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Biological conversion of the aqueous wastes from hydrothermal liquefaction of algae and pine wood by *Rhodococci*

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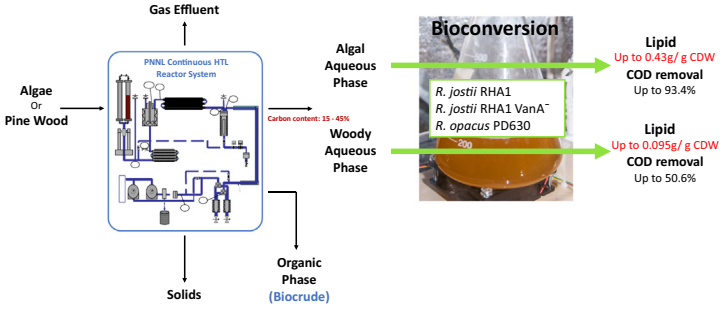
HIGHLIGHTS

- Hydrothermal liquefaction aqueous wastes (HTLAWs) were fully characterized by GC–MS.
- 93.4% COD of algae-HTLAW was effectively removed by *R. jostii* RHA1.
- Wood-HTLAW promoted lipid accumulation of 0.43 g lipid/g CDW in *R. opacus* PD630.
- Both of the single culture and coculture of *Rhodococci* strains were employed.
- HTLAWs can be potentially converted to lipids by *Rhodococci* for biofuel production.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, *R. opacus* PD630, *R. jostii* RHA1, *R. jostii* RHA1 VanA$^-$, and their co-culture were employed to convert hydrothermal liquefaction aqueous waste (HTLAW) into lipids. After 11 days, the COD reduction of algal-HTLAW reached 93.4% and 92.7% by *R. jostii* RHA1 and its mutant VanA$^-$, respectively. Woody-HTLAW promoted lipid accumulation of 0.43 g lipid/g cell dry weight in *R. opacus* PD630 cells. Additionally, the total number of chemicals in HTLAW decreased by over 1/3 after 7 days of coculture, and 0.10 g/L and 0.46 g/L lipids were incrementally accumulated in the cellular mass during the fermentation of wood- and algal-HTLAW, respectively. The GC–MS data supported that different metabolism pathways were followed when these *Rhodococci* strains degraded algae- and woody-HTLAW. These results indicated promising potential of bioconversion of under-utilized carbon and toxic compounds in HTLAW into useful products by selected *Rhodococci*.

1. Introduction

Intensified efforts have been applied in recent years to develop biological and thermochemical processes for biomass conversion to liquid fuels in order to reduce green-house gases, lower the nation's dependence on imported petroleum crude, create domestic biorefinery industries and employment opportunities. Hydrothermal liquefaction (HTL) is a thermochemical conversion process that is receiving considerable interest due to its inherent simplicity, ability to handle a wide range of biomass feedstock (i.e., lignocellulosics, agricultural residues, wet wastes, and algae), and high carbon efficiency (Elliott et al., 2015). In HTL processes, aqueous biomass slurries are heated (200–400 °C) under pressure (10–20 Mpa) and held at target temperature for sufficient time to break down the solid biopolymeric structure into a mixture of products, including biocrude, gas rich in CO_2, solid residue (primarily ash), and an aqueous byproduct with high concentrations of

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both organics and nutrients (Elliott et al., 2013, 2015). HTL can release nitrogen and oxygen from biomass into the aqueous and gaseous fractions. Carbon yields to biocrude often exceed 50% while carbon yields to the aqueous phase range from about 15–45% depending on the biomass source. The overall carbon efficiency of the HTL process can be significantly improved by converting the hydrothermal liquefaction aqueous waste (HTLAW) into useful products. Current options for utilization of HTLAW include gasification, anaerobic digestion, and nutrient recycle (for algal applications). However, the compositions of HTLAW present challenges to these options. The HTLAW streams contain high concentration of chemical oxygen demand (COD) and/or high concentration of ammonia (1.9–7.1 g/L) in addition to other characteristics that make HTLAW unsuitable for direct discharge to the environment (Pham et al., 2013). Therefore, it is important to develop noval approaches that effectively degrade HTLAW.

One widely applied approach of wastewater treatment is microbial degradation (Hollinshead et al., 2016; Li et al., 2013; Zeng et al., 2013). It is well-known that *Rhodococci* can catabolize short- and long-chain alkanes, aromatic (nitro-, hydroxyl-, halogenated substituted), heterocyclic and polycyclic aromatic compounds (Holder et al., 2011; Jena et al., 2011; He et al., 2012, 2013; Labana et al., 2005; Larkin et al., 2005; Nadaf and Ghosh 2014; Lenke and Knackmuss, 1996; Muller et al., 2014). *Rhodococcus* UKMP-5 M degraded 900 mg/L phenol in wastewater (Suhaila et al., 2013). The maximum phenol oxidation achieved about 34%, promoting an increase in 28% of *R. erythropolis* growth, 10% of respiration activity with shorter lag- and exponential-phase interval (Křiklavová et al., 2014). *R. erythropolis* UPV-1 grown in synthetic wastewater significantly decreased 90% of COD, 98.7% of phenol and 99.7% of formaldehyde (Hidalgo et al., 2002). *Rhodococcus* sp. NCIM 2891 was also evaluated in the pulp and paper industrial effluent remediation, resulting in 53.2% decrease of COD, 81.0% reduction of the total dissolved solid along with a decrease in heavy metal concentrations (Nadaf and Ghosh, 2014). *Rhodococcus* sp. DK17 was isolated from soil and analyzed for the ability to grow on *o*-xylene as the sole carbon and energy source. Although DK17 couldn't grow on *m*- and *p*-xylene, it was capable of growing on benzene, phenol, toluene, ethylbenzene, isopropylbenzene, and other alkylbenzene isomers (Kim et al., 2002). *Rhodococcus* sp. EH831 was reported to degrade alcohols, chlorinated hydrocarbons, cyclic alkanes, ethers, ketones, monoaromatic and polyaromatic hydrocarbons, and petroleum hydrocarbons (Lee et al., 2010).

Recently, it was also reported that *Rhodococcus* strains are strong candidates to produce lipids using aromatic compounds (Kurosawa et al., 2013; Kosa and Ragauskas, 2013). Eukaryotic organisms are mainly known to store lipophilic compounds, which have higher calorific value than carbohydrates and proteins. Lipid accumulation was observed in *Rhodococcus erythropolis*, *Rhodococcus fascians*, *Rhodococcus rhodochrous*, and *Rhodococcus ruber* etc., which were capable of synthesizing triacylglycerol (TAG) as their carbon and energy storage (Alvarez et al., 2000; Voss and Steinbüchel, 2001). Ethanol organosolv lignin and its ultrasonicated product were found to be sufficient carbon sources for *R. opacus* DSM 1069 to accumulate lipids up to 0.04 g lipid/g CDW (Kosa and Ragauskas, 2013). *R. opacus* PD630 could degrade vanillic acid via the β-ketoadipate pathway and biotransform vanillic acid to lipids (Kosa and Ragauskas, 2012; Zhao et al., 2013). Laccase and *R. opacus* PD630 cells synergized in lignin degradation, leading to a 17-fold increase of lipid production compared to that without laccase addition (Zhao et al., 2016). In contrast, few reported effective reduction of COD in HTLAW and bioconversion of HTLAW into lipids.

HTL is a promissing and carbon efficient approach to produce petroleum-like biocrude from biomass (Elliott et al., 2015). However, many polar organic compounds, including organic acids, alcohols, ketones, and biocrude constituents, are produced during HTL (Elliott et al., 2013, 2015). Biotransformation of HTL aqueous waste (HTLAW) to biofuel is highly challenging due to potential inhibition and toxicity from the biocrude constituents along with its high COD. Thus, the overall goal of this study is to develop a cost-effective biological process that converts the organic carbons, including aromatics and other organic compounds in the HTLAW, into microbial biomass enriched with lipids that can then be used for biofuel production. This strategy will improve the carbon efficiency of thermochemical conversion of various biomass feedstocks. In this study, three aerobic *Rhodococci* strains (e.g. *R. opacus* PD630, *R. jostii* RHA1 and its mutant *R. jostii* RHA1 VanA$^-$) with strong aromatics degradation and/or lipids biosynthesis capacities were selected for investigation to reduce the HTLAW COD as well as to produce lipids.

2. Materials and methods

2.1. Chemicals and wastewater

Tryptic Soy Broth (BD, cat #: 257107) was obtained from St. Becton, Dickinson and Company (USA). All other chemicals were also from commercial sources and of analytical grade. Aqueous wastewater generated during HTL conversion of loblolly pine (WD38) and marine *Tetraselmis* algae (Tet06) were kindly provided by the Pacific Northwest National Laboratory (PNNL). The bench-scale continuous HTL system, which was used to generate WD38 HTLAW and Tet06 HTLAW, was previously described by Elliott et al. (2013). Table 1 shows the selected characteristics of the WD38 HTLAW and Tet06 HTLAW.

2.2. Microorganisms

R. opacus PD630 was kindly provided by Dr. Joshua Yuan (Texas A&M University) while *Rhodococcus jostii* RHA1 and *R. jostii* RHA1 VanA$^-$ were generously supplied by Dr. Lindsay Eltis (University of British Columbia, Canada).

2.3. Fermentation of HTLAW

The strains were cultivated in Tryptic Soy Broth (BD, cat #: 257107) for 24 h at 30 °C and 180 rpm. The cells were harvested by centrifugation (8000×*g*, 10 min) at 4 °C and washed twice with sterilized potassium phosphate buffer (KPB) (100 mM, pH 7.0). The cells re-suspended in the same KPB at OD_{600} = 1.0 were used as inoculum while the supernatant was discarded after the final wash. To start single strain fermentation, 1% (v/v) inoculum was added to 100 ml HTLAW without added nutrition. To start fermentation of co-culture, 0.333 mL *R. opacus* PD630, 0.333 mL *R jostii* RHA1, and 0.333 mL *R. jostii* RHA1 VanA$^-$ were added to 100 ml HTLAW without added nutrition. The 100 ml nutrient medium was comprised of (per liter): 1.4 g $(NH_4)_2SO_4$, 1.0 g $MgSO_4{\cdot}7H_2O$, 1.0 ml $CaCl_2{\cdot}2H_2O$ (15 g/L), 1.0 ml sterile trace element solution (Stock A). Stock A solution contains: $NaMoO_4{\cdot}H_2O$ 2.0 g/L and FeNa-EDTA 5.0 g/L. Fermentation experiments were carried out at 30 °C and 180 rpm. 5 ml samples were taken during the cultivation for analysis.

2.4. Analysis of soluble compounds by GC–MS

After 10 ml fermentation broth was centrifuged at 9000 rpm for 30 min at 4 °C, the cells were discarded. The supernatant was filtered through a 0.2 μm hydrophilic filter (Millipore Express® SHC) and then analyzed by GC/MS. The analysis utilized an Agilent

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