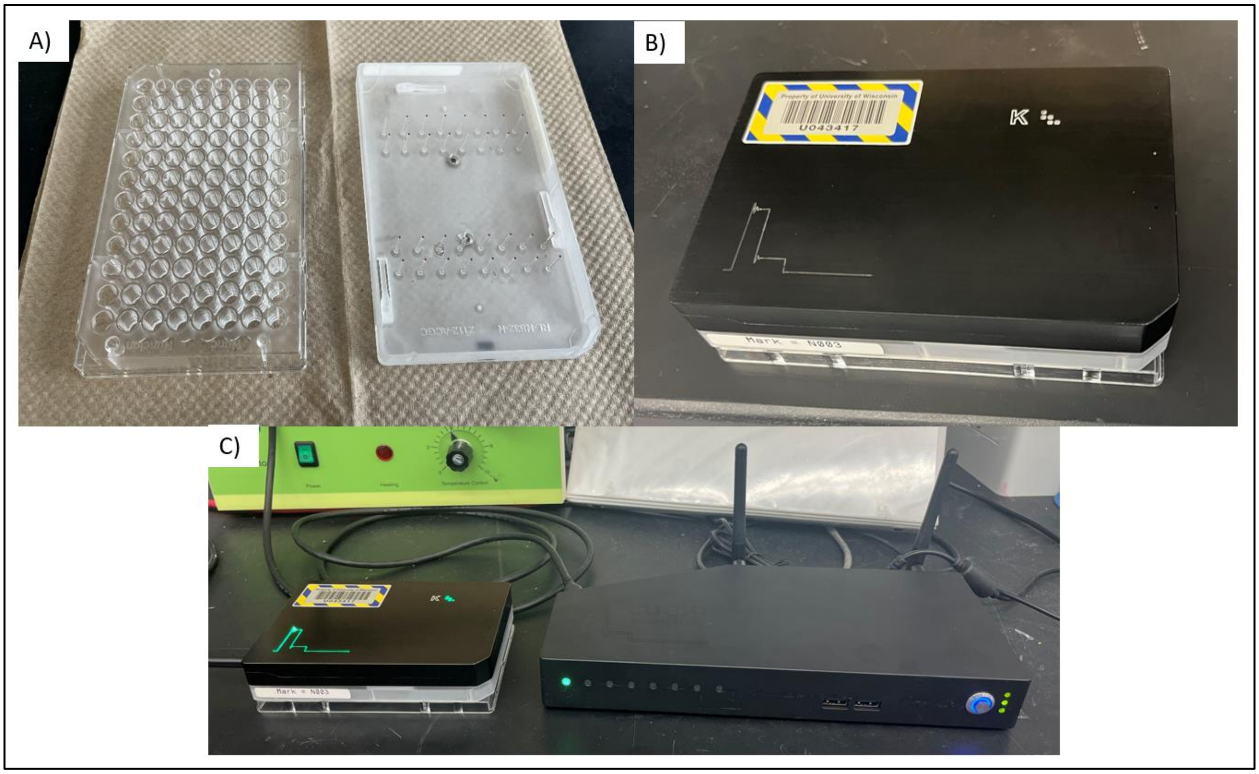

Figure 6. A) The Resipher device employs a standard 96 well plate and a lid with oxygen sensing probes to measure oxygen concentrations within the media. **B)** The Resipher device magnetically attaches to the oxygen sensing lid. Optical sensors measure oxygen concentrations through openings on the bottom side of Resipher. **C)** The Resipher device (left) attached to the lid and connected to a Resipher hub (right), capable of connecting to the internet and uploading live data from ongoing experiments.

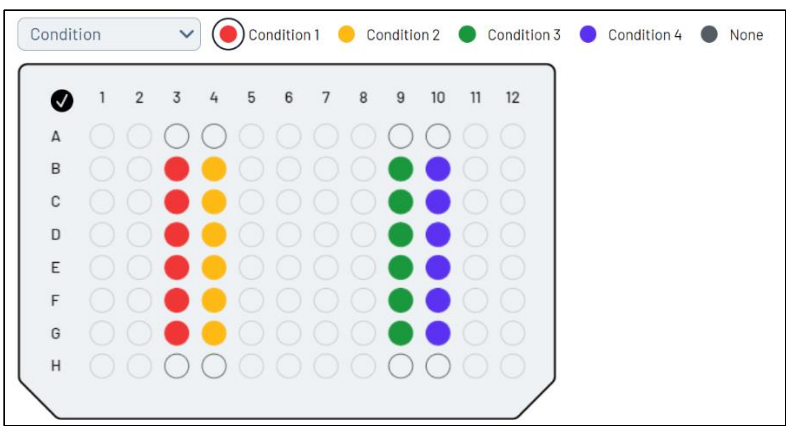

Figure 7. The 96 well plate format Resipher employs. 32 total cells are capable of being monitored at one time and can each be given its own individual condition that is desired. Each well as the option of assigning a specific condition labeling. Examples of 4 different conditions are seen. In this figure wells in rows A and H are not in use.

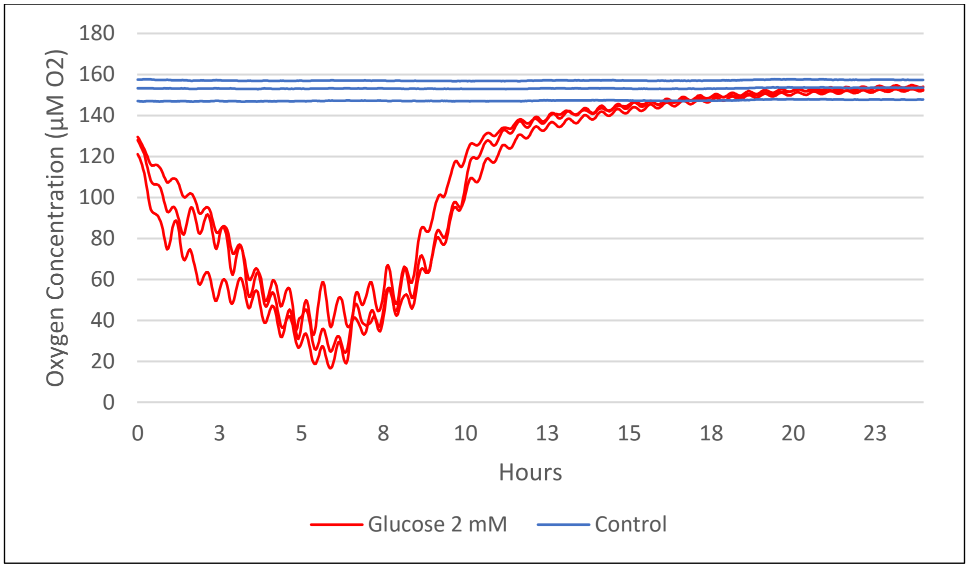

Figure 8. An experiment conducted using the Resipher instrument. SMB media containing 0 mM (control, blue traces) and 2 mM of glucose (red traces), when inoculated with *N. aromaticivorans* wild type cells.

Figure 8 shows an example of the dissolved oxygen concentrations recorded with the Resipher instrument during a batch experiment in which wild-type *N. aromaticivorans* was grown on glucose as the sole carbon and energy source and a control experiment in which the cells were incubated without glucose. Triplicate experiments were run for each condition. The control condition shows no sign of oxygen consumption, as oxygen measurement stays constant at ~155 µM for the duration of the experiment. This lack of oxygen consumption is associated with a lack of metabolic activity in cells contained within the control wells. In contrast, the experiments with 2 mM glucose show changes throughout the duration of the experiments. One characteristic of this data output is the undulations that are seen with a periodicity of about 20 minutes. This is the result of the oxygen probe moving up and down within each well and taking measurements at varying heights in the well. Because the dissolved oxygen concentration in the wells is the result of the balance between oxygen consumption by the microbial culture and oxygen diffusion from the top of the well, measurements taken closer to the top of the well have a higher reading of dissolved oxygen than measurements taken from the bottom of the well. In addition to the many smaller undulations that are recorded, one larger undulation can be seen throughout the experiment. At the beginning of the experiment the dissolved oxygen is high and starts to decrease as the microbial culture starts to grow and consume oxygen from the media. This overall drop in oxygen concentration reaches its maximum about 6 hours into the experiment. Throughout this period of time, the rate of oxygen consumed is higher than the rate of oxygen diffusing into the well, with the net result of an overall decrease in dissolved oxygen concentrations throughout the well. Approximately 6 hours into the incubation, the dissolved oxygen concentration in the well begins to increase. This is a sign that metabolic activity is slowing down and that the rate of oxygen diffusion into the well becomes larger than the rate of oxygen consumption in the well. As microbial activity begins to slow down, oxygen consumption no longer outpaces the influx of atmospheric oxygen into the wells and the oxygen concentration increases until it reaches the same levels as in the control well, after approximately 20 hours of incubation. One possible explanation for the changes in media oxygen concentration is that respiration ceases when cells run out of glucose at approximately 5-8 hours after inoculation (Figure 9).

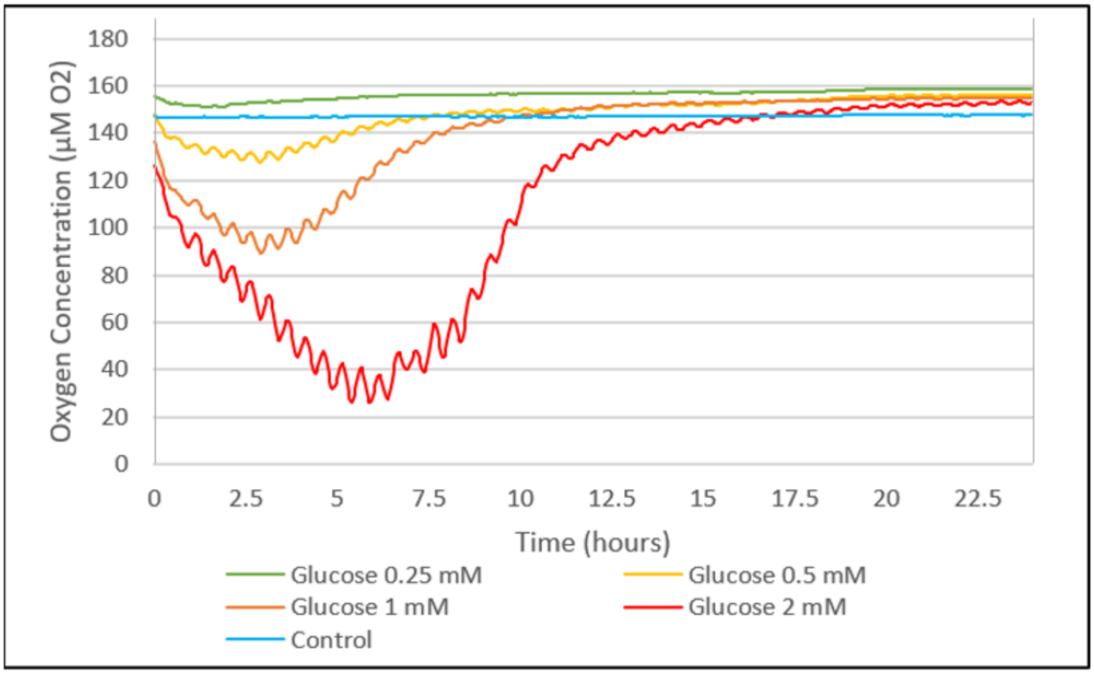

Figure 9. An experiment conducted using the Resipher instrument. Wild-type *N. aromaticivorans* was inoculated into SMB media containing 0 mM (control), 0.25, 0.5, 1.0, and 2.0 mM of glucose. The average of triplicate runs is displayed.

Effect of Substrate Concentration on Resipher's Output. Figure 9 shows the effect of different initial substrate (glucose) concentrations in the Resipher dissolved oxygen output. The shape of the curves is the same, but as concentrations of glucose increase within the media, the depletion in the dissolved oxygen concentration lasts longer. That is, the lowest point in the dissolved oxygen concentration is at ~ 2.5 hours of incubation when the initial substrate concentration is 0.50 mM versus ~ 6 hours with an initial substrate concentration of 2 mM. The drop in dissolved oxygen in the well also depends on the initial substrate concentration, with lower substrate concentrations resulting in less severe dips in the dissolved oxygen concentration.

Given the observed variation in the dissolved oxygen curves as a function of the initial substrate concentration (Figure 9), we can estimate the total oxygen consumption as the area above the dissolved oxygen curve, limited by a line representing the dissolved oxygen when no growth is observed. The average final oxygen concentration per each condition was chosen for this threshold. An example of the areas of integration for each condition is shown in in Figure 10. The data taken from calculating the integral of each condition is seen in Figure 11. These data show that there is a relationship between the amount of oxygen consumed and the initial glucose concentration.

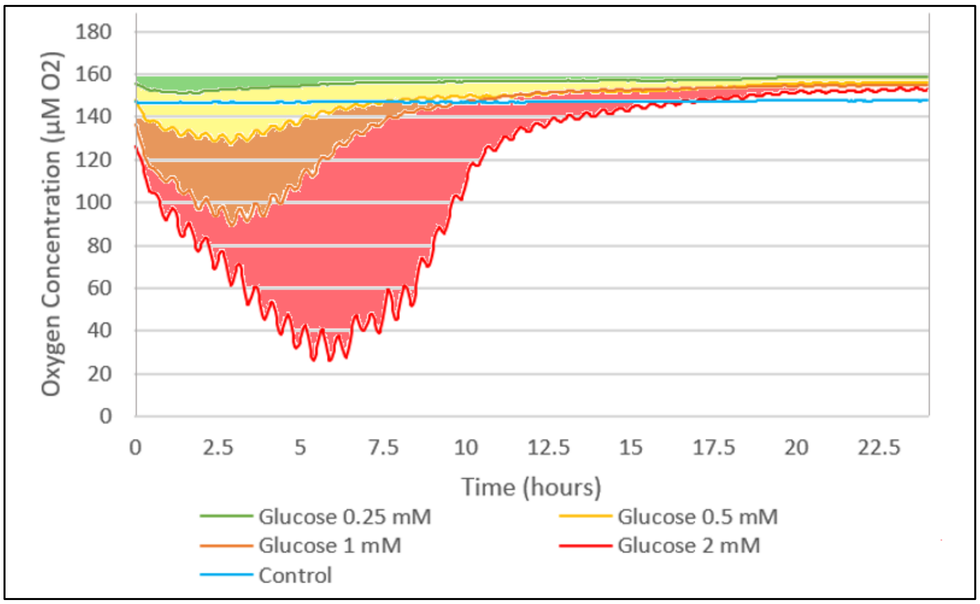

Figure 10. A visual diagram of the integration method used to obtain total oxygen consumption (µM-hr O_2) by *N. aromaticivorans* during a Resipher experiment.

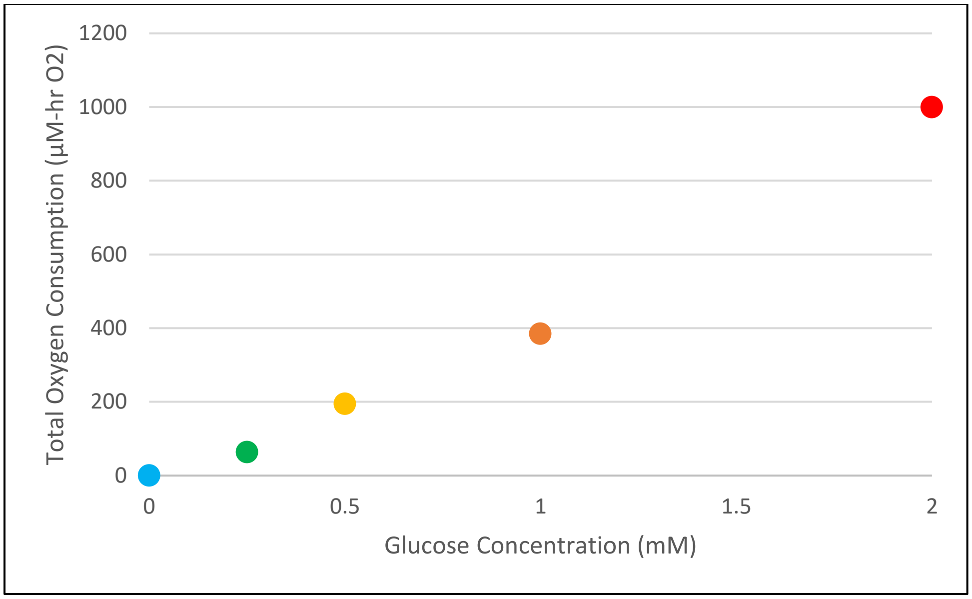

Figure 11. Total oxygen consumption values calculated integrating the Resipher curves (Figure 10). The correlation between initial glucose concentration and the total oxygen consumption by *N. aromaticivorans* is shown.

MATERIALS AND METHODS

SMB Media. Cultures were grown at 30 °C in SMB media (Stanier et al., 1966) supplemented with different carbon sources. SMB media contains 20 mM Na_2HPO_4, 20 mM KH_2PO_4, 7.5 mM $(NH_4)_2SO_4$, 0.167 mM $ZnSO_4$, 0.125 mM $FeSO_4$, 0.028 mM $MnSO_4$, 0.006 mM $CuSO_4$, 0.009 mM $Co(NO_3)_2$, 0.016 mM $Na_2B_4O_7$, 24.319 mM $MgSO_4$, 1.667 mM $CaCl_2$, 0.013 mM $(NH_4)_6Mo_7O_{24}$. For routine microbial culture and storage, the SMB media is supplemented with glucose to reach a 1 g L^{-1} concentration.

Synthetic Media for Resipher Oxygen Consumption Measurements. In this case, we used *p*-hydroxybenzoic acid (pHBA) as an aromatic substrate to test the tolerance of *N. aromaticivorans* to different solvents. A general description of how the solvent-containing media was prepared is shown in Figure 12. A total mass of 5.52 g of pHBA was dissolved in 20 mL of solvent (Figure 12). This produced a concentrated solution of 2,760 g pHBA per liter of solvent. To create growth media containing 20 mM pHBA and 1% solvent, 0.5 mL of the solvent containing pHBA were added to 30 mL of SMB media, the pH was adjusted with 10M KOH, and then more SMB was added to reach a total volume of 50 mL. Doubling the amount of pHBA-containing solvent and using the same procedure created growth media containing 40 mM pHBA and 2% solvent (Figure 12). Media was then filtered using 0.2 µm sterile polyether sulfone (PES) membrane filters (Thermo Scientific). Figure 12 depicts methanol (MeOH) as the solvent, but similar procedures were used to prepare media containing either ethanol or ethylene glycol as the solvent.

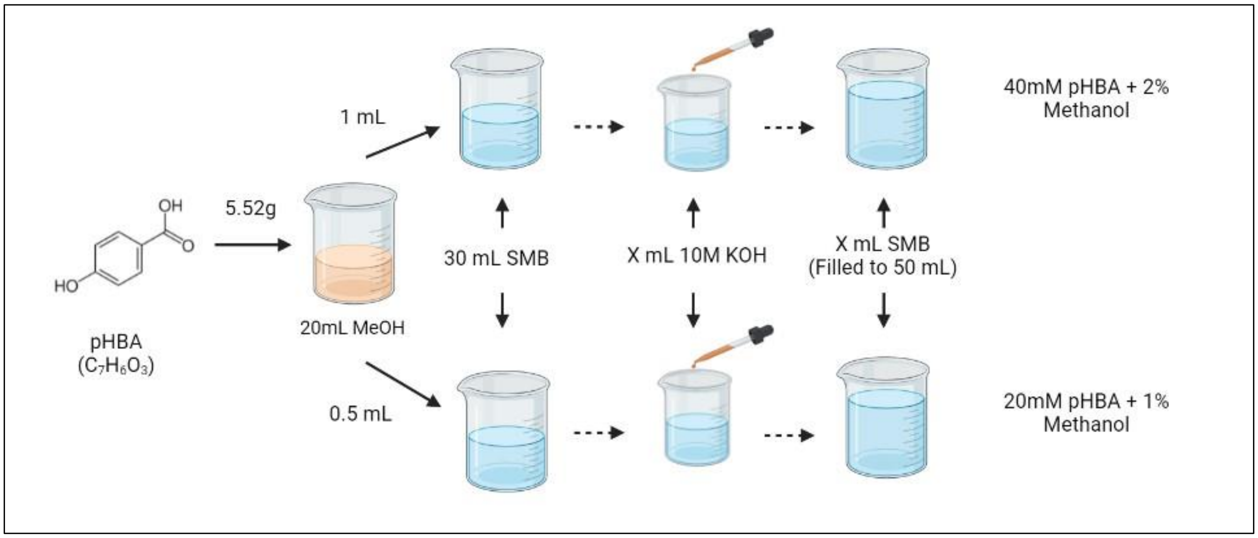

Figure 12. An illustrative overview of the creation of synthetic media for Resipher experiments.

Oxygen Consumption Quantification Using Resipher. For oxygen consumption experiments using Resipher, wild type (WT) cultures of *N. aromaticivorans* DSM12444 were pre grown overnight in SMB media supplemented with 1 g/L (5.55 mM) glucose, in an environmental growth chamber at 30° C and using a shaker at 200 rpm. For the preparation of the cultures that were incubated in the Resipher microtiter plates, aliquots (10 mL) of the media containing solvent and pHBA (Figure 12) were inoculated with 1-mL aliquots of the pre-grown *N. aromaticivorans* culture. This solution was thoroughly mixed, and then, 100 µL of this solution were aliquoted into experimental wells in the 96-well plate. The Resipher lid containing the oxygen measurement probes was then placed directly on top of the plate. An additional lid, rested on top of the probe lid, connects to a local Resipher computer hub, which connects to the cloud and instantly uploads obtained oxygen concentration levels. Upon the start of an experiment, the Resipher device automatically begins to create graphs similar to those shown in Figures 8 and 9.

Batch Experiments for PDC Production by N. aromaticivorans. Experiments were performed to assess the production of PDC by the *N. aromaticivorans* PDC strain when incubated with media containing aromatic mixtures from RCF depolymerization using different solvents (Figure 13). For these experiments, the *N. aromaticivorans* strain PDC culture was pre-grown overnight in 20 mL of SMB media containing 2 g/L (11.10 mM) glucose and incubated in an environmental growth chamber at 30° C with shaking at 200 rpm. After overnight incubation, to obtain a microbial pellet for inoculation of testing material, 1 mL culture was centrifuged at 5,000 RPM for 5 minutes; the supernatant was removed and discarded. For testing PDC production, 9.8-ml aliquots of SMB solution were mixed with 200 µL aromatic-containing solvent and inoculated with the microbial pellet. These cultures were incubated for 24 hours in an environmental growth chamber at 30° C with shaking at 200 rpm. After a 24-hour incubation period, 1-mL samples were collected and centrifuged at 5,000 RPM for 5 min, the supernatant was recovered and filtered, and samples were stored at −18°C until further analysis.

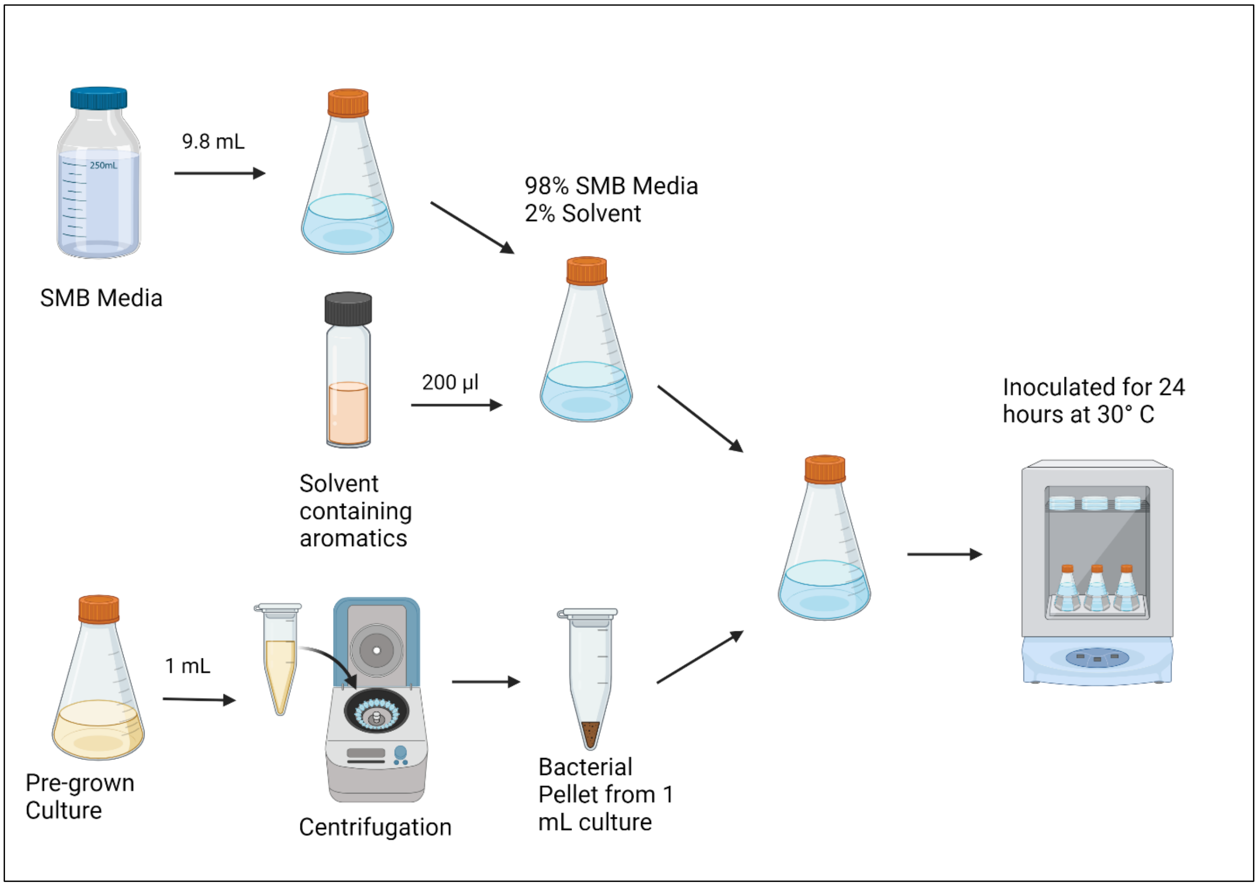

Figure 13. An overview of the inoculation of batch experiments for PDC production by *N. aromaticivorans.*

Analysis of Extracellular PDC. Quantitative analysis of PDC was accomplished by using a Shimadzu triple-quadrupole liquid chromatography-mass spectrometer. The mobile phase was a binary gradient that consisted of a mixture of water containing 0.1% formic acid and methanol. The stationary phase was a Kinetex F5 column (Phenomenex, 2.6 µm pore size, 2.1 mm ID, 150 mm length, P/N: 00F-4723-AN). PDC was detected by multiple-reaction-monitoring (MRM), quantified by use of a standard curve measuring the strongest MRM transition (Supplementary Table 1).

RESULTS

Solvent Tolerance in *N. aromaticivorans*

We used Resipher to evaluate the tolerance of *N. aromaticivorans* to different solvents used in RCF. For these experiments, we used wild-type *N. aromaticivorans* grown on SMB containing varying levels of solvent (0%-3%) and two different levels of pHBA as a model aromatic compound (Figure 12).

Evaluation of Methanol as a Solvent. Eight conditions were tested. In the first experiment, incubations were performed with media containing 1 mM pHBA and 0, 1, 2, and 3% methanol (Figure 14). A second experiment tested 2 mM pHBA and the same solvent concentrations (Figure 15). In the 1 mM pHBA conditions (Figure 14) respiration, as monitored by oxygen uptake, was inhibited at the 2% and 3% methanol concentrations, whereas at 2 mM pHBA inhibition of respiration was only seen at 3% methanol. It is possible that the use of 2 mM pHBA as a carbon source provides cells with more energy to combat a potential stress response generated by the methanol in the media. Indeed, in the absence of any solvent, cells provided with 2mM pHBA consume approximately twice as much oxygen as those grown in the presence of 1mM pHBA. To compare the results from the two experiments, we estimated the total oxygen consumption by integrating the area between the baseline oxygen concentration and the curve representing dissolved oxygen throughout the experiment (Figure 16). From this one can see that more oxygen was consumed in all of the cultures containing 2 mM pHBA compared to those grown in the presence of 1 mM pHBA as a carbon source.

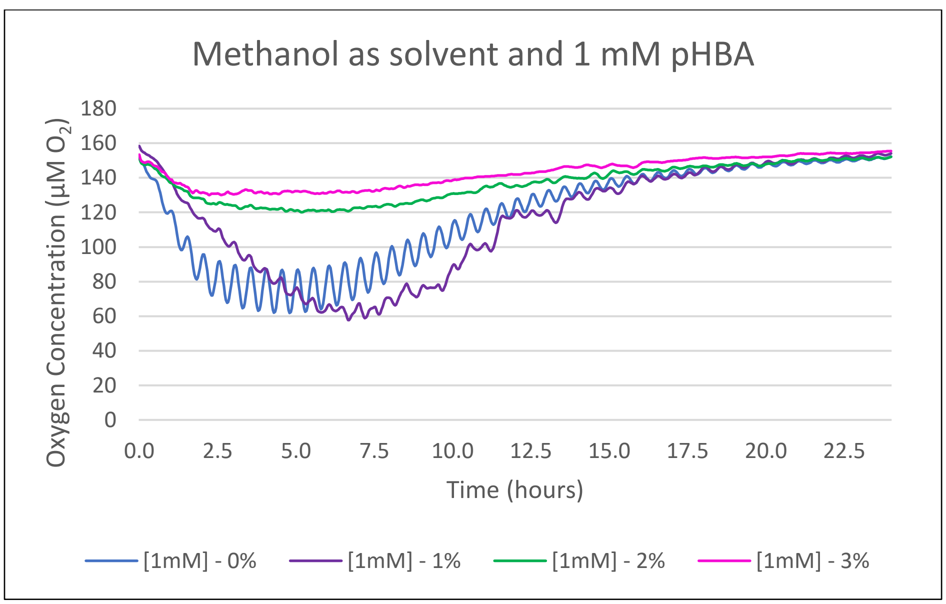

Figure 14. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of methanol with 1 mM *p*-hydroxybenzoic acid (pHBA).

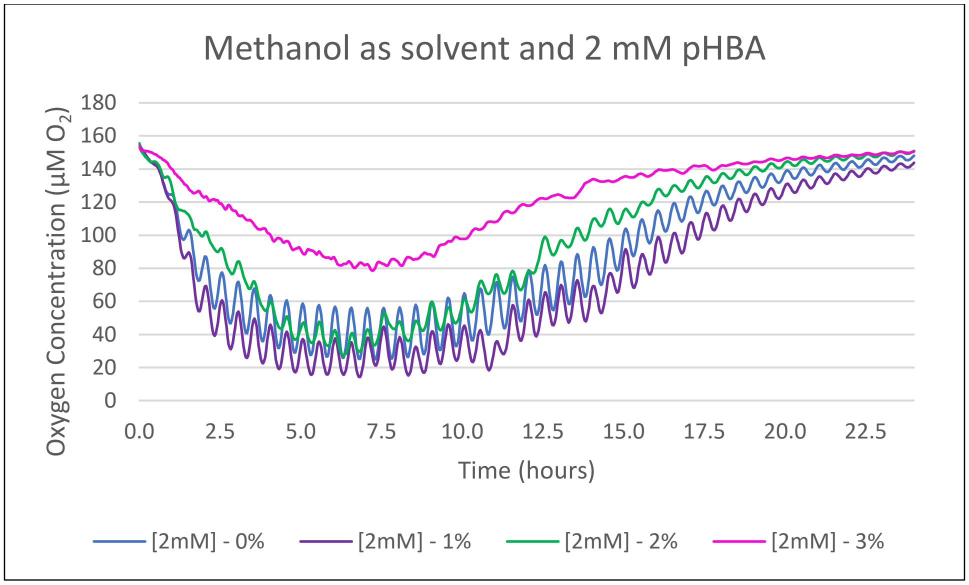

Figure 15. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of methanol with 2 mM *p*-hydroxybenzoic acid (pHBA).

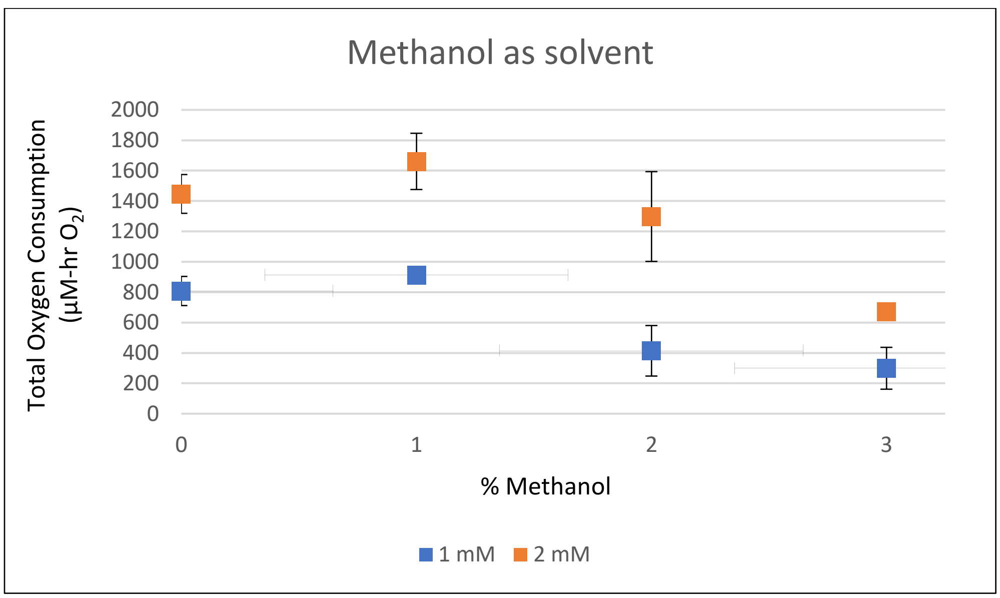

Figure 16. Resipher data comparing total oxygen consumption (µM) when media contains different methanol concentrations (% v/v) and 1 or 2 mM pHBA.

Evaluation of Ethanol as a solvent. The same pHBA and solvent concentrations were used to test the tolerance of *N. aromaticivorans* to ethanol (Figures 17-19) with two different concentrations of pHBA. In the cultures containing 1 mM (Figure 17) or 2 mM (Figure 18) pHBA, inhibition of respiration was observed with ethanol concentrations of 2% and 3%. The total oxygen consumption (Figure 19) data shows more inhibition of respiration with ethanol when compared to the concentration of methanol (Figure 16).

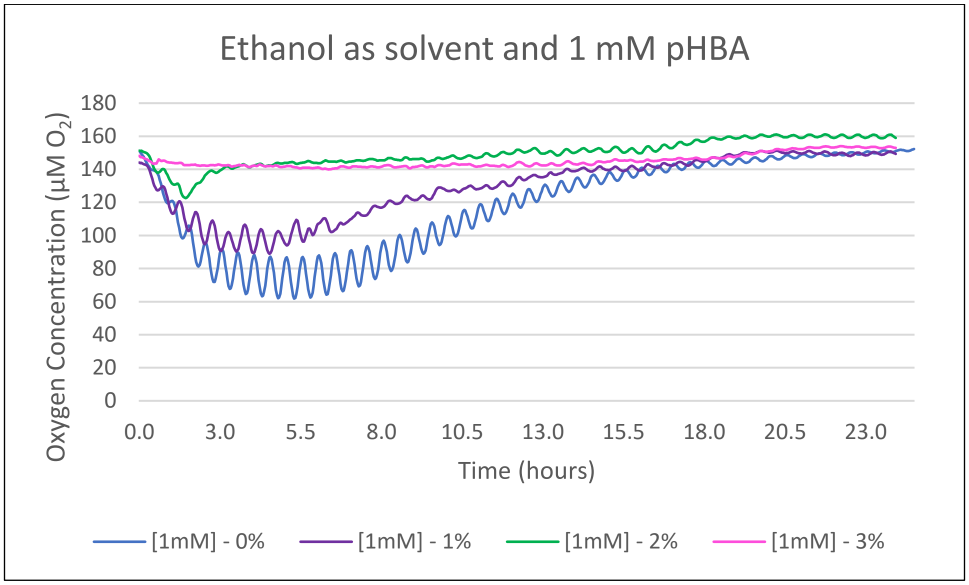

Figure 17. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of methanol with 1 mM *p*-hydroxybenzoic acid (pHBA).

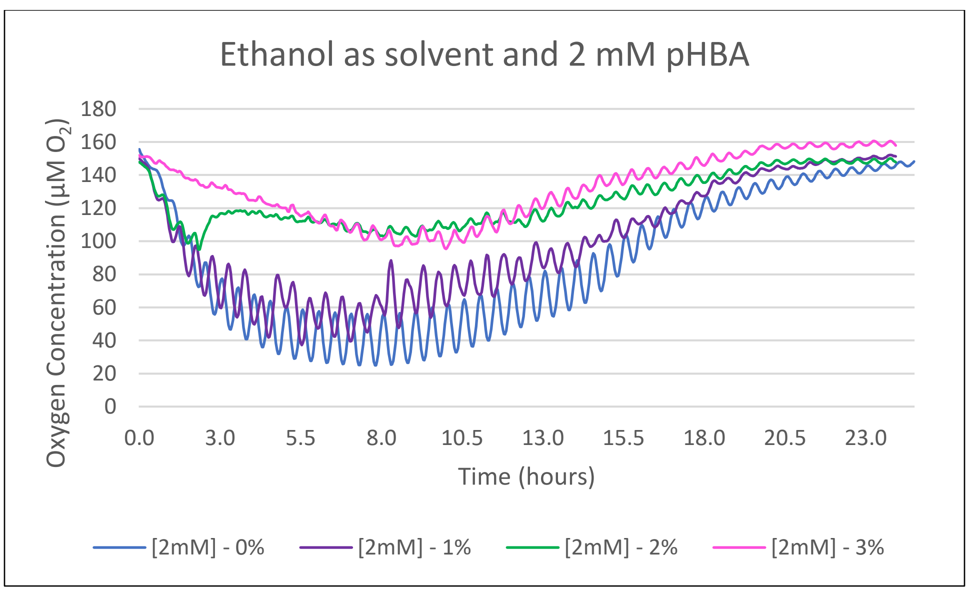

Figure 18. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of methanol with 2 mM *p*-hydroxybenzoic acid (pHBA).

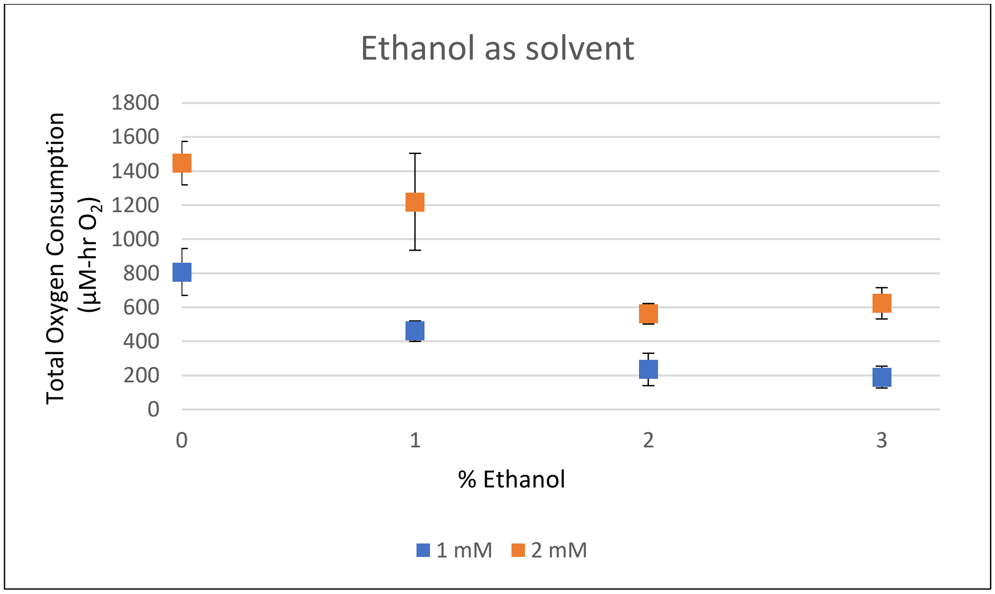

Figure 19. Resipher data comparing total oxygen consumption (µM) when media contains different ethanol concentrations (% v/v) and 1 or 2 mM pHBA.

Evaluation of Ethylene Glycol as a solvent. Similar experiments were conducted to test the effect of ethylene glycol on respiration. At the 1 mM pHBA level (Figure 20), similar respiration curves were obtained at 1 to 3% ethylene glycol, indicating that respiration by the cultures was not inhibited at these solvent concentrations. At the 2 mM pHBA level (Figure 21), little to no inhibition of respiration was observed with ethylene glycol concentrations as high as 3%.

Given the apparent lack of inhibition of *N. aromaticivorans* growth when ethylene glycol was present at concentrations of up to 3%, we conducted additional experiments with higher solvent concentrations. In these experiments (Figures 22 and 23), little to no inhibition of respiration was observed with ethylene glycol concentrations as high as 6%. As a result, further investigation was conducted, continuing up through 20% ethylene glycol as a solvent. In the 1 mM (Figure 24) and 2 mM (Figure 25) pHBA experiments, little to no significant inhibition of respiration was observed with ethylene glycol concentrations up to 20%.

Figure 26 summarizes the total oxygen consumption for all experiments conducted with ethylene glycol. When 1 mM pHBA was used, total oxygen consumption was about the same under all conditions. When 2 mM pHBA was used, there is more variability in the total oxygen consumption at different ethylene glycol concentrations, but the data did not show any specific trends.

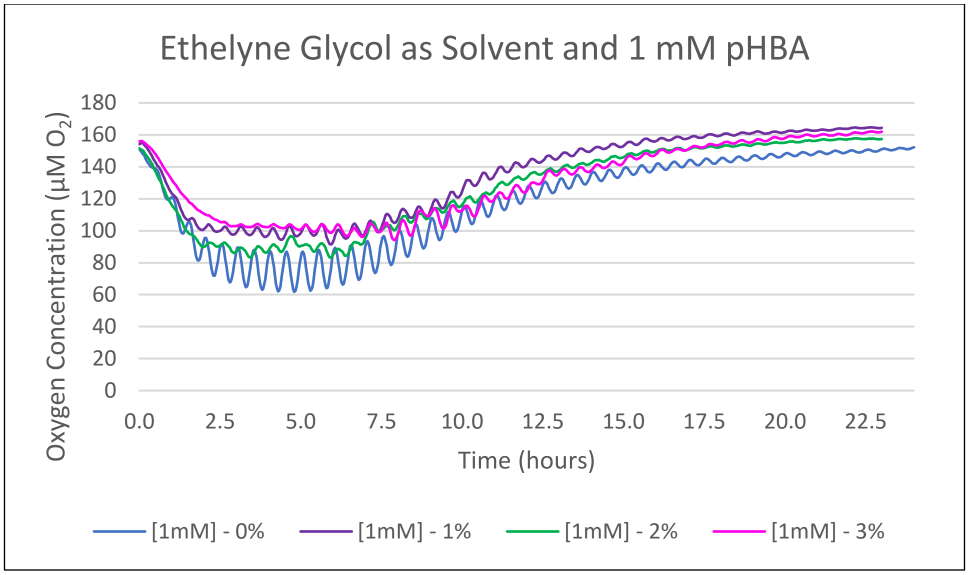

Figure 20. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of Ethylene Glycol with 1 mM *p*-hydroxybenzoic acid (pHBA).

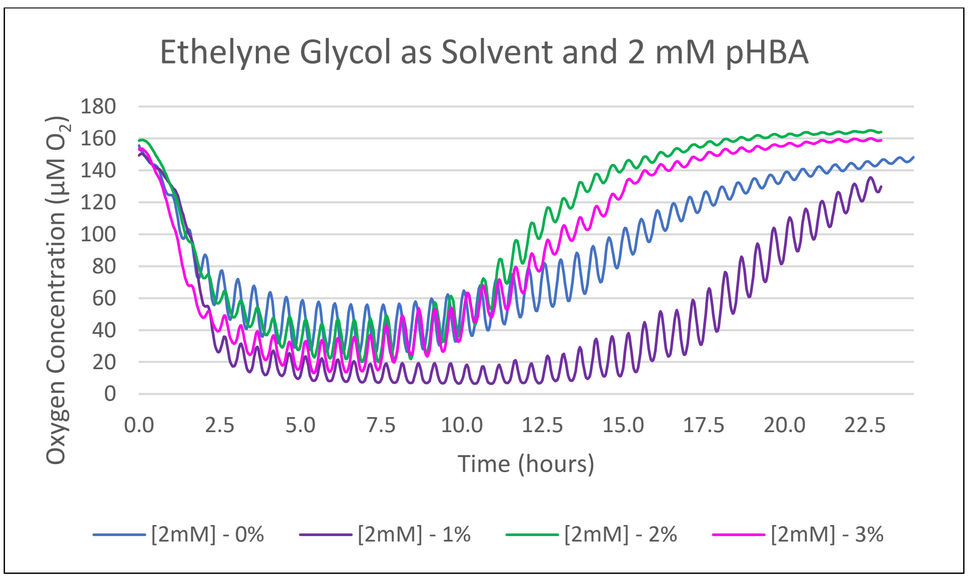

Figure 21. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of Ethylene Glycol with 2 mM *p*-hydroxybenzoic acid (pHBA).

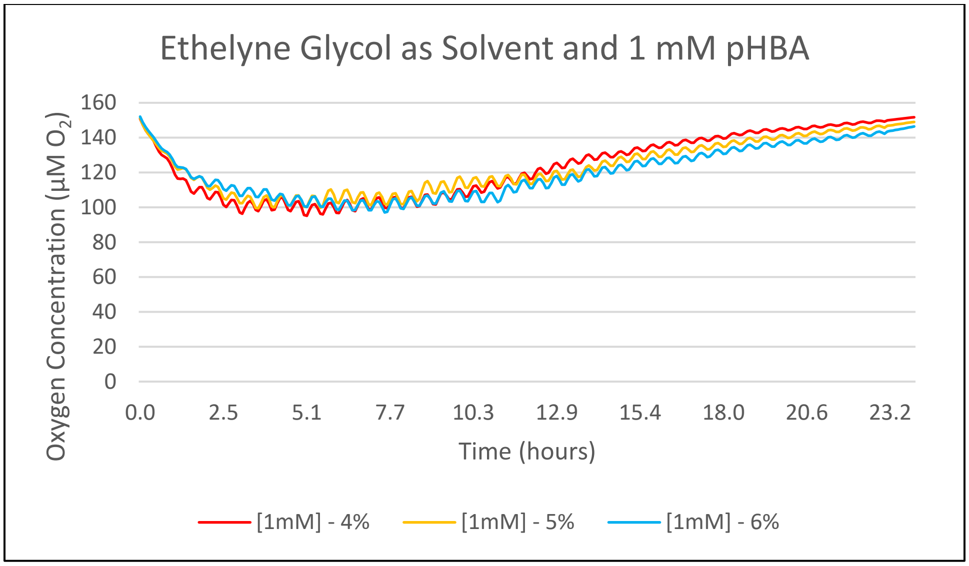

Figure 22. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of Ethylene Glycol with 1 mM *p*-hydroxybenzoic acid (pHBA).

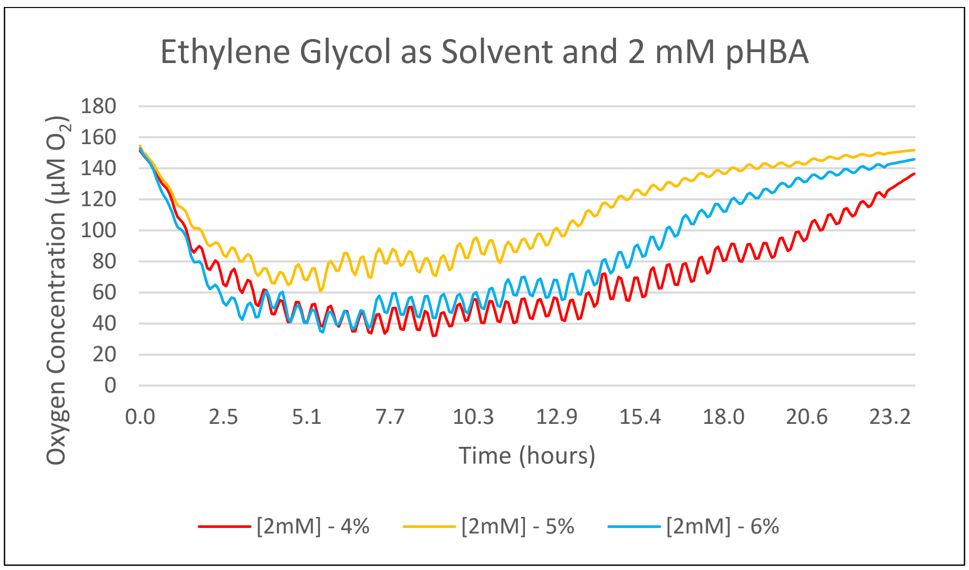

Figure 23. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of Ethylene Glycol with 2 mM *p*-hydroxybenzoic acid (pHBA).

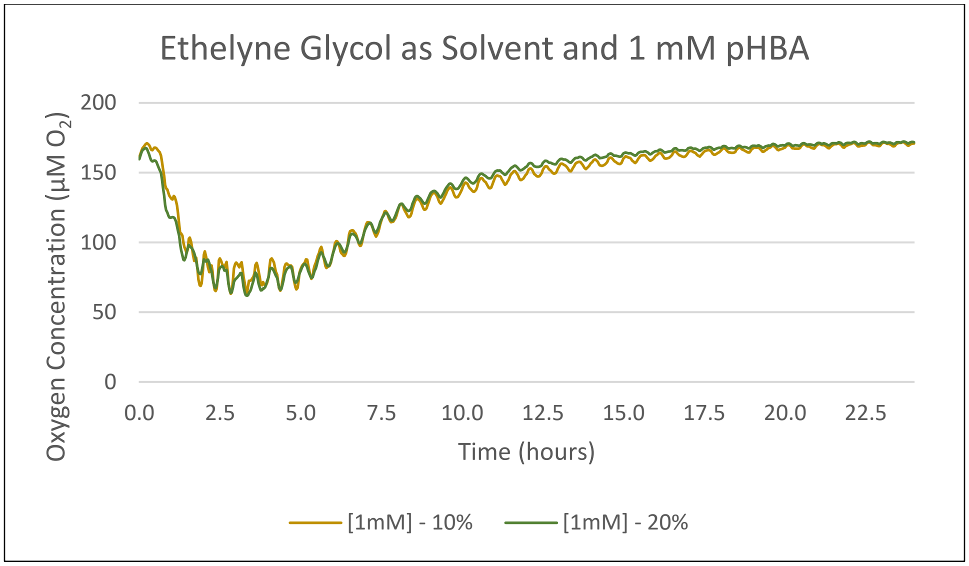

Figure 24. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of Ethylene Glycol with 1 mM *p*-hydroxybenzoic acid (pHBA).

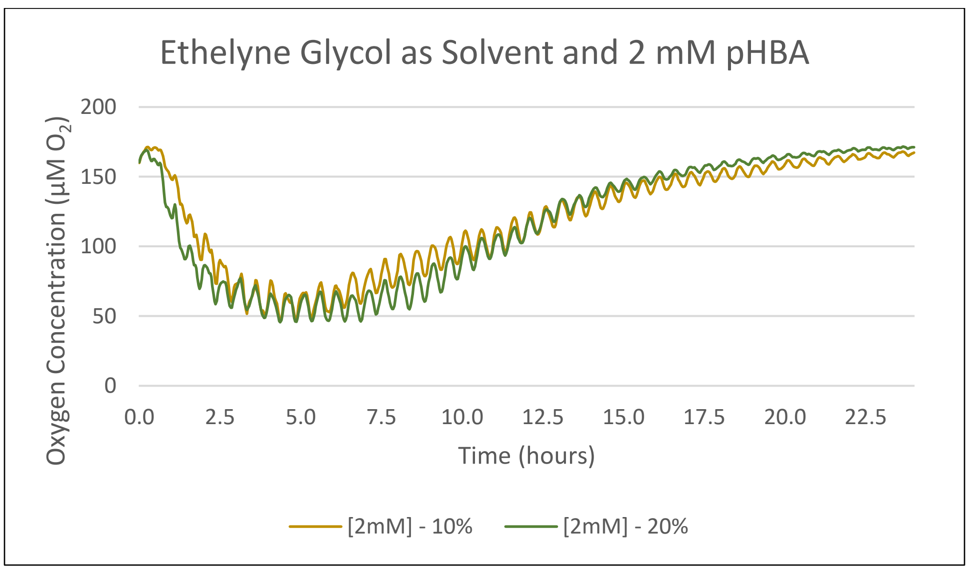

Figure 25. Resipher data tracking oxygen concentration over a 24-hour period. Varying levels of Ethylene Glycol with 2 mM *p*-hydroxybenzoic acid (pHBA).

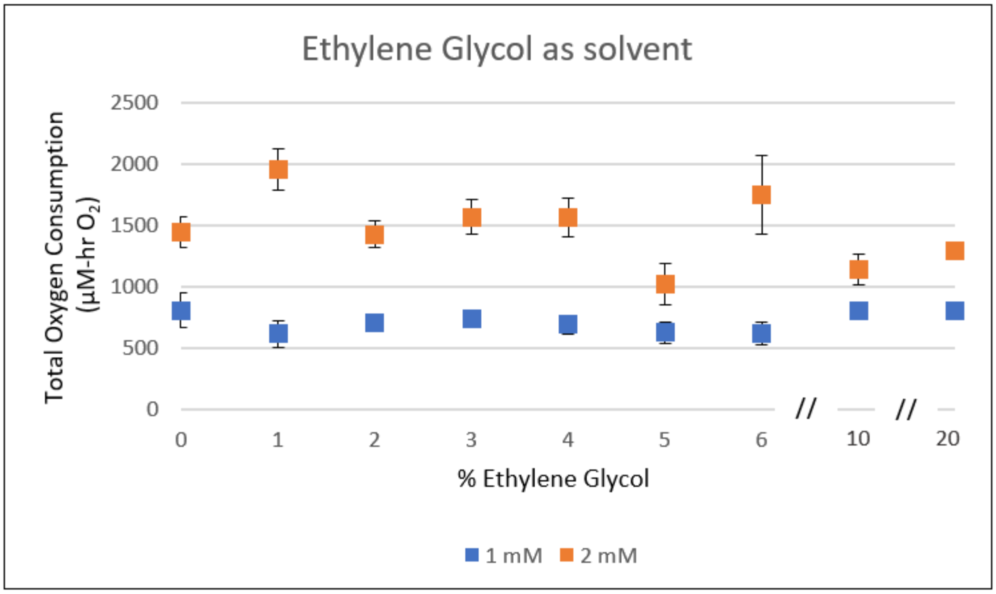

Figure 26. Resipher data comparing total oxygen consumption (μM) when media contains different ethylene glycol concentrations (% v/v) and 1 or 2 mM pHBA.

PDC Production by *N. aromaticivorans* from Biomass Treated by RCF

A second assessment of solvent compatibility with microbial funneling was performed to evaluate PDC production with the engineered *N. aromaticivorans* PDC strain when grown on media that contained the aromatics derived by the RCF using concentrations of solvents that did not significantly lower rates of oxygen consumption. For these experiments, the solvent concentration was maintained at 2% for all three solvents evaluated (methanol, ethanol, and ethylene glycol).

Cultures for these experiments were prepared with the procedure described above (Figure 13), which results in SMB media containing 2% solvent and aromatic diluted 50-fold after RCF. Triplicate 10 mL cultures were incubated for each condition, for 24 hours (Table 1).

The concentrations of PDC were quantified at the end of a 24-hour incubation period. To compare the PDC-producing performance under the different experimental conditions used, the PDC yield per gram of biomass used was quantified. Table 2 represents the step-by-step calculations to convert measured PDC concentrations to PDC yields per unit mass of biomass used for the RCF treatment.

Table 1. Reaction variables for each sample processed using reductive catalytic fractionation. Variables include; Mass of Biomass (mg), mass of catalyst (mg), solvent volume (mL), Reaction time (hours), and Solvent.

Sample Number	Mass of Biomass (mg)[1]	Mass of Catalyst (mg)[2]	Solvent Volume (ml)	Reaction Time (h)	Solvent	Gas[3]
1	1377.21	77.65	30	2	Methanol	Hydrogen
2	1377.90	77.35	30	2	Ethanol	Hydrogen
3	1377.47	75.55	30	2	Ethanol	Hydrogen
4	1377.41	77.49	30	2	Ethylene Glycol	Hydrogen
5	1377.55	77.27	30	2	Ethanol + Water	Hydrogen

[1]The biomass used for these experiments was from NM6 poplar.
[2]The catalyst used was palladium on carbon (Pd/C)
[3]Hydrogen is provided as an electron donor for catalytic reduction.

Table 2. Total PDC produced and calculation of PDC yield per gram of biomass dry weight.

Sample Number	PDC Concentration (mM)[1]	PDC Concentration (g/L)[2]	Max. PDC production (g)[3]	Biomass Used (g)[4]	PDC Yield (g PDC/ g Biomass)[5]	PDC Yield (%)[6,7]
	A	B	C	D	E	F
1	0.100±0.088	0.018	0.028	1.377	0.020	2.0±1.7
2	0.408±0.120	0.075	0.113	1.377	0.082	8.2±2.4
3	0.448±0.233	0.082	0.124	1.377	0.090	9.0±3.3
4	0.244±0.005	0.045	0.067	1.377	0.049	4.9±0.1
5	0.404±0.079	0.074	0.112	1.377	0.081	8.1±1.6

[1]PDC concentration (Column A) was quantified using LC-MS. Concentrations are given as the average of triplicate runs with standard deviations. Standard deviations are omitted in columns B-E for simplicity.
[2]PDC concentration (Column B): Column B = Column A * PDC Molecular Weight (184.10 g) / 1000 (mg/g)
[3]Maximum PDC production that could be attained if all the aromatic-containing solvent is used for PDC production (Column C) = Column B * Dilution for microbial incubation (10 mL/ 0.2 mL) * RCF reaction Volume (0.03 L)
[4]Column D taken from Table 1
[5]Column E = Column C / Column D
[6]Column F = Column E * 100
[7]Maximum PDC yields from poplar biomass ~24% (Perez et al. (2022)

Effect of Solvent on PDC Production. To evaluate PDC production, experiments with equivalent amounts of biomass (~1377 mg), catalyst (~75 mg), and reaction times (2 hours), and different solvents (Samples 1 to 4 in Table 1) were compared (Figure 27). All samples using either ethanol or ehtylene glycol as the solvent in RCF resulted in a greater PDC yield (~8%) compared with the sample originating from RCF performed with ethylene glycol (~4%) or methanol (~2%) as the solvent.

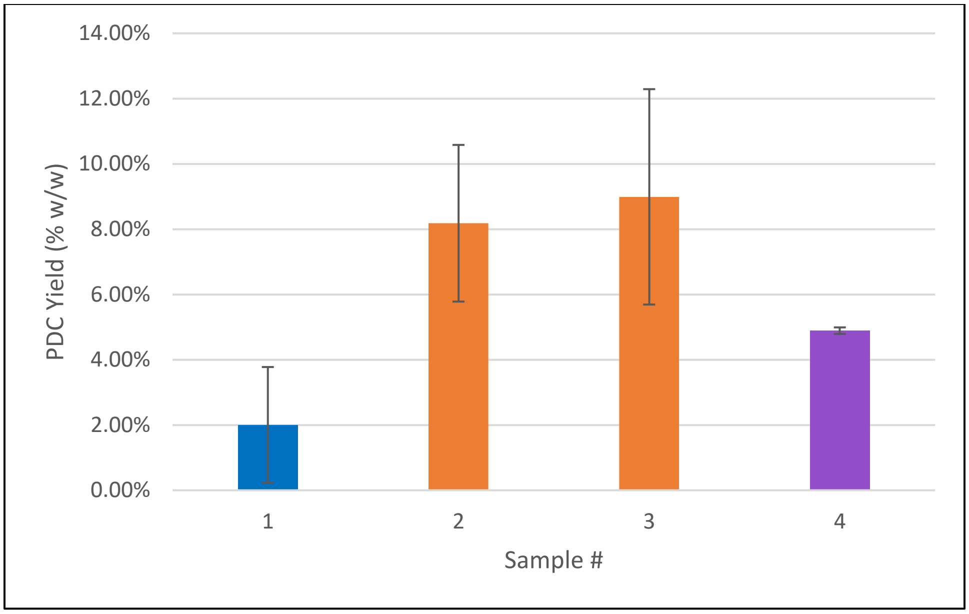

Figure 27. Comparison of PDC yields when using different solvents during RCF process. All variables kept constant except for the solvent used. Sample numbers listed on the x-axis correspond to sample numbers in Tables 1 and 2.

With ethanol as the solvent, an additional experiment was conducted to compare the PDC yields when a combination of ethanol and water, at a ratio of 85% to 15%, respectively, was used (Figure 28). The addition of water during the RCF reaction has the benefit of lowering the pressure of such reaction inside of the reactor. Indeed, the use of an ethanol and water mixture resulted in a reduced final pressure of 39 bar inside of the reactor, compared to 54 bar when ethanol only was used (Dr. Sener, personal communication). In these experiments, all conditions were kept similar, except for the solvent used (Samples 2, 3, and 5 in Table 1). The use of ethanol only or a mixture of water and ethanol did not significantly change the observed PDC production yield per unit of biomass.

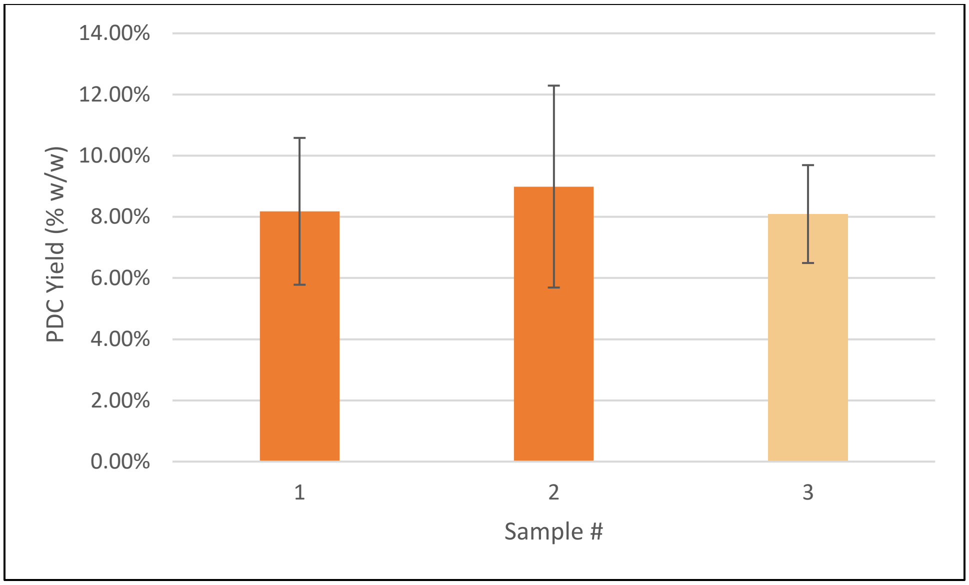

Figure 28. Comparison of PDC yields from 3 samples of RCF material. All variables kept constant except the use of solvent. Sample numbers listed on the x-axis correspond to *those* in Tables 1 and 2.

DISCUSSION

In a future lignocellulosic industry, the RCF process provides a promising solution to the acquisition of low molecular mass aromatic products from lignin, while also preserving the cellulose as a pulp material that can be recovered and used for biofuel production or other processes (Kuznetsov et al., 2023). Subsequent biological funneling of the solvent extracted aromatics during RCF will require solvent removal and reuse (to make the process economically feasible) and an approach to deliver the aromatics to the microbial culture. However, solvent recovery cannot be expected to be 100% effective, and thus, the microorganisms performing the biological funneling will be exposed to some concentration of solvent that is delivered with the aromatic stream. Although methanol has been the traditional solvent used in RCF, the pressures generated during the catalytic process at high temperature are thought to be too high for successful technoeconomic implementation of this process (Cooreman et al. 2020).

In this study, we evaluated ethanol and ethylene glycol as alternatives to methanol as RCF solvents. We investigated the tolerance to *N. aromaticivorans* to different concentrations of all three solvents. Two approaches were used for this analysis. First, using the WT *N. aromaticivorans* DSM12444 strain, we investigated respiration rates in the presence of different solvent concentrations. Second, using the PDC-producing *N. aromaticivorans* strain, we investigated the production of PDC from aromatics when the solvent concentration was maintained at 2% (v/v), a condition we know works when methanol is used as the solvent (Perez et al., 2022) and one that did not have a negative effect on respiration. This project was a collaboration with Dr. Canan Sener, who performed the RCF experiments and delivered the aromatic-containing product of the RCF process, without any solvent recovery steps. The selection of parameters to be tested during RCF were done by Dr. Sener.

For the experiments with WT *N. aromaticivorans*, respiration rates in cultures with solvent concentrations of 1, 2, and 3% were tested and compared to respiration rates in control cultures containing 0% solvent. The experiments with methanol as the solvent (Figures 14-16) confirmed earlier observations that *N. aromaticivorans* tolerated 2% methanol and that microbial activity was inhibited when higher concentrations were used. However, it was interesting to note that the concentration of pHBA appeared to influence the tolerance to methanol. When 1 mM pHBA was used, inhibition of respiration was observed at the 2% methanol condition (Figure 16). In experiments with the higher aromatic concentration (2 mM pHBA), 2% methanol did not appear to inhibit microbial respiration (Figure 17). Additional experiments could be conducted to determine the cause for this response and assess whether the tolerance to methanol increases when at higher concentrations of aromatics. This exploration, however, would be limited by the tolerance of *N. aromaticivorans* to high concentrations aromatic compounds (Chang, 2023).

The experiments with ethanol as the solvent (Figures 17-19) show that *N. aromaticivorans* tolerated 1% ethanol but that respiration was inhibited when higher concentrations were used. When 1 mM pHBA was used, inhibition of respiration was observed at the 2% ethanol condition (Figure 17). In experiments with the higher aromatic concentration (2 mM pHBA), inhibition of respiration was observed at the 2% ethanol condition (Figure 18). Additional experiments should be conducted to assess whether the tolerance to ethanol increases when higher concentration of aromatics is used.

The experiments with ethylene glycol (Figures 20-26) show that respiration by *N. aromaticivorans* was not inhibited by concentrations of this solvent as high as 20%. When 1 mM pHBA was used, inhibition of respiration was not observed through the 20% ethylene glycol condition (Figures 20, 22, 24). In experiments with the higher aromatic concentration (2 mM pHBA), inhibition of respiration was not observed through the 20% ethylene glycol condition (Figure 21, 23, 25).

In addition, the impact of variables in the RCF system on PDC production were quantified using batch cultures. RCF aromatic samples prepared using either ethanol or ethylene glycol resulted in greater PDC yield per unit of biomass when compared to PDC yield when using methanol (Figure 27), with samples from using ethanol during RCF achieving the highest PDC yields. These results were achieved while ethanol and ethylene glycol have lower pressure during RCF, compared to methanol. With methanol, the reactions take place at a starting pressure of 30 bar inside the reactor and have an average finishing pressure of 59 bar. With ethanol and ethylene glycol reactions begin at 30 bar and 10 bar, respectively, and finish at 54 bar and 20 bar, respectively (Dr. Sener, personal communication).

An additional experiment was conducted to compare the PDC yields when the RCF process used ethanol as the solvent or a combination of ethanol and water, at a ratio of 85 to 15%, respectively (Figure 29). Reducing the volume of solvent present in and RCF reaction can aid in achieving lower pressure requirements of the reactions. The use of an ethanol and water mixture resulted in a reduced final pressure of 39 bar inside of the reactor, compared to 54 bar when ethanol only was used (Dr. Sener, personal communication). Even with the reduction in solvent volume, there was no change in the PDC yield at the biological funneling stage (Figure 28). An increase in the volume of water to solvent used for aromatic delivery could also allow for a greater concentration of aromatics to be delivered to the biological funneling stage. However, the recovery of ethanol may leave the water in the sample, and then, the delivery of aromatic could be limited by the solubility of these aromatics in water.

Implications for Scale Up Lignin-Processing Pipeline

For the industrial production of a chemical from lignin in a lignocellulosic biorefinery, the product of biological funneling should have the highest possible titer since the cost of downstream product recovery largely depends on the product titer achieved in the biological reaction. In batch reactors, the product titer is limited by the initial concentration of substrate at the start of the batch.

In my experiments, the PDC titer derived from RCF material will be limited by the concentration of aromatics that could be delivered in an influent stream. In my batch experiments, the concentration of aromatics at the beginning of the incubation period was low because of the need to dilute the solvent to prevent inhibition of microbial growth. Consequently, the PDC titers at the end of incubation were less than 0.5 mM (Table 2). Since the solvent concentration was kept at 2% in the culture media, this means that aromatics were diluted 50-fold compared to the concentration of PDC-producing aromatics in the solvent after the RCF process. Furthermore, if a portion of the solvent is recovered after the RCF process, for example by distillation, the concentration of aromatics in the remaining solvent will increase, increasing the PDC production potential during the microbial funneling stage.

One can define the PDC-production potential (PPP) as the concentration of aromatics in the RCF product, after solvent recovery, that would be needed to achieve a desired PDC titer by the microbial community. Given the observed inhibitory solvent concentrations in this study, and a target of 100 mM PDC produced by the microbial community, we can estimate the PPP required and the corresponding solvent removal efficiency needed to achieve the PPP (Table 3).

Using 100 mM as a goal for PDC titer allows us to make two important calculations. The aromatic concentration that is required in the RCF product after solvent recovery, accounting for the required dilution to avoid microbial inhibition by the solvent (Column E in Table 3) and the solvent volume reduction needed to obtain these concentrations (Column F). Using the calculations in Table 3, the concentration of PDC-producing aromatics in the RCF product before solvent recovery (Column D) is obtained by dividing the PDC titer from each sample (Column A in Table 2) by the dilution factor that was used to reach a 2% solvent concentration. In a methanol RCF sample (sample 1), this value comes out to be 5 mM PDC-producing aromatics in the RCF solvent. To calculate the concentration of PDC-producing aromatics that would be needed after solvent extraction (Column E) considering the maximum levels of solvent permissible in the microbial culture (Column C), we divide the titer goal (100 mM PDC; Column B) by the maximum solvent tolerance (Column C) and applying the corresponding unit conversion. For the methanol RCF material, this value comes out to be 5,000 mM or 5 M. Lastly, to calculate how much solvent would be required to be removed to achieve 5,000 mM from the initial 5 mM solution estimated in Column D, we subtract the ratio of the observed PDC concentration in the RCF product (Column D) to the estimated PPP for each sample after solvent recovery (Column E) from one and multiplying by 100 to obtain the result in percentage units. For this sample, this calculation indicates that one would need to remove 99.9% of the methanol from the RCF product to reach a PPP compatible with 100 mM PDC titer in a batch microbial process (Column F).

We can use the same logic to calculate PPP values and solvent reduction requirements for different PDC target titers (Figures 29 and 30). The high concentration requirements in methanol and ethanol (Figure 29) could be limited by the solubility of aromatics in these solvents. If ethylene glycol is used as the solvent, the concentration of aromatics after solvent recovery is much lower (Figure 29) because the microbial culture is more permissive of high solvent concentrations (20% for ethylene glycol compared to 1% and 2% for ethanol and methanol).

Table 3. Required PDC production potential (PPP) in delivered aromatics (column E) and Solvent Removal requirement (column F) to achieve desired PPP.

Sample #	Solvent	PDC titer goal (mM)	Maximum Solvent Tolerance (% by volume)[1]	PDC Concentration (mM)[2]	Aromatics in RCF product (mM)[3]	PPP in Delivered Solvent (mM)[4]	Solvent Removal to Obtain Column F[5]
Column	A	B	C	D	E	F	G
1	Methanol	100	2	0.100	5.00	5000	99.9%
2	Ethanol	100	1	0.408	20.41	10000	99.8%
4	Ethylene Glycol	100	20	0.244	12.18	500	97.6%

[1] Experimentally determined tolerance of *N. aromaticivorans* to the different solvents tested (Column C).
[2]Column D taken from Table 2 (Column A).
[3] Concentration of PDC-producing aromatics in RCF product before solvent recovery. Based on experimental results of PDC production shown in Table 2. Column E = Column A (from Table 2) * solvent dilution for microbial incubations (10 mL/ 0.2 mL)
[4] Concentration of aromatics in RCF product after solvent recovery that would be necessary to achieve the target PDC titer of 100 mM, considering the dilution needed to prevent solvent inhibition of the microbial culture. Column F = (Column B/Column C) * 100
[5] Column G = 1 – (Column D / Column E) * 100

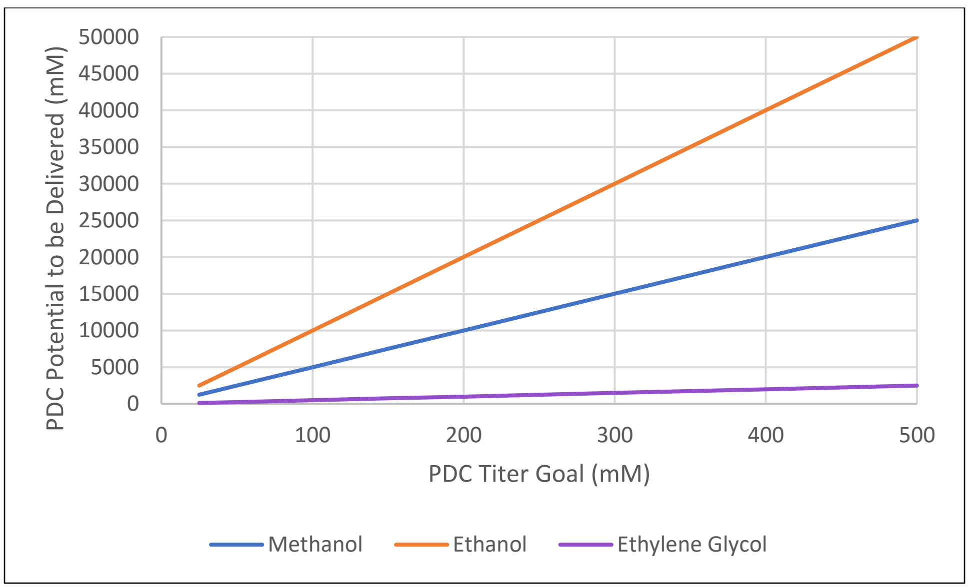

Figure 29. PDC Potential Concentration (column E, table 3) in Solvent as a function of PDC production goals.

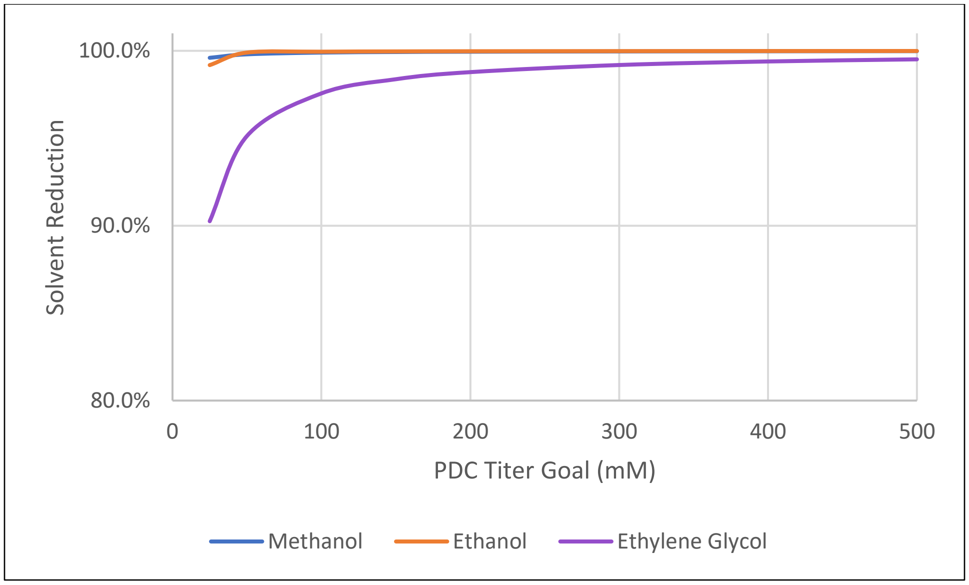

Figure 30. Solvent Reduction Required (Column F, Table 3) as a function of PDC production goals.

The data in Figures 29 and 30 predict that ethylene glycol presents the lowest values for both the initial aromatic concentration and efficiency of required solvent recovery. Although PDC production in samples from ethylene glycol (Figure 27) is not as great as observed in samples from ethanol, ethylene glycol may present many beneficial factors in the RCF to biological funneling pipeline. Low inhibition in *N. aromaticivorans,* increasing PDC yields when compared with methanol, reduced pressure requirements during RCF reactions, and lower requirements of solvent volume reduction give ethylene glycol a strong case for future exploration into the use as a solvent in the RCF process. Nevertheless, low solvent recovery has economic consequences that would need to be weighed versus the possibility of using this solvent for efficient aromatic delivery to the microbial funneling stage.

All microbial funneling experiments in this study were conducted using batch cultures. When considering the scale-up of PDC production, microbial incubations could be done in batch, continuous flow bioreactor systems, or fed-batch cultures (Figure 31). In a batch process (Figure 31A), if PDC production were to be scaled up with a goal of achieving a 100 mM PDC titer or higher, the concentration of PDC-producing aromatics present in the media at time zero should be close to the PDC target concentration (assuming a stoichiometry yield of PDC from the PDC-producing aromatics). However, in batch experiments *N. aromaticivorans* the limit of aromatic concentrations thus far has been established at about 40 mM (Chang, 2023) so achieving 100 mM PDC through batch incubations may not be feasible.

When considering fed-batch experiments (Figure 31B), it is possible to increase PDC titers by repeated addition of aromatic-containing substrates, provided that the aromatic concentration is high and additional feeding does not either dilute the product already made or create solvent toxicity in the culture. With this method, it may be possible to reach the 100 mM target when PDC-producing aromatics from RCF are delivered with the solvent, if the solvent concentration does not increase to inhibitory levels. It is possible that the continuous aeration of the cultures would contribute to the evaporation of a given solvent, and thus, allow for multiple feedings in the same batch. Moreover, fed-batch experiments would also be restricted by the *N. aromaticivorans* tolerance to high aromatic concentrations, as is the case in batch experiments.

Lastly, when considering continuously feed bioreactors (Figure 31C), we have shown that it is possible to achieve PDC titers near 100 mM with *N. aromaticivorans* grown with a single aromatic (Kim, 2023) if appropriate operational conditions are employed. Even though achieving these high titers requires that a feed with PDC-producing aromatics at similar concentrations as the target titer, the concentrations of aromatics in the bioreactor can be kept below inhibitory levels by the continuous transformation of the aromatics to PDC. Another advantage of the continuous flow system is that once the reactor reaches steady state operation, PDC production is continuous, thus eliminating idle times in production that are required when using batch or fed-batch reactors.

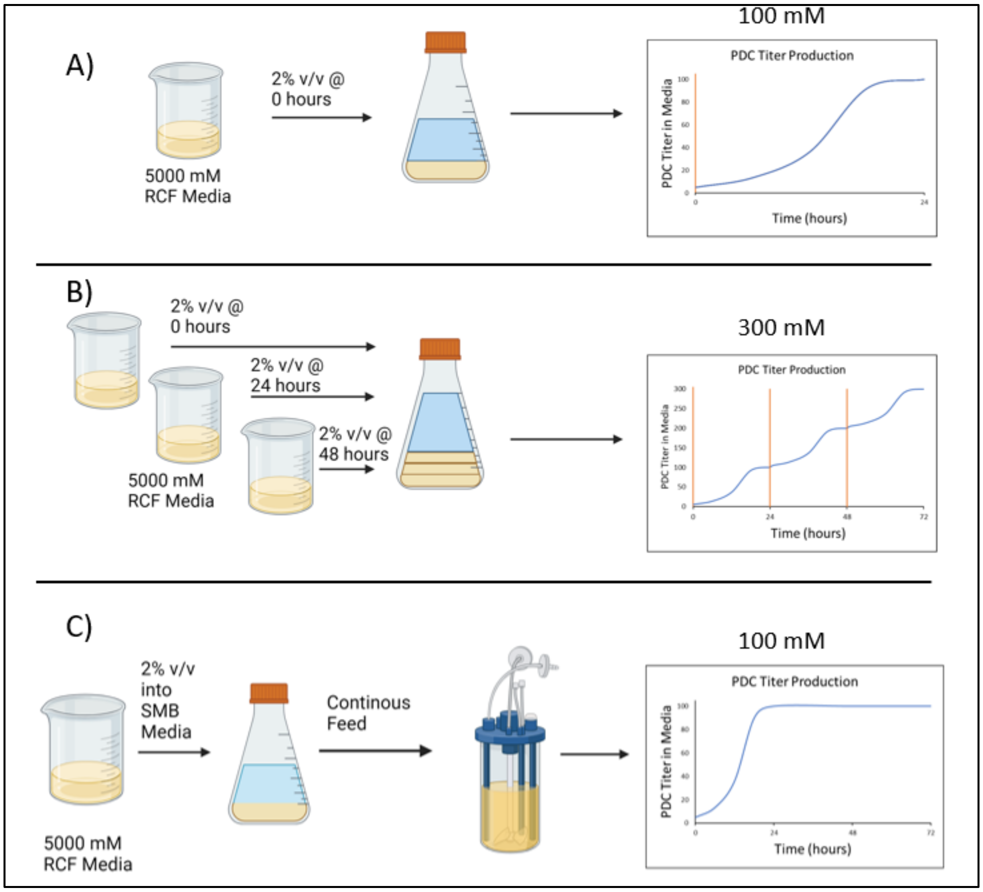

Figure 31. A) Illustration of a batch reaction to achieve 100 mM PDC titer after 24 hours. **B)** Illustration of a fed batch reaction to achieve 300 mM PDC titer after 72 hours. **C)** Illustration of continuous feed reactor to achieve a continuous production of 100 mM PDC titer.

In conclusion, this study of biological funneling by *N. aromaticivorans* in the presence of three different solvents, methanol, ethanol, and ethylene glycol, identifies thresholds that inform the requirements for using *N. aromaticivorans* to produce PDC and possibly other valuable products at titers that are higher than those achieved when using typical batch experiments when fed with different lignocellulosic biomass streams. Attaining the required concentrations of PDC-producing aromatics in feed streams may be difficult and may depend on the individual aromatics and the solvents used in upstream processes such as RCF. This study provides insight and a method to evaluate the feasibility of achieving a desired product titer that depends on the tolerance of *N. aromaticivorans* to the solvents used as well as the requirements for concentrations of PDC-producing aromatics that need to be delivered from the upstream processes.

In the case of RCF, future research should investigate the solubility of different RCF products on the solvent used for aromatic delivery after most of the solvent is extracted. Alternatively, if all the solvent is removed, an RCF oil is produced and aromatic delivery would, in theory, not be limited by solvent inhibition. However, efficient delivery of this RCF oil to a microbial culture will be needed to ensure that aromatics are dissolved in the culture media after delivery.

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Appendix

Supplementary Table 1. Multiple Reaction Monitoring (MRM) transitions used to quantify phenolics (table from (Umana et al., 2022).

Mass Spectrometer	**Shimadzu LCMS-8045**						
ESI Source	300ºC Ionization mode: DUIS ESI/APCI						
Operation Mode	Multiple Reaction Monitoring (MRM) Argon gas, 230 kPa						
MRM Transition Details Negative (-) mode							
Compound Name	**Retention Time**	**Transition 1**	**CE 1**	**Transition 2**	**CE 2**	**Transition 3**	**CE 3**
2-pyrone-4-6 dicarboxylic acid	1.89	183.1>111.0	15	183.1>139.0	12	183.1>95.1	13