Comparative Exploration of Biological Treatment of Hydrothermal Liquefaction Wastewater from Sewage Sludge: Effects of Culture, Fermentation Conditions, and Ammonia Stripping

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Abstract

Hydrothermal liquefaction wastewater from sewage sludge (sludge HTLWW) is an emerging waste stream that requires treatment before being discharged into the environment. Biological treatment of sludge HTLWW is an attractive option due to the low cost and operational flexibility. In this study, we investigated and compared the performance of three bacterial strains and four fungal strains for biodegradation of sludge HTLWW. Our screening experiments established pH and mineral supplementation (iron, magnesium, calcium, and phosphorus) conditions that greatly improved COD removal and chemical compound degradation by the microbes. An ammonia stripping pretreatment improved COD removal efficiency of *Rhodococci jostii* RHA1 by 44%. All tested bacterial strains can tolerate 10X dilution of HTLWW and remove 35-44% of COD in 2-15 days, while all tested fungal strains were able to tolerate 20X dilution and were better at degrading phenolic compounds than bacteria. HTLWW treatment with biomass pellets of fungus *Aspergillus niger* NRRL 2001 achieved the best COD removal efficiency of 47% in 12 days without the need of nutrient supplementation. Comparisons on chemical compound degradation by the tested microbes suggested that organic acids in HTLWW were highly degradable, followed by phenolic compounds. N-heterocyclic compounds were resistant to biodegradation and were removed by 38%. This study demonstrated pure culture biological treatment of sludge HTLWW with diverse types of microorganisms, which would guide the culture development and bioprocess parameter optimization for treating HTLWW of different compositions.

Keywords: hydrothermal liquefaction, sewage sludge, biodegradation, recalcitrant compound, bio-oil, sustainability

1. Introduction

Hydrothermal liquefaction (HTL) is a thermochemical conversion process conducted at 250-370°C and 4-22 MPa (Gollakota et al., 2018). Compared with pyrolysis, HTL uses lower operating temperature and has higher energy efficiency while it doesn't require drying or dewatering of the biomass feedstock. Therefore, HTL is a promising process for converting wet biomass (e.g., sewage sludge, microalgae, food waste, etc.) into biofuels (Watson et al., 2020). One of major barriers that limits the wide application of HTL is the wastewater generated during the process, which contains various recalcitrant organic compounds that are solubilized during HTL (Pham et al., 2013a; Gu et al., 2019). HTL wastewater (HTLWW) is considered an emerging waste stream with high concentrations of unrecovered nitrogen (around 1-50 g/L) and organic carbon (around 10-40 g/L) (Gu et al., 2019). Depending on the feedstock, nitrogen and organic carbon in HTLWW can be in the form of toxic compounds, such as phenol, phenol derivatives, and N-heterocycles (Watson et al., 2020).

HTLWW from sewage sludge, manure, and microalgae is considered to have higher toxicity than that from other feedstocks due to the presence of nitrogenous organics, such as N-heterocyclic compounds and aromatic amines that originate from the nitrogen-rich feedstocks (Chen et al., 2014; Li et al., 2019; Maddi et al., 2017; Usman et al., 2019; Yang et al., 2018a). It was reported that 50-75% of the feedstock nitrogen can be solubilized into HTLWW with 10-40% being nitrogenous organics and the rest being ammoniacal nitrogen (Ekpo et al., 2016; Yu et al., 2014). Total ammonia nitrogen (TAN) (i.e., free ammonia nitrogen + ammonium nitrogen) concentrations of above 1700 mg/L is known to greatly inhibit high-rate anaerobic digestion (AD) (Yenigün and Demirel, 2013). NH_4^+-N was also shown to inhibit the formation and metabolic activity of aerobic biofilm at concentration above 25 mg/L (Weng et al., 2022).

Therefore, high TAN in sludge HTLWW would pose a big challenge on the biological treatment of HTLWW.

Cultivation of microorganisms in HTLWW can degrade toxic compounds and recover nutrients in the form of electricity, heat, natural gas, microbial biomass, and value-added products (Watson et al., 2020). Using AD and microalga for HTLWW treatment usually requires extensive dilution (around 100-1000X) and/or pretreatment (extraction, adsorption, and/or partial oxidation) of HTLWW to reduce inhibition of the wastewater on microbes (Watson et al., 2020). As shown in Table S1, COD removal of AD was limited to 8-49% without pretreatment and required long incubation time due to a prolonged lag phase when exposed to HTLWW from nitrogen-rich feedstocks. Cultivation of microalgae alone in HTLWW from nitrogen-rich feedstocks showed poor COD removal of 17-20% but can be improved to 63% by using an algae and bacteria consortium (Table S1). Compared with AD and microalga, biological treatment of HTLWW with other microorganisms was less studied but existing findings have demonstrated promising potentials of some bacterial and yeast strains in treating HTLWW. Bacteria, including *Escherichia coli*, *Pseudomonas putida*, *Rhodococci opacus*, and *Rhodococci jostii* were shown to have higher tolerance to HTLWW and required less than 20X dilution without the need of pretreatment (Table S1). Oleaginous yeast species *Yarrowia lipolytica* was able to grow in HTLWW with 10X dilution while producing value-added compounds such as polymer precursors, lipids, and organic acids (Cordova et al., 2020).

Filamentous fungi, such as *Aspergillus niger*, *A. oryzae*, *Phanerochaete chrysosporium*, and *Trametes versicolor* are known to degrade a variety of problematic organic compounds, such as antibiotics and pharmaceuticals that are resistant to bacterial degradation, by means of the non-specificity of their fungal enzymes (e.g., laccase and peroxidase) (Naghdi et al., 2018). In

addition, fungi can survive and thrive under adverse conditions with high inhibitor concentrations and environmental challenges (More et al., 2010). It was shown that *Penicillium corylophilum* and *A. niger* can dominate 95-98% of the microbes in sludge treatment process with inoculum size above 10% (Mannan et al., 2005). In addition, filamentous fungi can form spherical pellets during cultivation, making it easier to separate the fungal biomass from the waste stream (Veiter et al., 2018; Zhang and Zhang, 2016). To the best of our knowledge, filamentous fungi have not been studied for HTLWW treatment.

Owing to the significant variations among HTLWW generated from different feedstocks and HTL operating conditions, it is challenging to compare the treatment performance among the microbes tested in different studies (Table S1). Therefore, this research aims at three objectives: 1) evaluate the impact of pH adjustment and nutrient supplementation on the cultivation of bacteria (*P. putida* and *R. jostii*), yeast (*Rhodotorula sp.*), and filamentous fungi (*A. niger*, *T. versicolor*, and *P. chrysosporium*) strains in HTLWW; 2) compare the overall COD removal and specific compound degradation with different microbial strains; and 3) examine the effect of TAN removal and filamentous fungal pellets on the biodegradation of HTLWW. The findings from this research will guide the selection of microorganisms for the optimization of operating conditions, and the design of the process scheme for treating HTLWW with specific compositions.

2. Materials and Methods

2.1 Materials

2.1.1 HTLWW of sewage sludge

HTLWW used in this study was kindly provided by the Great Lakes Water Authority (GLWA), Detroit, MI, USA. The HTL was conducted at 350°C and 200 atm using sewage sludge as a feedstock with solid content of 26 wt%. The primary conversion reaction happened in a plug flow reactor at a liquid hourly space velocity of 4 L/L/h (Cronin et al., 2022). After HTL, biocrude was separated by extraction, and phosphorus was removed through precipitation. The resultant effluent water (sludge HTLWW) was used in this study.

The HTLWW was pretreated in different ways prior to biological treatment to study the necessity of pretreatments. pH of HTLWW was adjusted to 5, 6, and 7 with 6 M hydrochloric acid. TAN removal was achieved through air stripping by agitating the HTLWW solution overnight at 150 rpm and 50°C after adjusting its pH to 12 with potassium hydroxide. All pretreated and raw HTLWW were sterilized by filtration through a 0.22-μm syringe filter before being used for biological treatment. All chemicals used in the study were purchased from Fisher Scientific (Hampton, NH, USA) unless noted, otherwise.

2.1.2 Microorganisms

P. putida ATCC 31800 was purchased from the American Type Culture Collection. *P. putida* NRRL 14878, and *A. niger* NRRL 2001 were obtained from the Agricultural Research Service Culture Collection (Northern Regional Research Laboratory, U.S. Department of Agriculture). White-rot fungi *T. versicolor* Mad-697 (also known as ATCC 12679) (Mad) and *P. chrysosporium* BKM-F-1767 (BKM) were kindly provided by the U.S. Department of Agriculture Forest Service. *R. jostii* strain RHA1 was a generous gift from Dr. Lindsay D. Eltis (Departments of Microbiology and Biochemistry, University of British Columbia, Vancouver, Canada). A yeast strain was isolated in lab from alkaline liquor with a high phenolic compound

concentration from NaOH treatment of corn stover. The strain was identified through sequencing the ITS region (Psomagen, MD, USA) followed by performing a BLAST search within the NCBI BLAST database (Johnson et al., 2008; Sayers et al., 2022). The isolated strain was shown to closely relate to *Rhodotorula mucilaginosa* and was abbreviated as RHM in the rest of the study (99.83% identity, Fig. S1).

All bacterial and fungal strains were cultivated on tryptic soy agar and potato dextrose agar (Becton Dickinson GmbH, Heidelberg, Germany), respectively, at 25°C, subcultured every month, and stored in a fridge as agar-plate cultures during the use for HTLWW treatment studies. To prepare inocula of bacterial strains, cells were harvested from 2-day-old agar plates, washed 3 times with 0.85% sodium chloride solution before being used to prepare cell suspension with OD_{600} of 0.5 and 1.0 for *P. putida* and *R. jostii* strains, respectively. Inoculum of yeast RHM was prepared with cells harvested from a 3-day-old agar plate that were washed 3 times with sterile water and adjusted to OD_{600} of 1. Two forms of inocula (small and large inocula) were investigated on the filamentous fungi (BKM, NRRL 2001, and Mad) used in this study (Fig. S2). The small inocula were prepared by harvesting the 10-day-old spores of the strain culture. The harvested spores were washed three times with sterile water and made into a suspension with 1×10^7 spores per mL as determined using a Neubauer hemocytometer (Figs. S2a, b). Since Mad did not produce any spore, all fungal cell biomass grown on a 10-day-old agar plate surface (diameter = 90 mm) was harvested, washed three times, and suspended in 10 mL sterile water (Fig. S2c). The large inocula were prepared with the 3-day-old fungal biomass established in potato dextrose broth (Becton Dickinson GmbH, Heidelberg, Germany) (Figs. S2d-f). The fungal biomass was harvested from the medium and washed 5 times with sterile water to remove any residual medium component. The bacterial, yeast, and small fungal inocula were applied at 1%

(v/v) in the subsequent screening and batch experiments, whereas the large fungal inocula were loaded at a concentration of 0.93 ± 0.01 g dry cell mass (DCM) per liter of diluted HTLWW.

2.1.3 Supplementary nutrient stocks

Supplementary nutrients used in the study were developed according to the compositions of Bold's basal medium and minimal medium for fungal spore germination (Ijadpanahsaravi et al., 2021). Concentrated stock solutions of carbon source (C), nitrogen source (N), phosphorus source (P), calcium source (Ca), magnesium source (Mg), and iron source (Fe) were prepared separately at 100X concentration, except for iron stock solution that was prepared at 1000X concentration. The individual stock solutions of C, N, P, Ca, Mg, and Fe contained (per liter): 100 g glucose, 40 g NH_4Cl, 10 g KH_2PO_4, 2.5 g $CaCl_2 \cdot 2H_2O$, 5 g $MgSO_4 \cdot 7H_2O$, and 5 g $FeSO_4 \cdot 7H_2O$, respectively. All the concentrated stocks were sterilized by filtration through a 0.22-μm syringe filter and stored at 4°C until used.

2.2 Cultivation of microorganisms in sludge HTLWW

2.2.1 Screening of cultivation conditions

Cultivation conditions of microorganisms in HTLWW were determined with a series of screening experiments conducted in sterile 12- and 24-well microplates. HTLWW was diluted by 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20X with tryptic soy broth and potato dextrose broth for cultivation of bacterial strains and fungal strains, respectively, to determine the overall inhibitory effect of HTLWW on individual microbes and identify dilution factor required to grow the strains in the wastewater without inhibition. To determine the optimum pH for different strains, the HTLWW pH was adjusted to pH 5, 6, 7, and 8.8 (the original pH) followed by dilution with sterile water and supplementation of all nutrients described in section 2.1.3. Lastly, with the

established dilution factor and pH, the effect of individual supplementary nutrient was evaluated by determining the change of microbial growth after removing one nutrient at a time from the full supplement scenario. All screening experiments were conducted in a humidified incubator operated at 25°C and 150 rpm.

2.2.2 Batch cultivation of microorganisms in HTLWW

Batch cultivation of the microorganisms in HTLWW was performed in 250-mL Erlenmeyer flasks containing 100 mL of diluted wastewater with pH and nutrient supplementation determined in section 2.2.1. Flasks were incubated at 25°C and 150 rpm in a humidified incubator. A control flask of each pH and dilution factor combination was also incubated and was not inoculated with microorganisms. Analytical results on the control served as a baseline for calculating COD and organic compounds removal efficiency by correcting the effect of evaporation during incubation on the composition of HTLWW. An aliquot of 5 mL of homogeneous sample was taken for all flasks at different sampling time points and centrifuged to obtain the supernatant for analysis. On the last day of incubation, flasks were harvested to determine DCM of the fungal cells (day 30). The cell density (OD_{600}) of bacterial strains was continuously monitored throughout incubation for 9-10 days (NRRL 14878 and ATCC 31800) and 15 days (RHA1).

2.2.3 Cultivation of large fungal inocula in HTLWW

Cultivation of large fungal inocula was conducted in 250-mL Erlenmeyer flasks with 100 mL of diluted wastewater at 25°C and 150 rpm, in a humidified incubator. HTLWW for cultivation of the filamentous fungi was adjusted to pH 5, with or without nutrient supplementation as described in section 2.2.1. Once the large fungal inocula were inoculated, flasks were allowed to

192 equilibrate for 2 h before a sample of time "0" was taken to exclude any unspecific adsorption of
193 wastewater components by the fungal biomass. Liquid samples were taken every 3 days for COD
194 determination until the COD change was negligible (day 12). Once the COD stabilized, 85% of
195 the liquid volume was replaced with freshly diluted HTLWW with the same composition as that
196 initially used in this test. This stepwise wastewater feeding was to evaluate continuous
197 performance of the fungal inocula.

198 **2.3 Analytical methods**

199 *2.3.1 Microbial growth analysis*

200 Bacterial growth during screening experiments was measured as OD_{600}, whereas fungal growth
201 was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay
202 as absorbance at 560 nm (Benov, 2019; Cui et al., 2015; Cui et al., 2018a; Liu et al., 2009). MTT
203 reacts with metabolically active cells to develop a purple color that can be solubilized and
204 quantified on a spectrophotometer. Intensity of the purple color is therefore directly related to the
205 concentration of active cells (Fig. S3). Since DCM of fungus cannot be reliably determined for
206 screening experiments conducted in microplates due to the small volume, MTT assay instead of
207 optical density was used. MTT assay was performed immediately at the end of incubation when
208 the tested filamentous fungi can grow into loose pellets consisting of intertwined hyphae,
209 whereas the yeast cells settled down at the bottom of the microplate. In both cases, about three
210 fourths of the supernatant in the microplate can be carefully replaced with potato dextrose broth
211 while leaving the fungal biomass largely intact. Then, MTT solution (5 mg MTT solubilized in 1
212 mL of saline phosphate buffer solution) was added at 50 µL MTT solution per mL of liquid in the
213 well. The microplate was incubated for 4 h at 25°C and 150 rpm in the dark, during which MTT

reacted with active cells to develop purple color. The purple color was solubilized with dimethyl sulfoxide and measured at 560 nm. The absorbance was measured on a microplate reader (BioTek Epoch 2, Agilent Technologies, CA, USA). Fungal DCM at the end of batch experiments was determined by capturing fungal biomass via repeated washing/filtration through a 0.45-μm filter paper and measuring the cell dry weight using oven drying at105°C for overnight.

2.3.2 Chemical analysis

Chemical compound analysis of HTLWW was performed on a gas chromatography-mass spectrometry (GC-MS) (Agilent GC 6890 coupled with a MS 5973, Agilent Technologies, CA, USA) and a high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). Chemical compounds were first identified by GC-MS with match score above 90, after which standards were purchased for major compounds in HTLWW. To quantify the compounds, a five-point standard curve was generated for each compound to cover its concentration in the HTLWW samples. Equations for the standard curves and detection limits of the quantified compounds are listed in Table S2. To quantify N-heterocyclic compounds, 1 mL of sample was adjusted to pH 2-4 with 20 μL of 1 M sulfuric acid and extracted twice with dichloromethane. The dichloromethane extract of 1 μL was then injected into GC-MS with split ratio of 10:1. Total retention time of the analysis was 17.5 min with helium as a carrier gas at 0.5 mL/min. Oven temperature was set at 50°C for 1 min and increased to 200°C at a rate for 20°C/min. After held at 200°C for 2 min, the oven temperature was further increased to 250°C at a rate of 10°C/min and held at the temperature for 2 min. The inlet, MS source, and MS quadrupole analyzer temperatures were set at 180, 230, and 150°C, respectively. The MS detector used electron impact ionization with total ion chromatogram and mass spectrum acquisition started at 3.4 min

to avoid solvent peaks. Spectrum acquisition was set at scan mode to cover mass range between 30 and 250. To quantify phenolic compounds, 5 mL of sample was adjusted to pH below 2 using 200 µL of 1 M sulfuric acid, and 30 µL internal standard (10 mg/mL trans-cinnamic acid-d6) was included into the sample. After the extraction with ethyl acetate twice, the sample extract was concentrated to 1 mL with a RapidVap N_2 Dry Evaporation System (Labconco, MO, USA) and derivatized with MSTFA + 1% TMCS (TS-48915, Thermo Fisher Scientific, MA, USA) at 60°C for 45 min. Following derivatization, 1 µL of sample was analyzed on the GC-MS. The total retention time for MSTFA-derived samples was 20 min with MS acquisition of 5.4 min to avoid solvent peaks. The oven temperature started at 50°C for 1 min, ramped to 150°C at a rate of 20°C/min, held for 2 min, increased to 250°C at 10°C/min, and held for 2 min. All other instrument parameters were the same as the analysis for N-heterocyclic compounds described above. A 30-meter long Zebron ZB-1701 column with 0.25 mm inner diameter and 0.25 µm film thickness (Phenomenex, CA, USA) was used for both N-heterocyclic compound and phenolic compound analyses. Organic acids were quantified using a HPLC equipped with an Aminex HPX-87H column (7.8 × 300 mm, Bio-Rad Laboratories, CA, USA) and a refractive index detector (RID). The sample injection volume was set 20 µL with the column and RID temperatures at 60 and 45°C, respectively. Mobile phase was 5 mM H_2SO_4 and the flow rate was 0.6 mL/min. The retention time for the HPLC analysis was 60 min.

Total organic carbon (TOC), chemical oxygen demand (COD), total nitrogen (TN), TAN, and total phosphorus (TP) were measured with test kits (Hach Company, IA, USA). The inorganic elements, including K, S, Na, Ca, Mg, Fe, and Mn were measured on an inductively coupled plasma optical emission spectroscopy (ICP-OES) (Agilent 5800, Agilent Technologies, CA,

USA). The HTLWW pH was measured using a pH meter (Accumet AE150, Fisher Scientific, NH, USA).

2.3.3 Adsorption assay

An adsorption assay was performed to determine if any COD removal resulted from the adsorption of pollutants onto microbial cell biomass. The adsorption assay was performed as described in a previous study (Ji et al., 2023). Specifically, the highest amount of microbial biomass achieved by each microbe during cultivation in HTLWW was inoculated into diluted HTLWW (10X for bacteria and 20X for fungi) that was used for their cultivation. The microbial biomass used for inoculation was freshly harvested from culture medium and was thoroughly washed as described in section 2.1.2. Upon inoculation of microbial biomass into HTLWW, the reactor was sealed and subjected to continuous nitrogen purging for 4 h, as shown in Fig. S4. Since all the microbes used in this study are obligate aerobes, the lack of oxygen in the reactors can deactivate the microbes. The adsorption assay was performed in duplicates with a pair of controls for HTLWW of each dilution factor that served as the baseline without any inoculation of microbial biomass. Samples were taken at 2 and 4 h to determine the COD.

2.4. Data analysis

One-way ANOVA test was used to determine overall statistical significance among COD removal of different treatments with $p = 0.05$, after which a multiple comparison with Tukey-Kramer HSD test was conducted to identify any significant differences between pairs of treatments ($\alpha = 0.05$) (Cui et al., 2018b). JMP Pro 16 software (SAS Institute, Cary, NC, USA) was used for all the statistical analyses.

3. Results and Discussion

3.1 Characteristics of Sludge HTLWW

Table 1 shows the characteristics of HTLWW used in this study. Inorganic elements K, S, and Na were present in the wastewater at 172.3, 156.4, and 34.5 ppm respectively, whereas Ca, Fe, Mg, and Mn were found at less than 1 ppm. This is consistent with the literature that K and Na in feedstock were transferred into the aqueous phase during HTL, whereas Ca, Mg, and Fe were accumulated in the solid residue (Lu et al., 2022). Since sewage sludge has high S content, the element likely migrates to HTLWW in the form of sulfate (Li et al., 2022; Shah et al., 2020). TOC and TN of HTLWW were 20.3 and 9.41 g/L respectively, giving a C/N ratio of 2.16. COD was 72.1 g/L, which is comparable to other HTLWW generated from sewage sludge (Watson et al., 2020). Around 45% of the TN were in the form of TAN (4.27 g/L). The low C/N ratio is responsible for the high pH (8.8) of HTLWW and is likely due to the low carbohydrate to protein ratio of the sewage sludge (Basar et al., 2023). The low TP value of HTLWW is probably due to the phosphorus precipitation conducted after the HTL process.

Table 1. Inorganic element concentration and water quality parameters of HTLWW

Inorganic element	Concentration (ppm)	Water quality parameter	Concentration
K	172.3	TOC	20.3 (g/L)
S	156.4	COD	72.1 (g/L)
Na	34.5	TN	9.41 (g/L)
Ca	< 1	TAN	4.27 (g/L)
Fe	< 1	TP	< 1 (ppm)
Mg	< 1	pH	8.80

Table 2 shows the concentration of major organic compounds in HTLWW. We were able to quantify eleven organic acids, four N-heterocyclic compounds, and four phenolic compounds. The quantified compounds account for around 50% of the TOC in HTLWW. The uncovered TOC

could be in the form of high molecular weight polymeric compounds, such as humic acids, which cannot be detected by HPLC or GC-MS. Sewage sludge is known to contain high humic acids that are difficult to degrade at HTL temperature due to the condensed structures (Gong et al., 2017). Therefore, some of the undegraded humic acid may end up in HTLWW. Another fourteen compounds in HTLWW were also identified in the HTLWW but were not quantified due to their low concentration, unavailability of proper standards, or inability to generate satisfactory standard curves (Table S3). The major compounds identified in HTLWW were similar to those in the literature for HTLWW generated from sewage sludge (Basar et al., 2023; Kapusta, 2018; Maddi et al., 2017). Specifically, HTLWW was reported to have around 3.2 g/L acetic acid, 0.56 g/L propanoic acid, 0.2 g/L pyrazine, 0.26 g/L methyl pyrazine, and 0.29 g/L pyrrolidinone (Maddi et al., 2017). In the HTLWW used in this study, acetic acid (9.04 g/L) was the most abundant compound in HTLWW, followed by propionic acid (3.43 g/L), 2-methylbutyric acid (1.24 g/L), and valeric acid (1.01 g/L). N-heterocyclic compounds, such as 2-piperidinone (1.45 g/L), methyl pyrazine (1.10 g/L), and 2-pyrrolidinone (1.01 g/L) were also shown to be in considerable concentrations. On the other hand, phenolic compounds were less abundant in HTLWW of which benzenepropanoic acid (0.79 g/L) and phoretic acids (0.38 g/L) were phenolic compounds with higher concentrations. The higher concentration of the organic acids and N-heterocyclic compounds in the HTLWW in this study could be due to the higher solid loading (26 wt%) and spacy velocity (4 L/L/h) used in HTL compared with the study by Maddi et al. (8-11.5 wt% and 1.2-2.1 L/L/h, respectively).

Table 2. Major organic compounds identified in HTLWW[a]

Organic acid	Concentration (g/L)	N-heterocyclic compound	Concentration (g/L)
acetic acid	9.04±0.02	2-piperidinone	1.45±0.03
propionic acid	3.43±0.08	methyl pyrazine	1.10±0.02
2-methylbutyric acid	1.24±0.01	2-pyrrolidinone	1.01±0.03
valeric acid	1.01±0.04	pyrazine	0.40±0.03
4-methylvaleric acid	0.93±0.02	**Total quantified N-heterocycles**	3.96±0.08
butyric acid	0.84±0.01		
formic acid	0.60±0.02	**Phenolic compound**	**Concentration (g/L)**
hexanoic acid	0.31±0.02	benzenepropanoic acid	0.79±0.03
succinic acid	0.20±0.02	phloretic acid	0.38±0.02
malic acid	0.18±0.01	p-cresol	0.18±0.00
glycolic acid	0.11±0.00	tyrosol	0.04±0.00
Total quantified organic acids	17.88±0.14	**Total quantified phenolic compounds**	1.39±0.05

[a]Data are presented as mean ± standard deviation of triplicate measurements.

3.2 Cultivation of microbes in HTLWW under screened conditions

3.2.1 Effect of dilution factor on microbial growth

The growth of bacteria and fungi in HTLWW diluted with commercial culture media is illustrated in Figs. 1a and b, respectively. The commercial culture media provided favorable nutrients conditions for the microbes and provided baselines for examination of the HTLWW toxicity on the tested strains. The bacteria grew well in HTLWW diluted more than 8X while the growth was significantly hampered at lower dilution factors for the substrate limitation (Fig. 1a). The fungi required higher dilution factor than the bacterial strains. In addition, the tolerance of fungi to HTLWW varied greatly. Specifically, NRRL 2001, RHM, BKM, and Mad could tolerate dilution factors of 8, 12, 18, and 20, respectively, without noticeable inhibition (Fig. 1b). Based on the results, HTLWW was diluted 10X for bacterial cultivation and 20X for fungal cultivation in the rest of the study.

3.2.2 Effect of pH on microbial growth

Figs. 1c and d show the growth of bacterial and fungal strains in HTLWW at different pH levels, respectively. Ultrapure water was used to dilute the wastewater and full nutrients were supplemented as described in section 2.1.3. Nutrient supplementation did not significantly affect pH of the diluted HTLWW. The bacteria showed a consistent growth in HTLWW without pH adjustment (pH 8.8) and adjusted pH (pH 6 and 7). However, HTLWW adjusted to pH 5 greatly inhibited the bacterial growth. On the contrary, the fungi preferred pH 5 with growth significantly inhibited in HTLWW at the unadjusted pH and pH 7. This result was expected since fungi are known to prefer acidic pH for growth whereas bacteria usually grow better at higher pH (Rousk et al., 2010; Sankaran et al., 2010). Therefore, pH adjustment could be necessary for successful biological treatment, depending on the pH of HTLWW. In this study, HTLWW with the original pH and adjusted pH 5 were used in the rest of the study for bacterial and fungal cultivation, respectively.

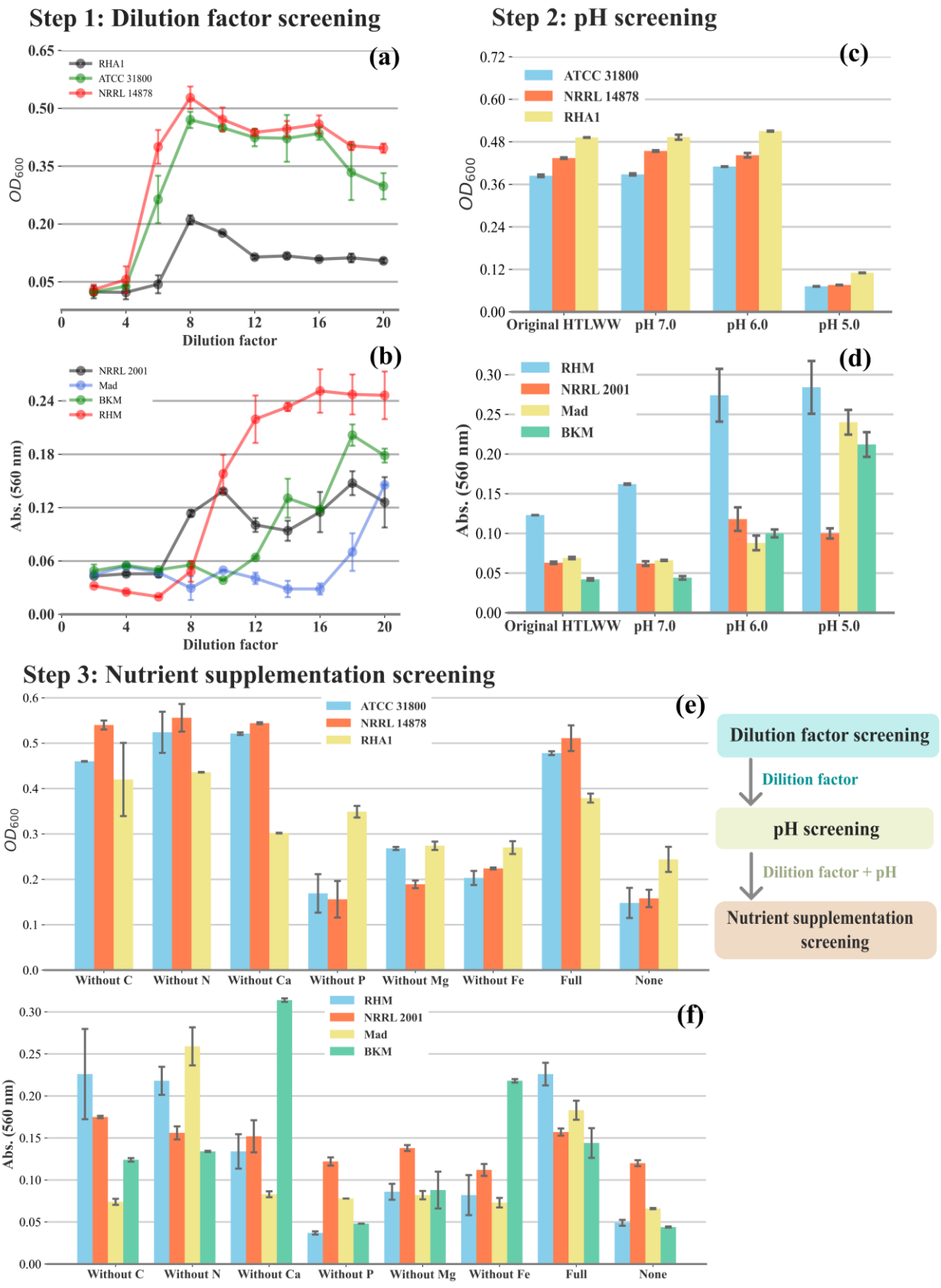
Step 1: Dilution factor screening
(a)
RHA1
ATCC 31800
NRRL 14878
OD600
Dilution factor
(b)
NRRL 2001
Mad
BKM
RHM
Abs. (560 nm)
Dilution factor
Step 2: pH screening
(c)
ATCC 31800
NRRL 14878
RHA1
OD600
Original HTLWW
pH 7.0
pH 6.0
pH 5.0
(d)
RHM
NRRL 2001
Mad
BKM
Abs. (560 nm)
Original HTLWW
pH 7.0
pH 6.0
pH 5.0
Step 3: Nutrient supplementation screening
(e)
ATCC 31800
NRRL 14878
RHA1
OD600
Without C
Without N
Without Ca
Without P
Without Mg
Without Fe
Full
None
(f)
RHM
NRRL 2001
Mad
BKM
Abs. (560 nm)
Without C
Without N
Without Ca
Without P
Without Mg
Without Fe
Full
None
Dilution factor screening
Dilition factor
pH screening
Dilition factor + pH
Nutrient supplementation screening

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Fig. 1. Growth of the tested microbes under screened HTLWW dilution factor (**a, b**), pH (**c, d**), and nutrient supplementation (**e, f**). Bacterial growth was quantified by measuring OD_{600} on day 3 of the incubation. Fungal growth was quantified using MTT assay as the absorbance (Abs.) at 560 nm on day 4 of the incubation. All the screening experiments were performed in duplicates.

3.2.3 Effect of nutrient supplementation on microbial growth

Nutrient supplementation showed different impact on the growth of microbial strains in HTLWW (Figs. 1e and f). P supplementation was required by all tested microbes in the study because the removal of P significantly impaired the growth of the microbes. Fe and Mg were also important nutrients for the tested strains, with six out of seven strains requiring Fe and/or Mg supplementation. P, Fe, and Mg are elements required for key biological processes, such as making genetic materials and serving as cofactors of essential enzymes (Sandy and Butler, 2009; Walker, 1994). The screening result suggested that P, Fe, and Mg concentrations in HTLWW were too low to support the normal microbial growth. Ca supplementation was also shown to be required for better growth of RHA1, RHM, and Mad. In contrast, supplementing additional N source did not improve the microbial growth, which was expected due to the high TAN concentration in the HTLWW (Table 1). Supplementing glucose (C source) was shown to be essential for Mad growth in HTLWW. Similar finding was also reported in *Saccharomyces cerevisiae* cultivation in HTLWW from HTL of microalgae, *Nannochloropsis oculate*, where glucose supplementation was required for the growth of the yeast in HTLWW (Nelson et al., 2013). To better evaluate the removal efficiency of HTLWW-originated COD, supplementation of glucose was avoided in the biological treatment of HTLWW. In summary, the screening results above suggested the following optimal conditions (Table 3) for cultivation of the selected microbes in HTLWW in this study.

Table 3. Dilution factor, pH, and nutrient supplementation for microbial cultivation in HTLWW

Microorganisms	Dilution factor	pH	Nutrient supplementation
Bacterial strains			
NRRL 14878	10	Original (pH 8.8)	Fe, Mg, P
ATCC 31800			Fe, Mg, P
RHA1			Fe, Mg, Ca, P
Fungal strains			
BKM	20	5.0	Mg, P
NRRL 2001			Fe, P
RHM			Fe, Mg, Ca, P
Mad			Fe, Mg, Ca, P

3.3 Growth and COD removal with batch microbial cultivation

Table 4 lists the removal efficiency of quantifiable (on GC-MS) organic acids during cultivation of the tested microbes under conditions listed in Table 3, which was used to determine the end point of biological treatment by each strain. After 2 days of cultivation, organic acid removal by *P. putida* ATCC 31800 and NRRL 14878 plateaued at 82.7 and 85.3%, respectively. This is likely due to the quick growth of *P. putida* strain in HTLWW with OD_{600} reached maximum on day 2 (Fig. 2a). Similarly, the growth of *R. jostii* RHA1 mostly happened on the first 3 days but showed some fluctuation afterwards (Fig. 2b). The removal efficiency of organic acids by RHA1 remained low throughout the 15-day cultivation period (14-19%, Table 4). Fungal strains required longer time for treatment of HTLWW, as suggested by the continuously increased organic acids removal efficiency throughout the 30-day incubation period (Table 4). The cultivation of fungal strains was stopped on day 30 due to negligible change of the organic acid removal efficiency between day 20 and 30 (Table 4).

Table 4. Removal efficiencies of quantified organic acids (via HPLC) at different time points of batch cultivation by tested microbes

Microorganisms	Removal efficiency (%)		
	Day 10	**Day 20**	**Day 30**
BKM	65.3±1.7	70.8±0.9	72.5±1.2
Mad	33.5±1.1	68.3±1.9	69.3±0.6
NRRL 2001	63.3±3.5	65.0±4.5	71.5±1.7
RHM	69.8±0.4	72.9±0.7	79.7±2.7
	Day 1	**Day 2**	**Day 3**
ATCC 31800	74.5±2.0	82.5±0.7	82.7±1.7
NRRL 14878	83.4±0.5	85.7±0.6	85.3±1.0
	Day 6	**Day12**	**Day 15**
RHA1	14.2±4.1	17.1±1.9	19.1±2.6

Data are presented as mean ± standard deviation (n=3).

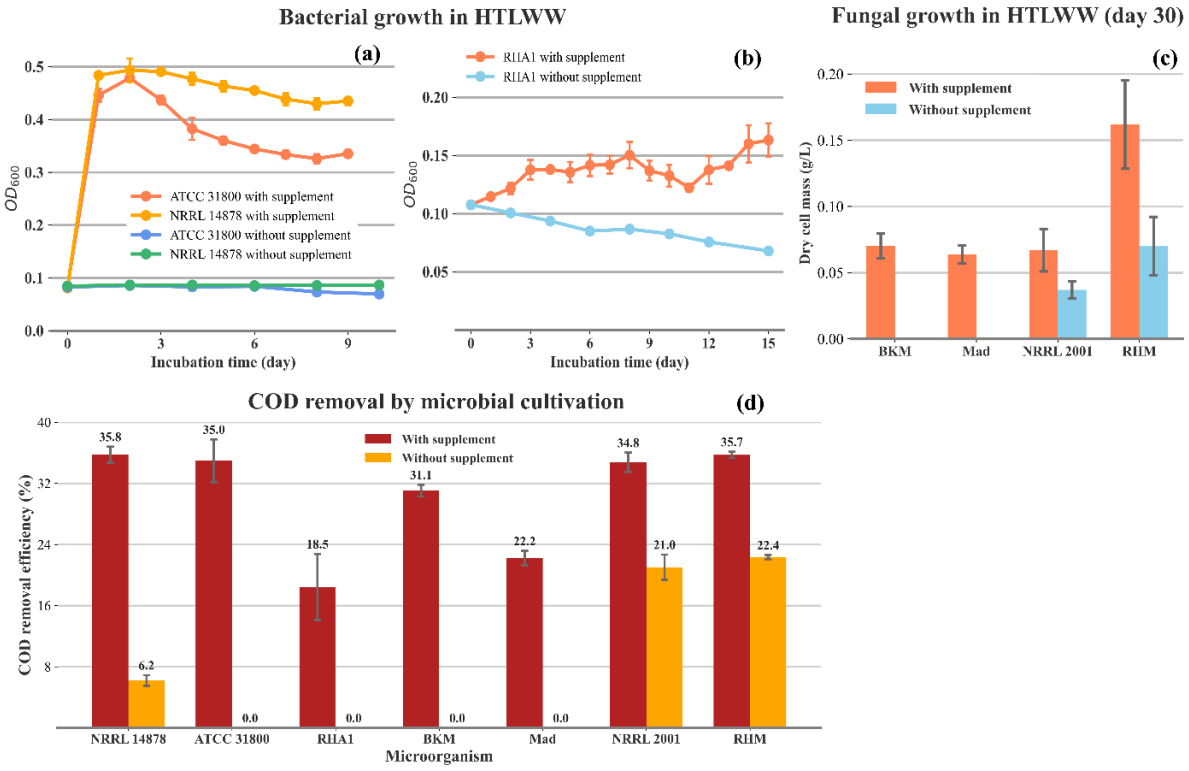


Fig. 2. Growth of the tested microbes in HTLWW with and without nutrient supplementation (**a-c**) and wastewater COD removal efficiency after the microbial cultivation (**d**). If the COD values at the end of cultivation are not significantly different from the untreated HTLWW as determined by Tukey-Kramer HSD test at α=0.05, removal efficiencies are shown as 0.0% on the graph (**d**). Data represent the average and standard deviation of triplicate analysis.

Nutrient supplementation may increase the cost and complexity of wastewater treatment. Therefore, we also evaluated biodegradation of HTLWW without nutrient supplementation. The growth of bacterial and fungal strains during batch cultivation in HTLWW with and without supplementary nutrients were shown in Figs. 2a-c. In general, supplementing nutrients significantly increased the growth of microbes, which confirmed the findings of screening experiments (Section 3.2). Specifically, 2 out of 4 tested fungal strains (BKM and Mad) and all tested bacterial strains (ATCC 31800, NRRL 14878, and RHA1) were unable to grow without nutrient supplementation and failed to remove any COD, whereas apparent growth of all the tested microbes was observed when nutrients were supplemented as described in Table 3. As shown in Fig. 2d, significantly higher COD removal efficiencies were achieved at the end of HTLWW biodegradation with nutrient supplementation as compared to those without additional nutrients ($p<0.05$, Table S4). The highest COD removal efficiency was achieved by NRRL 14878 (35.8%), followed by RHM (35.7%), ATCC 31800 (35%), NRRL 2001 (34.8%), and BKM (31.1%) in HTLWW with supplemented nutrients. The COD removal efficiencies of Mad (22.2%) and RHA1 (18.5%) were significantly lower even with nutrient supplementation ($p<0.05$, Table S4). Interestingly, NRRL 2001 and RHM were able to remove 21% and 22.4% COD, respectively, without nutrient supplementation, which were much higher than those achieved by other microbes under the same conditions ($p<0.05$, Table S4). Our results suggest that supplementation of Fe, Mg, Ca, and P when HTLWW lacks these minerals can greatly increase the COD removal efficiency by biodegradation. Although the 15.8-35.8% COD removal achieved with nutrient supplementation by the tested microbes is comparable to other biodegradation studies (Table S1), this COD removal efficiency is still too low and additional treatment steps (e.g., adsorption and ozone oxidation) may be needed.

3.4 COD removal with large fungal inocula

Unlike bacteria and yeasts, filamentous fungi can aggregate to form pellets of mm-to-cm in diameter. The pellets are formed due to coagulation of germ tubes and hyphae that grow from fungal spores or small pieces of hyphae (Grimm et al., 2004; Wittmann and Krull, 2010). All the filamentous fungi tested in this study (BKM, Mad, and NRRL 2001) were able to form spherical pellets when grown in potato dextrose broth (Fig. S2). Therefore, we also evaluated the performance of the fungal pellets for HTLWW treatment. To distinguish between fungal spore/hyphae inocula used in previous sections and the fungal pellet inocula, they were termed small inocula and large inocula respectively.

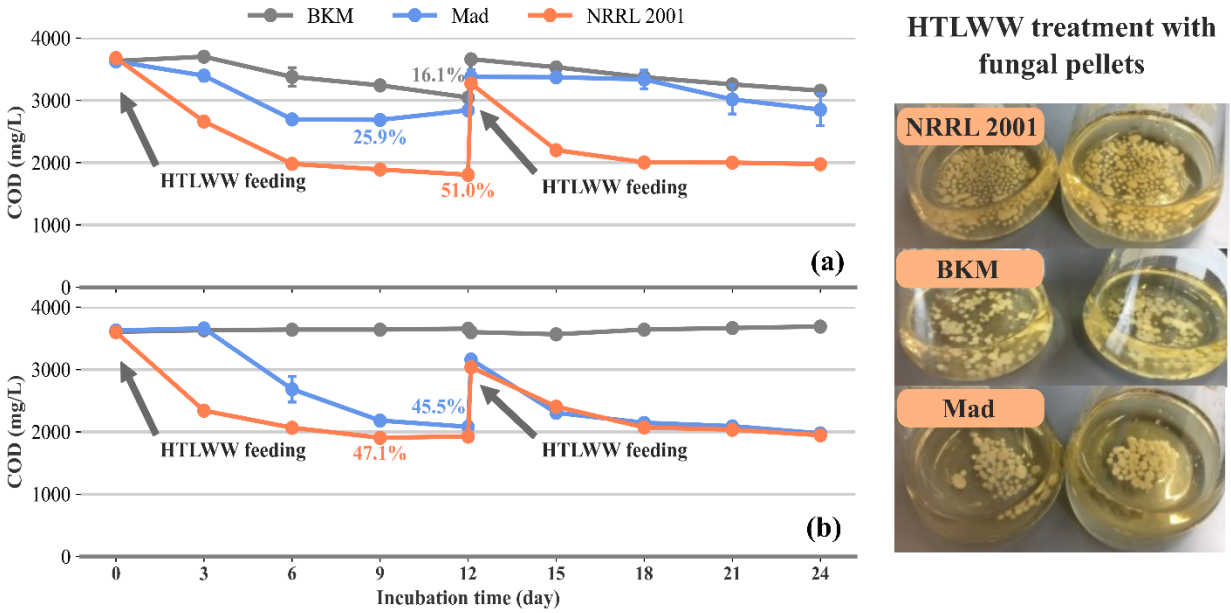


Fig. 3. COD variation of HTLWW during treatment with large inocula of BKM, Mad, and NRRL 2001 with (**a**) and without (**b**) nutrients supplementation (n = 2). The highest COD removal efficiency prior to feeding additional HTLWW on day 12 was labeled.

Fig. 3 illustrates the COD removal during cultivation of large fungal inocula in HTLWW with and without supplementing nutrients. On day 12, the COD removal efficiencies of most tested

426 treatments were comparable to that on day 9. Afterwards, additional HTLWW was fed as
427 described in section 2.2.3 on day 12, and COD was monitored for another 12 days. For Mad and
428 NRRL 2001, large inocula for HTLWW treatment significantly increased the COD removal
429 efficiency compared with that of small inocula even without nutrient supplementation ($p<0.05$,
430 Table S5). In addition, the higher COD removal efficiency was achieved in 12 days of
431 incubation, which was much shorter than the 30-day incubation time needed for small inocula
432 (Tables 4 and S5). The result suggested that nutrient supplementation was no longer necessary
433 when large inocula of Mad and NRRL 2001 were used for HTLWW treatment, since the two
434 strains achieved similar or higher COD removal efficiency without nutrient supplementation
435 compared with the nutrient addition (Table S5). Specifically, the highest COD removal efficiency
436 by Mad with and without supplementing nutrients were 25.9% and 45.5%, respectively (Figs. 3a
437 and b). For NRRL 2001, the highest COD removal efficiency with and without nutrients were
438 51% and 47.1%, respectively. After replacing 85% of the liquid volume in flask with freshly
439 diluted HTLWW on day 12, the large inocula of Mad and NRRL 2001 continuously reduced
440 COD of HTLWW without any nutrient supplementation (Fig. 3b). On day 24, the COD of
441 HTLWW treated with Mad and NRRL 2001 were comparable to that on day 12 prior to feeding
442 fresh HTLWW. Therefore, it appears that that large inocula of Mad and NRRL 2001 can be
443 continuously used for HTLWW treatment without the need of additional nutrient addition. On
444 the contrary, BKM large inocula only achieved a COD removal efficiency of 16.1% with nutrient
445 supplementation (Fig. 3a), which was significantly lower than the 31.3% COD removal when
446 small inocula was used ($p<0.05$, Table S5). Without nutrient supplementation, BKM was not able
447 to remove COD at all (Fig. 3b). The lower COD removal efficiency by BKM large inocula
448 compared with the small inocula could be due to the shorter incubation time or different

449 metabolic states of the small and large fungal inocula (Tegelaar et al., 2020). It is well-accepted
450 that the macromorphological structure of production fungal strains greatly affects the yield of
451 bioprocess (Tegelaar et al., 2020; Veiter et al., 2018). However, no simple relationship has been
452 identified that could describe the connection between the morphology of filamentous fungi and
453 their productivity (Veiter et al., 2018). In agreement with this, our study also suggested that the
454 biodegradation effectiveness of fungal spores and pellets when cultivated in HTLWW is strain
455 dependent.

456 The different performance of small and large fungal inocula during HTLWW treatment with and
457 without nutrient supplementation suggested that the nutrient requirement for small and large
458 fungal inocula are different. Specifically, sporulation and hyphae growth of the small fungal
459 inocula may require higher levels of nutrients, such as P, Mg, Ca, and Fe, which were deficient in
460 HTLWW (Table 1). Therefore, supplementation of these nutrients greatly benefited the HTLWW
461 treatment performance of the small fungal inocula. However, the growth of small inocula in
462 HTLWW were too slow for effective treatment of HTLWW even with nutrient supplementation,
463 resulting in lower COD removal efficiencies after prolonged incubation (Fig. 2d). On the other
464 hand, the large fungal inocula was well developed fungal biomass that may not require the
465 presence of P, Mg, Ca, and Fe at high concentrations. Therefore, the large inocula can be
466 continuously used for HTLWW treatment without nutrient supplementation. In addition, since
467 the growth of bacterial and yeast strains tested in this study were shown to heavily rely on the
468 addition of P, Mg, and Fe, it will be less likely for them to outcompete the large inocula of Mad
469 and NRRL 2001 when these nutrients are not supplemented, suggesting that large fungal inocula
470 may not be prone to contamination. Further research is needed to study the behavior of large
471 fungal inocula during HTLWW treatment, such as how long the large fungal inocula can be used

for effective removal of COD without nutrient supplementation and whether the large fungal inocula can tolerate less diluted and unsterilized HTLWW. A sequential batch reactor with selective settling velocity has been used to perform aerobic granulation of single culture protist which was able to retain microbial granules of larger particle size (An et al., 2021). Similar reactor setting could be employed to further study the morphology and biodegradation performance of the long-term fungal cultivation at different HTLWW feeding rates. Unlike microalga cultivation and AD that have well-known benefits of reducing carbon emission or producing biogas during biodegradation of HTLWW, value-added products of fungus cultivation remain to be identified. It is of interest in future studies to identify any platform fungal-originated chemicals, enzymes, and biomass generated during fungal treatment of HTLWW to provide economic incentives for the process. Nevertheless, our findings demonstrate that using large inocula of filamentous fungus for HTLWW treatment has some attractive benefits over bacterium and yeast, including higher COD removal efficiency, with no need of nutrient supplementation and easier separation of the fungal biomass from treated HTLWW.

3.5 Adsorption of COD by microorganisms

COD removal efficiency due to adsorption by microbial biomass is shown in Table 5. The percentage of COD removal efficiency due to adsorption of pollutants onto microbial biomass was below 2% for all the tested microbes after incubation for 2 and 4 h. No statistically significant difference was identified between the COD values of HTLWW after adsorption assay and their corresponding controls. HPLC and GC-MS chromatograms of the HTLWW samples after adsorption assay also showed no decrease of peak area for each identified chemical compound as compared with the controls (data not shown). This result suggests that COD

adsorption to microbial biomass is neglectable and all the COD and chemical compound removal during biological treatment of HTLWW was due to biodegradation.

Table 5. COD removal efficiencies due to microbial biomass adsorption

Microorganism	Inoculated biomass density	Incubation time (h)	COD removal efficiency (%)
ATCC 31800	OD_{600}=0.5	2	-1.52±0.68
ATCC 31800	OD_{600}=0.5	4	0.47±0.22
NRRL 14878	OD_{600}=0.5	2	-0.88±0.23
NRRL 14878	OD_{600}=0.5	4	-1.17±0.55
RHA1	OD_{600}=0.2	2	-2.00±0.68
RHA1	OD_{600}=0.2	4	-1.25±0.88
BKM	1 g/L dry cell mass	2	0.48±1.36
BKM	1 g/L dry cell mass	4	1.72±0.44
Mad	1 g/L dry cell mass	2	0.64±1.59
Mad	1 g/L dry cell mass	4	0.78±0.89
NRRL 2001	1 g/L dry cell mass	2	-0.32±1.14
NRRL 2001	1 g/L dry cell mass	4	-0.47±0.89
RHM	0.2 g/L dry cell mass	2	-1.77±0.91
RHM	0.2 g/L dry cell mass	4	0.31±0.66

Data are presented as mean ± standard deviation (n=2)

3.6 Biodegradation of chemical compounds

Besides COD removal, the removal of different chemical compounds in HTLWW by the tested microbes was also monitored (Fig. 4). Since large inocula of NRRL 2001 and Mad were better in COD removal even without nutrient supplementation, chemical compound degradation results of the large inocula cultures instead of the small inocula cultures were plotted in Fig. 4 for NRRL 2001 and Mad. Apart from RHA1, all other strains were able to remove the majority of acetic acid and propionic acid, which represented around 70% of the quantified organic acids in HTLWW (Table 2, Fig. 4a). Valeric acid, 2-methlybutyric acid, 4-methylvaleric acid, and butyric acid were also organic acids of high concentration in HTLWW (Table 2). The removal of these acids was strain dependent, with the best removal achieved by large inocula of NRRL 2001 and

Mad which completely removed 2-methlybutyric acid, 4-methylvaleric acid, and butyric acid (Fig. 4a). Benzenepropanoic acid and phloretic acid are the two major phenolic compounds in HTLWW (Table 2). As shown in Fig. 4a, both were well removed by all the tested fungal strains and the bacterial strain RHA1. However, *P. putida* NRRL 14878 and ATCC 31800 lacked the ability to degrade these two phenolic compounds. *P. putida* and *P. chrysosporium* are known to degrade N-heterocyclic compounds (Padoley et al., 2008; Singh et al., 2020). However, in this study, N-heterocyclic compounds were removed poorly by all the tested microbes, with removal efficiency less than 30% in most cases (Fig. 4a). This could be due to the high concentration of these compounds in HTLWW (Table 2) such that they were not effectively removed by the tested microbes. N-heterocyclic compounds were shown to have higher mammalian cell cytotoxicity but only 30% of toxicity can be removed by algal cultivation (Pham et al., 2013b). Therefore, it is of importance to develop microorganisms and treatment processes that can better remove N-heterocycles and/or remove these compounds with pysico-chemcial methods such as zeolite adsorption (Li et al., 2019) and ozone treatment (Yang et al., 2018b).

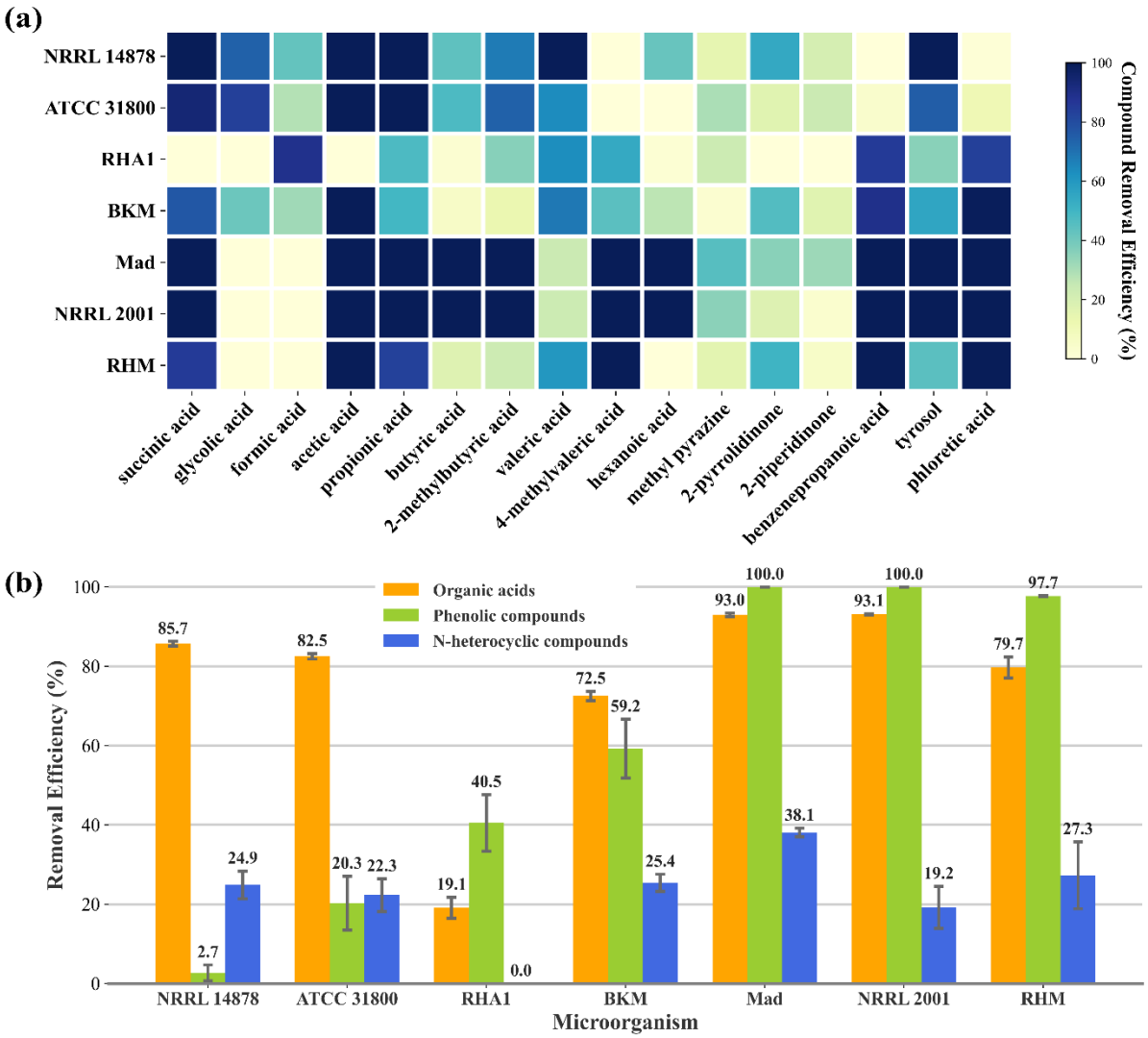


Fig. 4. The removal efficiencies of individual compounds (**a**) and compounds in the categories of organic acids, phenolic compounds, and N-heterocyclic compounds (**b**) in HTLWW through biodegradation with different microbes (n=3 for all microbes, except for Mad and NRRL 2001 that have n=2).

The removal efficiencies of compounds under total organic acid, phenolic, and N-heterocycle categories were shown in Fig. 4b. *P. putida* ATCC 31800 and NRRL 14878 degraded over 80% of quantified organic acids. However, both strains removed only 20.3% and 2.7% phenolic compounds, respectively, which were lower than that achieved by RHA1 and the tested fungal strains that can degrade 59-100% of the quantified phenolic compounds. ATCC 31800 is a strain

532 isolated from textile wastewater and is known to degrade phenol (Annadurai et al., 2002; Juang
533 and Tseng, 2010). In accordance with the literature, ATCC 31800 were able to degrade phenol
534 present in HTLWW (Table S3) (Annadurai et al., 2002; Juang and Tseng, 2010). In addition,
535 ATCC 31800 also effectively removed other phenolic compounds, including tyrosol, p-cresol,
536 and benzyl alcohol. NRRL 14878 is an isolate from municipal sewage sludge (Maryland, U.S.).
537 The compound removal profile of this strain was similar to ATCC 31800 (Fig. 4a), except that
538 the strain was able to remove hexanoic acid and benzeneacetic acid but not *p*-cresol and benzyl
539 alcohol (Fig. 4a, Table S3). Our results suggested that *P. putida* strains ATCC 31800 and NRRL
540 14878 can only degrade a small fraction of the phenolic compounds in HTLWW. The bacterial
541 strain RHA1 has been used for degrading HTLWW from algae and pine wood, during which the
542 strain removed over 90% of COD and most of the organic compounds (He et al., 2017). In
543 addition, genetic and biochemical studies have demonstrated that RHA1 can degrade a variety of
544 phenolic compounds including biphenyls, aromatic acids, and lignin (Yam et al., 2011).
545 However, when cultivated in HTLWW, RHA1 removed only 19.1% of organic acids, 40.5% of
546 phenolic compounds, undetectable percentage of N-heterocyclic compounds, and 18.5% of COD
547 (Figs. 2d and 4b). The significantly lower COD removal efficiency in our study by RHA1 as
548 compared with the previous study suggested that some inhibitors (e.g., TAN) might be present in
549 HTLWW that greatly reduced the performance of RHA1. Therefore, in the next section, we
550 examined the effect of TAN removal from HTLWW on its biological treatment with RHA1.

551 Compared with the bacterial strains, fungal stains were better at removing phenolic compounds,
552 with BKM having the lowest removal efficiency, 59.2%, and all other fungal strains having
553 removal efficiencies above 97% (Fig. 4b). In addition, all the fungal strain effectively removed
554 phenylpropanamide, which failed to be removed by the bacterial strains (Table S3). The better

555 degradation of phenolic compounds by fungal strains is likely due to the non-specificity of
556 fungal enzymes which allow the microbes to target a wider variety of compounds (Naghdi et al.,
557 2018). Among the tested fungal strains, RHM is closely related to *Rhodotorula mucilaginosa.*
558 Yeast isolates identified with close relationship with *R. mucilaginosa* have been used to treat
559 olive mill wastewater that is high in phenolic compound toxicity and were known to produce
560 lipids and biosurfactants as value-added products (Derguine-Mecheri et al., 2021; Jiru et al.,
561 2016). Both NRRL 2001 and Mad large inocula were able to remove over 93% of the organic
562 acids and all the phenolic compounds quantified in this study, making them the most effective
563 strains for treatment of HTLWW in this study (Fig. 4b). *A. niger* NRRL 2001 had been used for
564 production of citric acid from agro-industrial waste (Dhillon et al., 2013). In addition, the strain
565 was better than other organic acid-producing *A. niger* strains (NRRL 3122 and NRRL 567) for
566 bioleaching of loblolly pine, suggesting that NRRL 2001 is less dependent on nutritious
567 substrates for growth and can better tolerate inhibitory phenolic compounds (Liu et al., 2023).
568 White-rot fungi (e.g., *T. versicolor* and *P. chrysosporium*) are known to degrade a wide range of
569 pollutants, such as PAHs, pharmaceutical waste, and synthetic dyes (Gao et al., 2010). Between
570 the two white-rot fungi examined in this study, BKM had stronger tolerance to HTLWW than
571 Mad (Fig. 1b). However, the strain may require supplementation of Mg and P to achieve
572 satisfactory bioremediation, especially if the feedstock is deficient in them (Figs. 2d and 3). On
573 the other hand, when established fungal pellets (large inocula) of Mad were used for treatment of
574 HTLWW, the fungal biomass can effectively and continuously degrade a variety of organic acids
575 and phenolic compounds without the need of supplementary nutrients (Figs. 3 and 4). Similarly,
576 immobilized biomass of *T. versicolor* on sorghum grains have been used for treatment of

577 wastewater rich in humic acids and was able to continuously remove color for four batches
578 (Zahmatkesh et al., 2018).

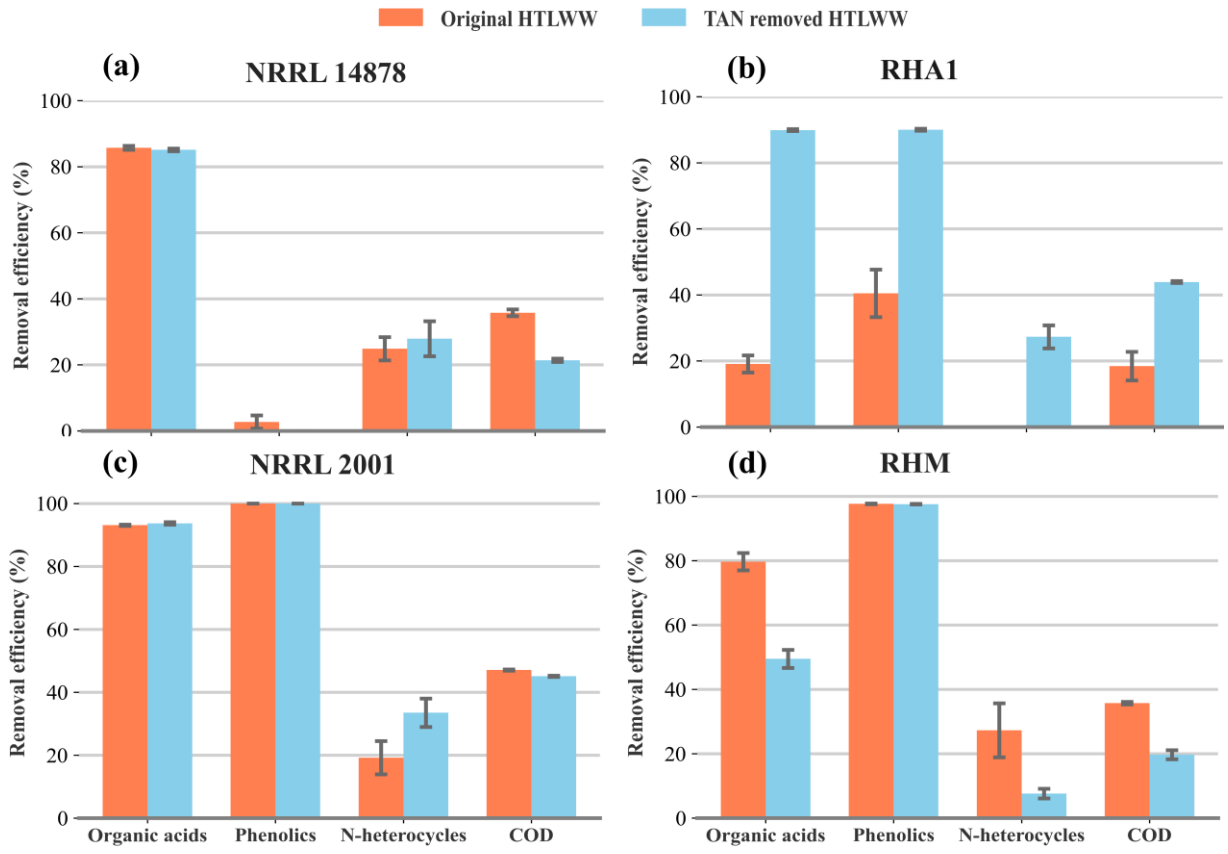
579

580 **3.7 Effect of TAN removal on biological treatment of HTLWW**

581 TAN is a well-known inhibitor of AD and was shown to inhibit the formation and metabolism of
582 aerobic biofilm (Weng et al., 2022; Yenigün and Demirel, 2013). Ammonium salts inhibited the
583 growth of some fungi but stimulated the growth of others (DePasquale and Montville, 1990;
584 Veverka et al., 2007). In this study, TAN removal was conducted by stripping the HTLWW at pH
585 12 and 50°C overnight until TAN of HTLWW was reduced to undetectable level (Table 6). The
586 TAN stripping was also shown to remove volatile organic and inorganic contaminants, which led
587 to COD reduction (Poveda et al., 2016; Toth and Mizsey, 2015). In this study, the COD of
588 HTLWW was reduced by 20.1% along with the TAN removal (Table 6).

Table 6. Water quality parameters before and after TAN stripping

Parameter	Before treatment (g/L)	After treatment (g/L)
COD	72.1	57.6
TN	9.48	4.67
TAN	4.27	< 0.02



589

590 **Fig. 5.** Removal efficiencies of organic acids, phenolic compounds, N-heterocyclic compounds, and COD by NRRL
591 14878 (**a**), RHA1 (**b**), NRRL 2001 (**c**), and RHM (**d**) when cultivated in original and TAN removed HTLWW. n=3
592 for organic acids, N-heterocycles, and COD removal data for NRRL 14878, RHA1, and RHM, and n=2 for all other
593 data.

594 The organic compound and COD removal efficiencies upon biological treatment of HTLWW
595 with and without TAN removal are shown in Fig. 5. TAN stripping increased the removal
596 efficiency of N-heterocyclic compounds by NRRL 14878 and NRRL 2001 but did not improve
597 the removal efficiency of organic acids and phenolic compounds (Figs. 5a and c). Specifically,
598 TAN stripping increased the removal of 2-pyrrolidinone for both strains and increased the
599 removal of methyl pyrazine and 2-piperidione for NRRL 2001 only (Fig. S5). TAN stripping
600 improved the treatment performance of RHA1 greatly, with the removal efficiencies of organic
601 acids, phenolics, and N-heterocycles, and COD being increased by 4.7, 2.2, 9.1, and 2.4 folds,

602 respectively (Fig. 5b). With the TAN removal, RHA1 was able to completely degrade acetic acid,
603 2-methylbutyric acid, hexanoic acid, and methyl pyrazine, which were poorly degraded by the
604 strain in the original HTLWW (Fig. S5). Therefore, TAN appeared to be a strong inhibitor of
605 RHA1 when the concentration is high. On the contrary, TAN removal hampered the
606 biodegradation of HTLWW by RHM. Final dry cell mass of the strain was reduced by half, and
607 COD removal efficiency decreased from 35.7% to 19.7% (Fig. 5d). Although RHM still
608 degraded over 97% of phenolic compounds in HTLWW with TAN removal, the strain removed
609 less organic acids and N-heterocycles. Our result suggested that RHM might lack the metabolic
610 pathway for effective degradation and utilization of N-heterocyclic compounds since it appeared
611 to require the presence of TAN as nitrogen source.

612 **4. Conclusions**

613 A comparative study was conducted on the biodegradation of sludge HTLWW with three
614 bacterial strains and four fungal strains. COD removal during biological treatment was greatly
615 improved through pH adjustment and nutrient supplementation of P, Mg, Ca, and Fe in a strain
616 dependent manner. Bacterial strains were able to tolerate 10X dilution of HTLWW without pH
617 adjustment and required shorter time for biodegradation. Among bacterial strains, *P. putida*
618 ATCC 31800 and NRRL 14878 demonstrated rapid growth in HTLWW and removed 35% of
619 COD. Biodegradation by *R. jostii* RHA1 was greatly inhibited by the high TAN concentration in
620 sludge HTLWW. TAN stripping improved the COD removal efficiency of the strain by 2.4 folds
621 to 44% COD removal. HTLWW treatment with fungal strains required 20X dilution, pH
622 adjustment to 5, and longer treatment time of 12-30 days. However, fungal strains were shown to
623 degrade a wider range of compounds, especially phenolic compounds which can be degraded for
624 59-100% by the tested fungal strains. HTLWW treatment with pellets of filamentous fungi *A.*

niger NRRL 2001 and *T. versicolor* (Mad) continuously achieved 45-47% COD removal without the need of nutrient supplementation. Most organic acids in HTLWW were readily biodegradable, with over 70% removal efficiency achieved by all the microbes under their specific optimal conditions. On the other hand, N-heterocyclic compounds were highly recalcitrant and removed by at most 38% through biodegradation.

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