

Aerobic fermentation to treat and upcycle hydrothermal liquefaction aqueous phase

by

Meicen Liu

B.S., Purdue University, 2017

B.S., China Agricultural University, 2018

M.S., Iowa State University, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2024

Abstract

Hydrothermal liquefaction (HTL) is a promising wet thermochemical process for converting biomass feedstocks into biofuels. HTL aqueous phase (HTLAP), as one of major by-products of HTL, retains a significant portion of the biomass energy and requires treatment before discharge to the environment. Developing a method to treat HTLAP and recover its energy content is crucial for the economic viability of HTL, while the complexity, variability, microbial toxicity, and recalcitrance of HTLAP make it a challenging task.

Biological treatment methods, such as algae cultivation, anaerobic digestion (AD), and aerobic treatment are environmentally friendly and straightforward options for treating HTLAP. Compared to algae cultivation and AD, aerobic treatment can tolerate higher concentrations of HTLAP and generally requires a shorter incubation time. Despite these advantages, aerobic treatment is less studied in literature. Therefore, it is of interest to develop an aerobic fermentation process for HTLAP that can effectively remove pollutants while producing valuable bioproducts.

This work addresses the need to advance HTLAP treatment technology by developing an effective aerobic fermentation process while further promoting the sustainability of HTL for biofuel production. It begins with a comprehensive review of HTL technology, HTLAP characteristics, existing treatment technologies, and highlighting the benefits and drawbacks of each technology. The research journey of this dissertation embarks upon a comparative evaluation on the biological degradation of HTLAP with various bacterial and fungal strains. The study emphasized the significance of pH adjustment and mineral supplementation in enhancing COD removal during HTLAP fermentation. Bacterial strains tolerated a 10× dilution of HTLAP, while most fungal strains required a higher dilution of 20×. However, the fungal strains

demonstrated effective degradation of a wide range of compounds, particularly phenolic compounds, achieving removal efficiencies between 60-100%. Among them, the filamentous fungi *A. niger* and *T. versicolor* were identified as the most promising strains, achieving approximately 50% COD removal in HTLAP fermentation. The research also highlighted the recalcitrance of sludge HTLAP, which was attributed to the presence of N-heterocycles, as these compounds were removed by less than 40% across all microbes tested.

Building on these findings, we further evaluated three filamentous fungal strains in the fermentation of corn stover HTLAP. Among the tested fungi, *A. niger* showed the best biodegradation performance, converting approximately 50% of its consumed organic carbon into oxalic acid as a value-added product. To enhance HTLAP biodegradation, *R. jostii* was co-cultured with *A. niger* during fermentation. The oleaginous bacterium *R. jostii* consumed organic acids that were left behind by *A. niger* and accumulated cellular lipids. Together, this co-culture achieved consistent COD removal of approximately 70% in diluted HTLAP with COD values between 3.8 and 7.8 g/L. These findings demonstrated the promising fermentation capabilities of *A. niger* and *R. jostii* co-culture, highlighting their potential for HTLAP valorization to improve the sustainability of HTL processes.

Lastly, we assessed the impact of HTL operating conditions on the chemical composition and biodegradability of the resultant HTLAP in *A. niger* and *R. jostii* fermentation. This study elucidated the variation in chemical composition of HTLAP derived from different HTL conditions and established a quantitative relationship between HTL operating parameters (temperature, retention time, and feedstock loading) and the biodegradability of HTLAP, highlighting that HTLAP derived from higher severities exhibited lower biodegradability. This reduced biodegradability was attributed to the increasing concentration of specific phenolic

compounds in HTLAP produced at higher severities. Additionally, the research demonstrated the versatility of the *A. niger* and *R. jostii* co-culture in fermenting HTLAP derived from a wide range of HTL conditions, achieving COD removal rates of 45-70% depending on the specific HTL parameters used.

In conclusion, this dissertation advances HTL technology by developing a promising aerobic fermentation process for HTLAP upcycling. The aerobic fermentation process outlined in this dissertation represents a significant step forward in making HTL technology more economically and environmentally sustainable.

Aerobic fermentation to treat and upcycle hydrothermal liquefaction aqueous phase

by

Meicen Liu

B.S., Purdue University, 2017

B.S., China Agricultural University, 2018

M.S., Iowa State University, 2019

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2024

Approved by:

Major Professor
Yonghui Li

Copyright

© Meicen Liu 2024.

Abstract

Hydrothermal liquefaction (HTL) is a promising wet thermochemical process for converting biomass feedstocks into biofuels. HTL aqueous phase (HTLAP), as one of major by-products of HTL, retains a significant portion of the biomass energy and requires treatment before discharge to the environment. Developing a method to treat HTLAP and recover its energy content is crucial for the economic viability of HTL, while the complexity, variability, microbial toxicity, and recalcitrance of HTLAP make it a challenging task.

Biological treatment methods, such as algae cultivation, anaerobic digestion (AD), and aerobic treatment are environmentally friendly and straightforward options for treating HTLAP. Compared to algae cultivation and AD, aerobic treatment can tolerate higher concentrations of HTLAP and generally requires a shorter incubation time. Despite these advantages, aerobic treatment is less studied in literature. Therefore, it is of interest to develop an aerobic fermentation process for HTLAP that can effectively remove pollutants while producing valuable bioproducts.

This work addresses the need to advance HTLAP treatment technology by developing an effective aerobic fermentation process while further promoting the sustainability of HTL for biofuel production. It begins with a comprehensive review of HTL technology, HTLAP characteristics, existing treatment technologies, and highlighting the benefits and drawbacks of each technology. The research journey of this dissertation embarks upon a comparative evaluation on the biological degradation of HTLAP with various bacterial and fungal strains. The study emphasized the significance of pH adjustment and mineral supplementation in enhancing COD removal during HTLAP fermentation. Bacterial strains tolerated a 10× dilution of HTLAP, while most fungal strains required a higher dilution of 20×. However, the fungal strains

demonstrated effective degradation of a wide range of compounds, particularly phenolic compounds, achieving removal efficiencies between 60-100%. Among them, the filamentous fungi *A. niger* and *T. versicolor* were identified as the most promising strains, achieving approximately 50% COD removal in HTLAP fermentation. The research also highlighted the recalcitrance of sludge HTLAP, which was attributed to the presence of N-heterocycles, as these compounds were removed by less than 40% across all microbes tested.

Building on these findings, we further evaluated three filamentous fungal strains in the fermentation of corn stover HTLAP. Among the tested fungi, *A. niger* showed the best biodegradation performance, converting approximately 50% of its consumed organic carbon into oxalic acid as a value-added product. To enhance HTLAP biodegradation, *R. jostii* was co-cultured with *A. niger* during fermentation. The oleaginous bacterium *R. jostii* consumed organic acids that were left behind by *A. niger* and accumulated cellular lipids. Together, this co-culture achieved consistent COD removal of approximately 70% in diluted HTLAP with COD values between 3.8 and 7.8 g/L. These findings demonstrated the promising fermentation capabilities of *A. niger* and *R. jostii* co-culture, highlighting their potential for HTLAP valorization to improve the sustainability of HTL processes.

Lastly, we assessed the impact of HTL operating conditions on the chemical composition and biodegradability of the resultant HTLAP in *A. niger* and *R. jostii* fermentation. This study elucidated the variation in chemical composition of HTLAP derived from different HTL conditions and established a quantitative relationship between HTL operating parameters (temperature, retention time, and feedstock loading) and the biodegradability of HTLAP, highlighting that HTLAP derived from higher severities exhibited lower biodegradability. This reduced biodegradability was attributed to the increasing concentration of specific phenolic

compounds in HTLAP produced at higher severities. Additionally, the research demonstrated the versatility of the *A. niger* and *R. jostii* co-culture in fermenting HTLAP derived from a wide range of HTL conditions, achieving COD removal rates of 45-70% depending on the specific HTL parameters used.

In conclusion, this dissertation advances HTL technology by developing a promising aerobic fermentation process for HTLAP upcycling. The aerobic fermentation process outlined in this dissertation represents a significant step forward in making HTL technology more economically and environmentally sustainable.

Table of Contents

List of Figures.....	xiv
List of Tables	xix
Acknowledgements	xx
Chapter 1 - Introduction	1
References	3
Chapter 2 - Review of Hydrothermal Liquefaction Technology and the Aqueous Phase	
Treatment Approaches	5
2.1 Hydrothermal liquefaction technology and the thermochemical conversion of hydrothermal liquefaction aqueous phase	5
2.1.1 Hydrothermal liquefaction technology	5
2.1.2 Hydrothermal liquefaction aqueous phase	11
2.1.3 Thermochemical conversion of hydrothermal liquefaction aqueous phase	13
2.2 Biological treatment of hydrothermal liquefaction aqueous phase	15
2.2.1 Biological systems for treatment of hydrothermal liquefaction aqueous phase	15
2.2.2 Pretreatment of hydrothermal liquefaction aqueous phase	16
2.2.3 Algae cultivation.....	23
2.2.4 Anaerobic digestion	25
2.2.5 Aerobic treatment	27
2.3 Summary	30
References	31
Chapter 3 - Comparative Exploration of Biological Treatment of Hydrothermal Liquefaction	
Wastewater	48
Abstract.....	49
3.1 Introduction.....	49
3.2 Materials and Methods	52
3.2.1 Materials.....	52
3.2.1.1 HTLWW of sewage sludge	52
3.2.1.2 Microorganisms	53
3.2.1.3 Supplementary nutrient stocks.....	54

3.2.2 Cultivation of microorganisms in sludge HTLWW	55
3.2.2.1 Screening of cultivation conditions	55
3.2.2.2 Batch cultivation of microorganisms in HTLWW	55
3.2.2.3 Cultivation of large fungal inocula in HTLWW.....	56
3.2.3 Analytical methods	56
3.2.3.1 Microbial growth analysis	56
3.2.3.2 Chemical analysis	57
3.2.3.3 Adsorption assay	59
3.2.4 Data analysis.....	60
3.3. Results and Discussion	60
3.3.1 Characteristics of Sludge HTLWW	60
3.3.2 Cultivation of microbes in HTLWW under screened conditions	62
3.3.2.1 Effect of dilution factor on microbial growth.....	62
3.3.2.2 Effect of pH on microbial growth.....	63
3.3.2.3 Effect of nutrient supplementation on microbial growth	65
3.3.3 Growth and COD removal with batch microbial cultivation	66
3.3.4 COD removal with large fungal inocula	69
3.3.6 Biodegradation of chemical compounds.....	73
3.3.7 Effect of TAN removal on biological treatment of HTLWW	78
3.4 Conclusions.....	80
Acknowledgements	81
References	81
Chapter 4 - Harnessing Fungal-Bacterial Co-culture for Treatment and Valorization of	
Hydrothermal Liquefaction Aqueous Phase.....	91
Abstract.....	92
4.1 Introduction.....	92
4.2 Materials and Methods	95
4.2.1 Materials.....	95
4.2.1.1 Hydrothermal liquefaction aqueous phase of corn stover	95
4.2.1.2 Microorganisms	95
4.2.1.3 Supplementary nutrient stocks.....	96

4.2.2 Aerobic fermentation of corn stover hydrothermal liquefaction aqueous phase.....	96
4.2.2.1 Optimization of fungal fermentation conditions.....	96
4.2.2.2 Fungal cultivation followed by <i>R. jostii</i> inoculation.....	97
4.2.2.3 Co-culture of <i>A. niger</i> with <i>R. jostii</i>	98
4.2.3 Analytical methods	99
4.2.3.1 Chemical analysis	99
4.2.3.2 Microbial cell analysis	100
4.3 Results and Discussion	101
4.3.1 Characteristics of corn stover hydrothermal liquefaction aqueous phase.....	101
4.3.2 Optimization of fungal fermentation conditions in hydrothermal liquefaction aqueous phase	103
4.3.3 Effect of <i>R. jostii</i> inoculation following fungal fermentation	105
4.3.4 Sequential batch fermentation with <i>A. niger</i> and <i>R. jostii</i> co-culture.....	107
4.3.5 Value-added product profiles during hydrothermal liquefaction aqueous phase fermentation	110
4.3.5.1 Oxalic acid production	110
4.3.5.2 Cellular fatty acid profile	115
4.3.6 Effect of hydrothermal liquefaction aqueous phase loading on <i>A. niger</i> and <i>R. jostii</i> co-culture fermentation.....	116
4.3.7 Outlook on using <i>A. niger</i> and <i>R. jostii</i> co-culture in hydrothermal liquefaction aqueous phase fermentation	118
4.4 Conclusions.....	119
References	119
Chapter 5 - Impact of Hydrothermal Liquefaction Conditions on the Chemical Composition and Biodegradability of Aqueous Phase	127
Abstract.....	128
5.1 Introduction.....	129
5.2 Materials and Methods	132
5.2.1 Hydrothermal liquefaction aqueous phase preparation.....	132
5.2.2 Microbial inoculum preparation	133
5.2.3 Determination of IC ₅₀	134

5.2.4 Fermentation assay	135
5.2.5 Analytical methods	136
5.2.6 Experimental design and statistical analysis	137
5.3 Results and Discussion	138
5.3.1 Effect of hydrothermal liquefaction conditions on the aqueous phase chemical compositions	138
5.3.2 Characterization of hydrothermal liquefaction aqueous phase used for biodegradability assay.....	142
5.3.3 Impact of hydrothermal liquefaction conditions on IC ₅₀ of the aqueous phase	145
5.3.4 Biodegradation of hydrothermal liquefaction aqueous phase via aerobic fermentation	148
5.3.5 Perspectives on integrating aerobic fermentation into hydrothermal liquefaction technology.....	154
5.4 Conclusions.....	155
Acknowledgement	156
References	156
Chapter 6 - Conclusions and Future Work.....	162
Appendix A - Supplementary Figures and Tables from Chapter 3	165
Appendix B - Supplementary Figures and Tables from Chapter 4.....	173

List of Figures

Figure 2.1 Different hydrothermal processing routes, reaction conditions, and products. Reproduced with [3]	6
Figure 2.2 Representative depiction of the HTL process at the bench scale (a) and the pilot scale (b) [6].	8
Figure 2.3 Influence of the reaction conditions on the product mass balance of the HTL process [6].	10
Figure 2.4 Potential reaction pathway of the biomass macromolecules to various end-products via HTL process as a function of temperature [19].	13
Figure 2.5 AD biomass decomposition stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) and key compounds [83].	27
Figure 3.1 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.	64
Figure 3.2 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 $\alpha=0.05$, removal efficiencies are shown as 0.0% on the graph (d). Data represent the average and standard deviation of triplicate analysis.	67
Figure 3.3 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.	69
Figure 3.4 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).	75

Figure 3.5 Removal efficiencies of organic acids, phenolic compounds, N-heterocyclic compounds, and COD by NRRL 14878 (a), RHA1 (b), NRRL 2001 (c), and RHM (d) when cultivated in original and TAN removed HTLWW. n=3 for organic acids, N-heterocycles, and COD removal data for NRRL 14878, RHA1, and RHM, and n=2 for all other data.79

Figure 4.1 Cell activity of the tested fungi under screened HTLAP dilution fold (a), pH (b), and nutrient supplementation (c), and COD removal efficiencies by the three fungi on day 12 of fermentation in HTLAP with and without nutrient supplementation (d). In (c), “Full” denotes the supplementation of all nutrient sources including N, Ca, P, Mg, and Fe. “None” indicates the absence of any nutrient supplementation. The remaining treatments represent the supplementation of all nutrient sources except for one (e.g., “No N” indicates supplementation of all nutrients except N). Each bar represents the average and standard deviation of duplicate analyses. In (d), the initial COD and pH were 1900 mg/L and 6.0, respectively, for all three fungi. The nutrient supplementation for each fungus is labeled in the figure.104

Figure 4.2 Concentrations of acetic acid (a), glycolic acid (b), malic acid (c), succinic acid (d), catechol (e), and hydroquinone (f) during fungal fermentation stage (day 0-12) and bacterial fermentation stage (day 12-18; shaded in gray) in HTLAP (n=2) with or without nutrient supplementation. HTLAP was the only COD source in the media. Each data point represents the average and standard deviation of duplicate measurements.106

Figure 4.3 COD profile during sequential batch fermentation with *A. niger* and *R. jostii* co-culture when subjecting to increasing initial COD loading (cycle 1: 3765 mg/L; cycle 2: 4500 mg/L; cycle 3: 6040 mg/L; and cycle 4: 7835 mg/L) from HTLAP (a), HPLC chromatogram of HTLAP before (pink line) and after (black line) fermentation with *A. niger* and *R. jostii* co-culture (peaks of major compounds were labeled in the graph) (b), COD profile during batch cultivation of *A. niger* with different initial fungal mass concentration (dry weight basis) of 0.1 (green triangle), 0.5 (blue square), and 1 g/L (red diamond), as well as during co-culture of *A. niger* at 1 g/L initial cell mass concentration and *R. jostii* (black circle) (c), and COD removals under various fermentation conditions with and without consideration of COD contributed from oxalic acid production (d). COD variation during the last cycle of sequential batch cultivation in (a) is overlaid with those in (c) as a dotted gray line. The COD removal at the end of each cycle of sequential batch

fermentation is labeled in (a). The COD removal before and after excluding oxalic acid production is labeled in (d). Both N and Fe were supplemented for all cultivations. Each data point represents the average and standard deviation of duplicate measurements.....108

Figure 4.4 Variation of oxalic acid concentration during HTLAP fermentation with and without N and Fe supplementations (a), oxalic acid concentrations at the end of different cycles of sequential batch fermentation (b), and percentage of HTLAP-originated TOC conversion to oxalic acid and the corresponding TOC concentration at the end of fungal fermentation stage, sequential batch fermentation cycles 1 to 4, and batch fermentation with 1 g/L *A. niger* biomass (c). Oxalic acid precipitated due to the presence of calcium in HTLAP is represented by gray bars while oxalic acid in solution is depicted by blue bars in (b). Predominant oxalic acid production was illustrated in the GC-MS chromatogram of post-fermentation HTLAP (d) compared to untreated HTLAP (e). Relevant metabolic pathways contributing to oxalic acid production in HTLAP fermentation with *A. niger* are outlined in (f). Key enzymes of the pathways are numbered as follows: 1. cytosolic acetyl-CoA synthetase (ACS); 2. cytosolic carnitine acetyltransferase; 3. mitochondrial carnitine acetyltransferase; 4. malate dehydrogenase (Mdh); 5. oxaloacetate acetyl hydrolase (OahA); and 6. intradiol ring cleavage dioxygenases.112

Figure 4.5 Fatty acid profile (a), cellular lipid content (b), and dry weight concentration (g cell mass/L HTLAP) (c) of microbial cell mass at the end of batch fermentation with single *A. niger* culture, and different cycles of sequential batch fermentation with *A. niger* and *R. jostii* co-culture.....117

Figure 5.1 Concentration variation of short-chain organic acids (group 1, blue), dihydroxybenzene isomers and derivatives (group 2, purple), phenolic acids (group 3, green), phenolic aldehydes (group 4, red), and furanic compounds (group 5, gray) when subjected to increased HTL reaction severity. CS loadings during HTL (5, 10, and 15%, w/v) are illustrated with the increasing saturation of the corresponding colors.139

Figure 5.2 Schematic diagram of the twenty HTLAP samples used for subsequent biodegradability analyses, illustrating the HTL conditions used to generate the HTLAP samples (a). Water quality parameters including COD (b), TOC (c), and TN (d), and concentration of calcium (e), magnesium (f), iron (g), and manganese (h) elements of the selected HTLAP samples. Since samples 8 to 13 were subsets taken from pooled HTLAP

produced under the same HTL condition, their water quality parameters and elemental compositions were measured together rather than individually.....	144
Figure 5.3 Response surface plots showing the effects of HTL reaction temperature and time on the IC ₅₀ (in g/L COD) of the resulting HTLAP for <i>A. niger</i> (a) and <i>R. jostii</i> (b). The actual IC ₅₀ data points are plotted as black-edged circles along with the response surfaces. The statistically significant (p<0.05) Pearson correlation coefficients between the concentrations of different compounds in HTLAP and the IC ₅₀ of HTLAP for <i>A. niger</i> and <i>R. jostii</i> are shown in (c).....	148
Figure 5.4 Concentrations of monitored compounds in HTLAP expressed as percentages of TOC (a), and their degradation during fermentation with <i>A. niger</i> (day 0-15) and <i>R. jostii</i> (day 15-25) in each of the twenty samples selected via CCD (b-h). The overall COD removal during the fermentation is shown in (i). Starting concentrations of COD and individual compounds for each sample are marked with a cross, while removal efficiencies are illustrated with bars colored according to the fermentation time points.	150
Figure 5.5 Schematics of three types of compound degradation profiles during aerobic fermentation with <i>A. niger</i> (day 0-15) followed by <i>R. jostii</i> (day 15-25). Each type of degradation profile is separated by dashed lines, with the corresponding sample number and severity range labeled at the bottom. The width of each node on the diagram quantitatively represents the average percentage of an individual compound in terms of TOC, with nodes representing zero percentage removed from the diagram. Compounds categorized as organic acids and phenolics are illustrated separately, with the corresponding total percentage of TOC labeled on the graphs.	154
Appendix Figure A.1 ITS region sequence of the lab isolated strain RHM and its BLAST search result	165
Appendix Figure A.2 Small (a-c) and large (d-f) inocula of filamentous fungal strains: spore stock of NRRL 2001 (a), spore stock of BKM (b), small hyphal clumps of Mad (c), and fungal pellets of NRRL 2001 (d), BKM (e), and Mad (f).	166
Appendix Figure A.3 Direct relationship between fungal dry cell mass and the absorbance at 560 nm determined by MTT assay.....	167

Appendix Figure A.4 Reactor setup for adsorption assay. Nitrogen gas was continuously blown into the tubes	168
Appendix Figure A.5 Comparison of compound biodegradation in sludge HTLWW with and without TAN removal (TANRM).	168
Appendix Figure B.1 GC-MS chromatogram of the corn stover HTLAP used in this study. Major compounds identified with a match score over 90 were labeled.	173
Appendix Figure B.2 COD variation during batch fermentation with <i>A. niger</i> pure culture and co-culture of <i>A. niger</i> with <i>R. jostii</i> (n=2). The COD removal at the end of 12-day fermentation were labeled on the graph. To ensure favorable conditions for <i>R. jostii</i> growth and activity, we inoculated the bacterium on day 2 of the incubation period, thereby avoiding exposure to potentially inhibitory levels of phenolic compounds.	174
Appendix Figure B.3 Cell activity of <i>R. jostii</i> under screened HTLAP dilution folds (n=2). The control represents the cell activity of <i>R. jostii</i> grown in 15× diluted HTLAP that has been detoxified by removing various phenolic compounds, including 4-methylcatechol, catechol, and hydroquinone, by 34%, 89%, and 79%, respectively. Detoxification was performed via overliming by adjusting the pH of HTLAP to 10 with Ca(OH) ₂ followed by incubation at 60°C for 5 h with hourly manual shaking. After incubation, any precipitates were removed by centrifugation at 5000 rpm. The pH of overlimed HTLAP was then adjusted to match that of untreated HTLAP using 6 M HCl and sterilized by filtration through a 0.22-μm syringe filter before being used for microbial cultivation.	175

List of Tables

Table 3.1 Inorganic element concentration and water quality parameters of HTLWW	61
Table 3.2 Major organic compounds identified in HTLWW ^a	62
Table 3.3 Dilution factor, pH, and nutrient supplementation for microbial cultivation in HTLWW	66
Table 3.4 Removal efficiencies of quantified organic acids (via HPLC) at different time points of batch cultivation by tested microbes	67
Table 3.5 COD removal efficiencies due to microbial biomass adsorption.....	73
Table 3.6 Water quality parameters before and after TAN stripping	78
Table 4.1 Inorganic element concentrations (a), water quality parameters (b), and organic compound concentrations (c) of HTLAP ^a	102
Table 5.1 Central composite design with the selected HTLAP samples and their IC ₅₀ results ..	147
Table 5.2 ANOVA results and estimated coefficients for IC ₅₀ models	148
Appendix Table A.1 Biological treatment of wastewater from HTL of nitrogen-rich feedstocks in other studies and this study	169
Appendix Table A.2 Standard curve equations and detection limits of organic compounds quantified in the study	170
Appendix Table A.3 Unquantified compounds in sludge HTLWW and their degradation by different microbes.....	171
Appendix Table A.4 Significantly different COD removal rate as determined by Tukey-Kramer test at $\alpha=0.05$	172
Appendix Table A.5 Significantly different COD removal rate after treatment with small and large fungal inoculums as determined by Tukey-Kramer test at $\alpha=0.05$	172
Appendix Table B.1 Unquantified compounds in HTLAP and their degradation by single <i>A. niger</i> culture and co-culture of <i>A. niger</i> and <i>R. jostii</i>	176

Acknowledgements

I would like to express my heartfelt gratitude to my former major professor, Dr. Yi Zheng, for his guidance and support during my Ph.D. program. I regret that he had to leave his position to better care for his mother. Dr. Zheng has been incredibly supportive throughout my studies, always ensuring I was making good academic progress while fostering a healthy and happy working environment. I greatly appreciate the countless pieces of advice he provided on experimental design and paper writing, which significantly enhanced the quality of my work.

I am also deeply thankful to Dr. Yonghui Li for kindly accepting the role of my major professor after Dr. Zheng's departure. Dr. Li has been consistently supportive, always letting me know that I can rely on his help.

My sincere appreciation extends to my committee members. Thank you, Dr. Zhiwu Wang, for your guidance, resources, and support throughout my Ph.D. journey. I feel fortunate to have collaborated with your group on the project presented in this dissertation. Your insights during our numerous project meetings and email communications inspired me to explore new ideas and further improve my work. I would also like to thank Dr. Donghai Wang and Dr. Mark Wilkins for their unwavering support, thoughtful discussions, and valuable guidance as part of my committee.

I extend my sincere thanks to Dr. Sandeep Kumar and his Ph.D. student Isamu Umeda, who contributed greatly to the project presented in this dissertation. Without their efforts, I would not have had the high-quality aqueous phase samples used in my studies or gained the valuable insights on the hydrothermal liquefaction process.

A special thanks to my wonderful lab mates: Sang Li, Xuexue Wang, Shubhangi Arvelli, Maddalena Logrieco, Dr. Jun Liu, Dr. Donovan Mafukidze, and Dr. Yuandi Zhang. The

discussions and laughter we shared in the lab were invaluable to me. I also want to thank Xueyao Zhang, Chandan Mahata, Asmita Mahara, Jing Qi, Youhan Chen, Rañia Marie E. Buenavista, Zhenjiao Du, Thomas Weiss, Ketan Shende, Dr. Jiefu Wang, Dr. Di Chen, Dr. Xueli Chen, Dr. Haiwei Lou, Dr. Jikai Zhao, and many others who I spent time with and/or studied with during my Ph.D. journey. I cherish the memories we created together and appreciate the encouragement and inspiration you provided throughout this time.

I would also like to thank our department and the National Science Foundation for funding my Ph.D. research through scholarships and grants. I am especially grateful to Dr. Hulya Dogan, Jordan Tilley, Liz Savage, Brenda Heptig, Scott Graber, and Mary Kaufman for their invaluable support in making my Ph.D. experience as enjoyable and smooth as possible.

Finally, my deepest gratitude goes to my parents for their unconditional love and encouragement. Thank you for fueling my passion for science ever since I was a child. I could not have reached this point without your support. I love you both dearly.

Chapter 1 - Introduction

Due to the increasing demand of human society for energy and over depletion of fossil fuels, there is a need to look for alternative energy sources that are sustainable and renewable to alleviate climate change [1]. Wind, solar, and biomass energy are three types of renewable energy with promising applications. Compared with wind and solar energy, biomass energy is based on the chemical energy of carbon containing compounds and therefore can be made into fuels of the forms of gas, liquid, or solid, exhibiting increasing versatility. As a basic element making up living organisms, carbon is the second most abundant element in nature. In addition, a significant fraction of carbon is wasted or has even become pollutants due to human activities, with examples including lignocellulosic residues, sewage sludge, municipal solid waste, and food waste. Developing technologies that harvest this biomass-derived carbon in the form of fuels has been of high interest, and a lot of research efforts have been made to improve the process efficiency and economy of these technologies, while ensuring reduced carbon dioxide emission against conventional fossil fuels.

Technical strategies, such as gasification, pyrolysis, and hydrothermal processing can concentrate the energy in biomass, producing energy dense fuels for easier transportation and storage. Hydrothermal liquefaction (HTL) is a type of hydrothermal processing that can utilize a wide variety of biomass feedstocks without the need for drying. HTL works like a speeded-up version of petroleum formation and is usually conducted at 250-374°C and 2-25 MPa [2]. During HTL, water is in its subcritical condition, allowing complex reactions to occur and leading to the formation of four production phases in a short period of time, including biocrude, gas, solid residue, and an aqueous phase (known as hydrothermal liquefaction aqueous phase, HTLAP).

Due to the water-rich nature of HTL, HTLAP is a by-product generated at high volume. It was reported that HTLAP consists of roughly 45% of the organic carbon and 80% of other nutrients from the feedstock [3]. Without proper treatment, HTLAP cannot be discharged directly into the environment due to its high nutrient content and potential toxicity to the environment and human health [4]. Recovering the abundant nutrients in HTLAP is also crucial for improving the process economy of HTL technology. Consequently, it is of high importance to develop methods that can treat HTLAP while recovering the nutrients as value-added products.

The development of HTLAP upcycling technologies is challenging due to the high variability of HTLAP composition, which varies significantly with the type of biomass feedstocks as well as HTL reaction parameters, such as temperature, time, and feedstock solid loading. The high variability of HTLAP complicates the method development for its utilization, as the understanding of the relationship between HTL feedstock and reaction conditions and the resultant HTLAP compositions is very limited.

Biological valorization, such as anaerobic fermentation (AD), microalgal cultivation, and aerobic fermentation represents a series of environment-friendly and simple options for HTLAP valorization. Among them, aerobic fermentation demonstrates a superior tolerance to microbial inhibitors in HTLAP and requires shorter incubation time, making it an attractive option for HTLAP valorization [3,5]. Compared to AD and microalgal cultivation, aerobic fermentation is less studied.

The goal of this research is to develop an aerobic fermentation process for the treatment of HTLAP, which is a promising technology for the valorization of HTLAP generated from HTL of various feedstocks under different reaction conditions. The objectives aim at: 1) developing

and optimizing an aerobic fermentation process for HTLAP; 2) identifying and quantifying value-added products yield during HTLAP fermentation; and 3) elucidating the correlation between HTL conditions and the HTLAP biodegradability.

Following this introduction, a comprehensive review is conducted on the HTL technology and the current HTLAP treatment approaches and challenges. Subsequently, three projects are presented to address the three objectives followed by the outline for conclusions and suggestions for future research.

References

- [1] L. Amjith, B. Bavanish, A review on biomass and wind as renewable energy for sustainable environment, *Chemosphere* 293 (2022) 133579.
<https://doi.org/10.1016/j.chemosphere.2022.133579>.
- [2] J. Watson, T. Wang, B. Si, W.-T. Chen, A. Aierzhati, Y. Zhang, Valorization of hydrothermal liquefaction aqueous phase: pathways towards commercial viability, *Prog. Energy Combust. Sci.* 77 (2020) 100819. <https://doi.org/10.1016/j.pecs.2019.100819>.
- [3] Y. Gu, Biological systems for treatment and valorization of wastewater generated from hydrothermal liquefaction of biomass and systems thinking_ A review, *Bioresour. Technol.* (2019) 17.
- [4] M. Pham, L. Schideman, J. Scott, N. Rajagopalan, M.J. Plewa, Chemical and Biological Characterization of Wastewater Generated from Hydrothermal Liquefaction of *Spirulina*, *Environ. Sci. Technol.* 47 (2013) 2131–2138. <https://doi.org/10.1021/es304532c>.
- [5] M. Nelson, L. Zhu, A. Thiel, Y. Wu, M. Guan, J. Minty, H.Y. Wang, X.N. Lin, Microbial utilization of aqueous co-products from hydrothermal liquefaction of microalgae

Nannochloropsis oculata, Bioresour. Technol. 136 (2013) 522–528.

<https://doi.org/10.1016/j.biortech.2013.03.074>.

Chapter 2 - Review of Hydrothermal Liquefaction Technology and the Aqueous Phase Treatment Approaches

Abstract

Over the past few decades, significant advancements have been made in hydrothermal liquefaction (HTL) technology, with a focus on the improvement of process economy and sustainability. However, the treatment and upcycling of hydrothermal liquefaction aqueous phase (HTLAP) remain key challenges to making HTL technology economically viable. To address these challenges, we developed an aerobic fermentation process that effectively converted most organic compounds in HTLAP into oxalic acid and microbial lipids. By thoroughly characterizing HTLAP and establishing correlations between HTL conditions, HTLAP composition, and its biodegradability, we identified the robustness of this process across a wide range of HTL conditions. The findings of this project support the integration of HTLAP aerobic fermentation into the HTL system, enhancing both process economy and sustainability. This section reviews HTL technology and the latest advancements in HTLAP treatment approaches, highlighting the benefits of treating HTLAP through aerobic fermentation.

2.1 Hydrothermal liquefaction technology and the thermochemical conversion of hydrothermal liquefaction aqueous phase

2.1.1 Hydrothermal liquefaction technology

HTL is a versatile wet thermochemical conversion process that can transform a wide range of biomass or waste into biocrude. As a type of hydrothermal process, HTL involves the degradation and reaction of biomass in hot and pressurized water, generally in the ranges of temperature and pressure of 250-370°C and 4-22 MPa [1,2]. As shown in Figure 2.1, compared

to hydrothermal carbonization and hydrothermal gasification, HTL is a hydrothermal process with intermediate reaction severity that falls within the subcritical range of water. HTL generates four product phases, including biocrude oil, gas, biochar, and HTLAP, with biocrude oil being the primary high-value product [3]. The biochar is known to have similar properties to biochar produced through hydrothermal carbonization [3]. In HTL, water serves as both a reaction medium and a reactant, allowing a complex series of reactions to happen during the reaction and eventually lead to the production of the four phases [4]. Since HTL involves 50~95% (v/v) water during the reaction, it can utilize wet feedstocks, such as sewage sludge, microalgal biomass, and food waste without the need of an energy-intensive drying step as in the case for pyrolysis and gasification.

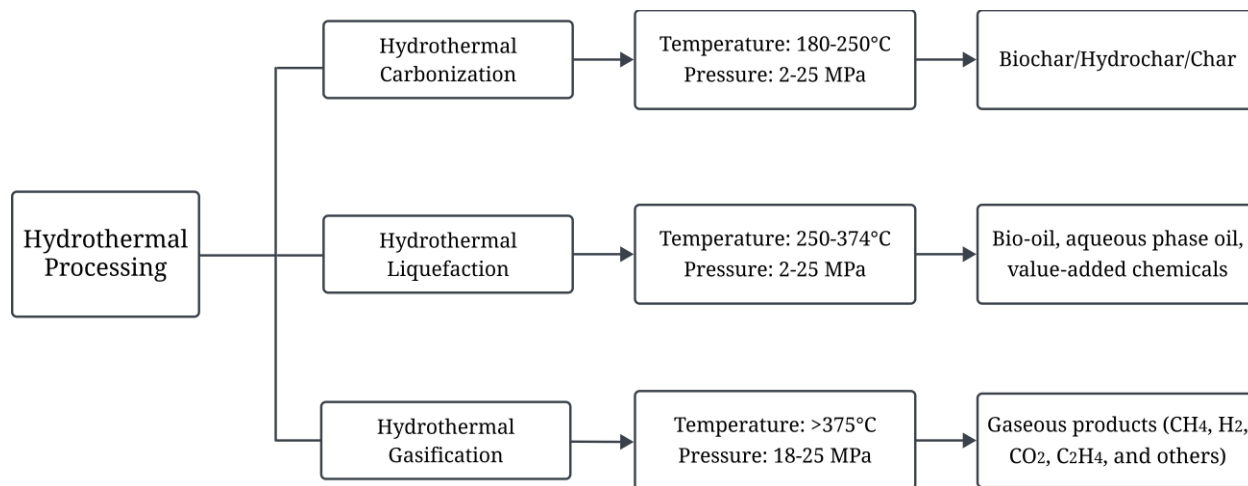


Figure 2.1 Different hydrothermal processing routes, reaction conditions, and products.

Reproduced with [3]

Generally speaking, the process of biofuel production works by removing the oxygen element from biomass because biomass contains approximately 40~60% oxygen while commercial fuels only have less than 1% oxygen. In HTL, oxygen removal is achieved through reactions (e.g., dehydration and decarbonization) at the elevated temperature and pressure of the

process [5]. It is widely accepted that the lipid content in feedstock greatly contributes to bio-oil yield and its higher heating value, whereas carbohydrates and protein contents contribute to the bio-oil yield to a less extent and tend to negatively affect the quality of bio-oil [6]. Therefore, the feedstock of high lipid content is preferred in HTL.

The HTL workflow is outlined in Figure 2.2, highlighting the downstream separation of the four product phases. The gas phase of HTL represents only a small fraction of HTL products (<10% in mass) and mainly consists of carbon dioxide (>90%) [7]. The remaining phases are generally separated differently on lab scale and pilot scale. For lab-scale HTL, the remaining phases are usually subjected to extraction with solvents, such as acetone, dichloromethane, and toluene to separate biocrude oil from the solid residue and HTLAP. For pilot-scale HTL, instead of using solvent for the separation, it mainly applies decantation and lets the remaining phases separate by density. The solvent soluble and lower density fraction is the biocrude oil fraction for lab-scale and pilot-scale HTL, respectively. After separating out biocrude oil and solid residue, the remaining fraction is HTLAP.

The solid residue of HTL is also known as biochar or hydrochar. Formation of the solid residue is mainly through precipitation of inorganic matter as the form of ash and the accumulation of incomplete degradation of lignocellulosic and protein-containing compounds [6]. It has several promising properties, including high hydrophobicity, high calorific value, good adsorption capacity, and potentially favorable mineral and organic content for plant growth, enabling possible applications in soil enhancement, direct combustion, energy storage, and/or adsorbent production [8-11]. The solid residue yield varies significantly from 5% to 60 wt% based on the biomass feedstock composition, as well as the HTL conditions [3]. Studies have shown that the yield of solid residue in HTL increased as the temperature and reaction time

decreased, likely due to the accumulation of incompletely degraded feedstock components in the form of solid residue under lower reaction temperatures [12].

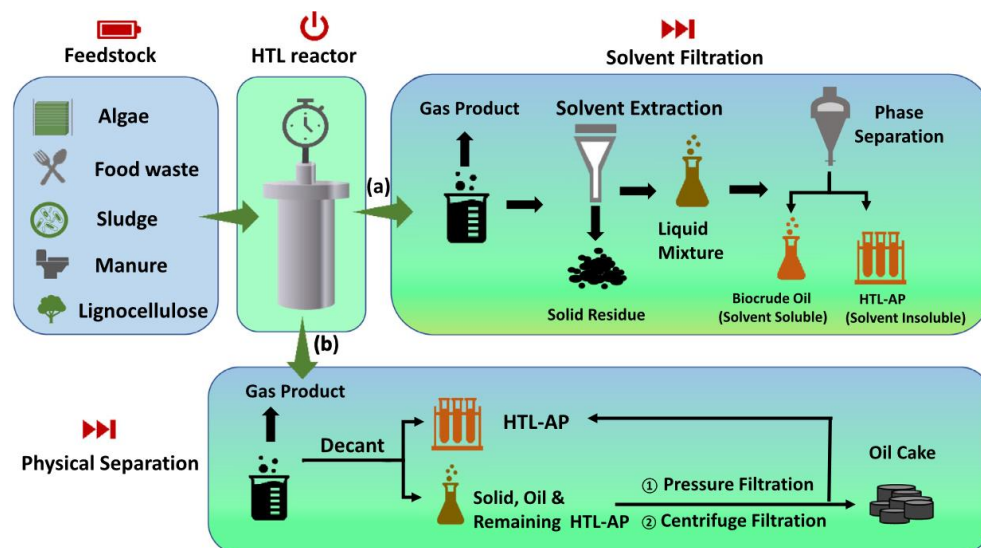


Figure 2.2 Representative depiction of the HTL process at the bench scale (a) and the pilot scale (b) [6].

As a primary high value product of HTL, biocrude oil has been the focus of many studies, aiming to improve the yield and quality of this fraction. Li et al. examined 24 different batches of HTL using microalgal biomass as a feedstock and found that the increasing biocrude oil yield was associated with higher feedstock lipid content, highlighting the benefit of using lipid-rich feedstock in HTL [13]. It is possible to blend biocrude oil and conventional diesel fuel to achieve satisfactory engine performance [14]. However, it is more desirable to upgrade biocrude oil into a stand-alone diesel fuel replacement for better sustainability. The goal of upgrading is to improve the physical and chemical properties of biocrude oil to meet the standard of biodiesel. Some frequently examined properties include viscosity, density, heating value, and the contents of oxygen, nitrogen, and sulfur [15]. Among them, heating value quantitatively represents the energy content of biocrude and can be used to evaluate the efficiency of biomass feedstock to fuel conversion in HTL. Heating value was found to positively correlate with the carbon and

hydrogen content in biocrude oil whereas increasing oxygen and nitrogen content in biocrude oil led to lower heating value. The oxygen content of biocrude oil is around 10 wt% which is significantly higher than that of petroleum diesel, leading to reduced heating value and consequently lowering the peak engine power. On the other hand, the higher oxygen content in biocrude oil can lower emission of pollutants, such as CO and particulate matter in combustion [16]. The nitrogen content in biocrude oil varies significantly with the composition of HTL feedstock, and the feedstock with higher protein content, such as sewage sludge and microalgal biomass generally produces biocrude oil with nitrogen content of around 5 wt%, whereas biocrude oil originates from lignocellulosic biomass has lower nitrogen content of less than 1wt% [6,15]. Existing methods for upgrading biocrude oil include distillation, hydrogenation, hydrodeoxygenation, catalytic cracking, and esterification, which covers a broad area of research interest but is not the primary focus of this dissertation. More detailed information on biocrude upgrading technologies has been comprehensively reviewed by Ramirez et al. [15].

HTLAP is the aqueous by-product of HTL that is generated in large quantities. As a reaction medium, nearly one third to 45% of the organic carbon in biomass feedstock is extracted into HTLAP. In coherence with this, HTLAP represents the largest fraction in most HTL batches in terms of mass balance, as illustrated in Figure 2.3. Therefore, if HTLAP is left untreated, it represents significant energy loss in the HTL process. For more information on HTLAP, please refer to section 5.2.1.2.

HTL has been operated in a wide range of operating conditions for optimizing biocrude oil yield [17]. Some of the important parameters of HTL, include heating rate (2-900°C/min), temperature (200-380°C), time (5-120 min), feedstock solid loading (5-50%), and catalysts (e.g., Na₂CO₃, KOH, Na₂CO₃, Ru/C, etc.) [6,17]. Among them, temperature and time are known to be

the most important operating parameters [6]. Temperature and time can be combined into a single parameter, severity, as shown in Eq. 2.1 [18]. All HTL phases are significantly affected by HTL severity (Figure 2.3), highlighting the variability of HTL process depending on the operation conditions. Typically, higher biocrude oil yields are obtained at the reaction temperature, pressure, and time of 300-350°C, 24-27 MPa, and 15-25 min, respectively [19].

$$Severity = \log \left[\int_0^t e^{\frac{T(t)-T_b}{\omega}} dt \right] \quad (2.1)$$

t : time (min)

$T(t)$: temperature (°C) at a given time

T_b : base temperature of 100°C

ω : fitted parameter of 14.75°C

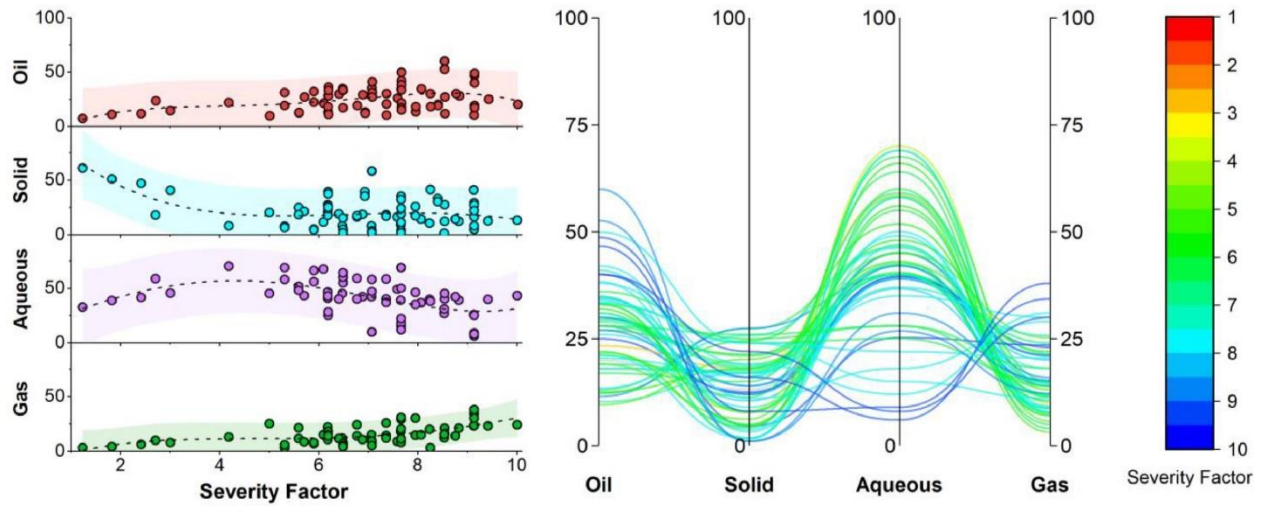


Figure 2.3 Influence of the reaction conditions on the product mass balance of the HTL process [6].

2.1.2 Hydrothermal liquefaction aqueous phase

To ensure the economic viability of HTL refineries, it is crucial to valorize HTLAP to recover the significant energy it contains. The composition of HTLAP varies widely with factors, such as temperature, reaction time, TS loading, and the type of biomass feedstock used in HTL. HTLAP is typically categorized into two groups based on the feedstock: protein-rich and carbohydrate-rich biomass, each having distinct characteristics. Generally, HTLAP from lignocellulosic biomass has a pH ranging from 3.5 to 5, while HTLAP from protein-rich biomass (e.g., sludge, manure, and algae) tends to be more alkaline, with a pH of 7.8-8.5 [6,20]. During HTL, the degradation of proteins produces ammonia through deamination reactions and N-containing organic compounds through condensation and Maillard reactions. These processes contribute to an increased pH in HTLAP and result in higher levels of ammonia nitrogen and total nitrogen (TN) in the aqueous phase [6,19]. In HTL, the degradation of both proteins and carbohydrates produces short-chain organic acids, including acetic acid, glycolic acid, lactic acid, levulinic acid, propionic acid, and formic acid. These acids are extracted into HTLAP and constitute a significant portion of the organic compounds present in this aqueous phase [6,20].

Although both HTLAP from carbohydrate-rich and protein-rich feedstocks contain substantial amounts of organic acids, they can be differentiated by the presence of unique compounds. Notably, HTLAP derived from lignocellulosic biomass feedstocks is distinguished by a diverse array of aromatic compounds which result from the degradation of lignin [19]. Common aromatic compounds found in HTLAP derived from lignocellulosic biomass include catechol, 4-methylcatechol, hydroquinone, and pyrogallol [21]. In contrast, HTLAP derived from protein-rich feedstocks contains significant concentrations of N-heterocyclic compounds which result from the Maillard reaction [19]. For example, N-heterocyclic compounds were identified

as the most abundant group in HTLAP from sewage sludge subjected to HTL conditions with severities of 6.6, 7.8, and 8.8 [22]. Notably, compounds, such as methyl pyrazine, 2-piperidinone, and 1,3-diazine were found to be more prevalent in HTLAP compared to other N-heterocyclic compounds [22].

To quantify the total organic content in HTLAP, measurements of chemical oxygen demand (COD) or total organic carbon (TOC) are typically used. The high COD concentration in HTLAP indicates its rich organic compositions. Depending on the type of biomass feedstock and the operating conditions of HTL, the COD of HTLAP can vary significantly, ranging from 20 to 100 g/L [6]. Generally, HTLAP derived from lignocellulosic biomass has a lower average COD concentration (around 20 g/L) compared to HTLAP derived from protein-rich feedstocks which typically has a higher average COD concentration (around 80 g/L) [6].

HTL is a process consisting of three major steps: depolymerization (150-250°C), decomposition (180-340°C), and recombination (above 300°C) [19]. Depending on the operating conditions of HTL, the extent to which incompletely degraded fragments or recombined molecules are extracted into HTLAP can vary. It has been reported that HTLAP can contain significant amounts of high molecular weight compounds (>1 kDa) [23-25]. In HTLAP derived from sewage sludge, the average molecular weight of dissolved organic matter was found to be 361 and 443 Da, suggesting an average formula of $C_{18}H_{18}N_1O_7$ and $C_{21}H_{23}N_4O_7$, respectively [24]. Therefore, only 40-70% of the total organic carbons in HTLAP can be quantitatively measured by using current mass spectrometry and chromatography methods, resulting in additional difficulty in monitoring HTLAP biodegradation process [24,26].

Figure 2.4 summarizes the fates of the aforementioned biomacromolecules during HTL, highlighting the temperature at which each reaction occurs. It also emphasizes that HTL products

are differentially distributed across the four product phases. The numerous products entering the aqueous phase illustrate the complexity of HTLAP.

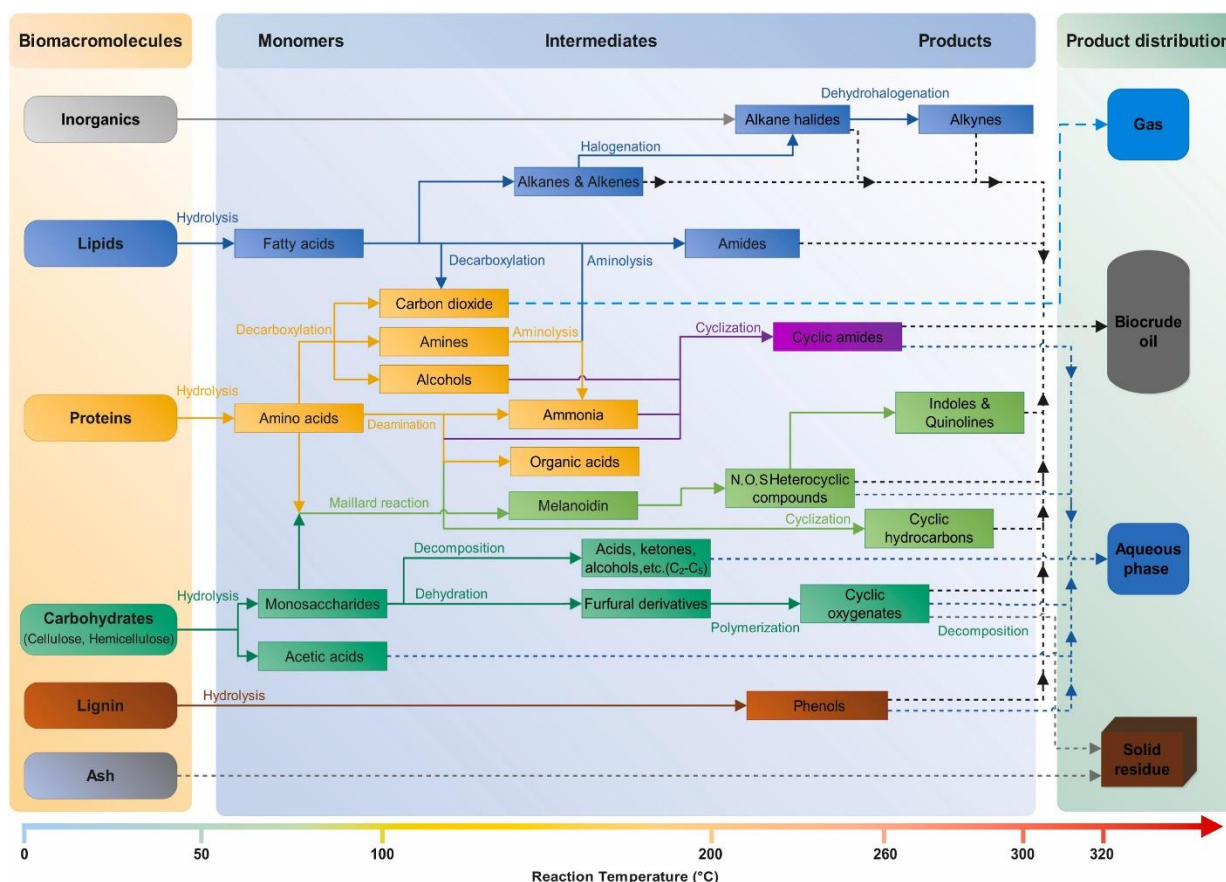


Figure 2.4 Potential reaction pathway of the biomass macromolecules to various end-products via HTL process as a function of temperature [19].

2.1.3 Thermochemical conversion of hydrothermal liquefaction aqueous phase

Several thermochemical methods have been explored for the treatment of HTLAP, including recirculation, gasification, and aqueous phase reforming (APR). The recirculation method involves reusing HTLAP as the reaction medium in HTL, which not only reduces the need for freshwater but also can enhance biocrude oil yield. Compared to other HTLAP treatment methods, recirculation is simpler to implement and more cost-effective due to its straightforward process and reduced freshwater requirements. Specifically, recycling HTLAP for

subsequent HTL has been shown to increase biocrude oil yield by 8.1% to 283%, while reducing HTLAP yield by 5.1% to 40.2% [27]. However, limited number of HTLAP recycling cycles can be performed before it starts to exhibit negative impact on biocrude oil yield and quality [28-30]. The resulting HTLAP after a series of recycling was shown to have 2- to 3-fold increased TOC and 4-fold increased TN [30]. Multiple studies found that the concentration of short chain organic acids (C_2 - C_5) increased significantly after each recycling, which could explain the increased TOC of the recycled HTLAP [28,30,31]. However, it remains to be elucidated which components of HTLAP are transformed into biocrude oil during recycling.

Hydrothermal gasification is another thermal chemical way to reform HTLAP. This method is conducted at temperature and pressure above the critical point of water, i.e., 374.3°C and 22.1 MPa, respectively, transforming HTLAP into hydrogen rich syngas. The syngas yield and COD removal by hydrothermal gasification of HTLAP was shown to be affected by the chemical composition of HTLAP, organic loading, catalyst selection, temperature, and retention time [6]. Specifically, a higher gasification temperature, lower retention time, and lower organic loading were associated with higher hydrogen yield [32–34]. Increasing the gasification temperature and reducing the organic loading also benefited organic compound removal from HTLAP. At temperatures of 400-500°C, the COD removal by gasification ranges from 10% to 80%, whereas increasing temperature further to 600°C increased the COD removal to above 90% [34-36]. Similarly, reducing TOC loading from 11,000 to 2000 mg/L were reported to increase the TOC removal after gasification from 53% to 95% [32]. From the process economy standpoint, increasing the gasification temperature and reducing organic loading is not desirable as it requires more energy to heat the water and increases the operation cost.

APR represents a promising method for valorizing HTLAP that has been developed recently. It applies lower reaction temperatures (225-270°C) than gasification thereby reducing the energy need for heating. APR commonly uses noble metal catalysts, such as Pt. Similar to gasification, APR also yields hydrogen that can be used for upgrading HTL-derived biocrude oil, allowing coupling of APR to HTL process for improved environmental friendliness [37]. However, both gasification and APR are conducted at high temperatures (>200°C) and can experience significant catalyst deactivation when encountering the aromatic compounds present in HTLAP [38,39]. In addition, the organic compound removal by APR has not been fully understood. Existing results suggested that APR poorly degraded compounds, such as acetic acid, propionic acid, and phenol in HTLAP [39]. It will be of value to examine APR-treated HTLAP to ensure the satisfactory removal of COD and harmful compounds in future studies.

2.2 Biological treatment of hydrothermal liquefaction aqueous phase

2.2.1 Biological systems for treatment of hydrothermal liquefaction aqueous phase

Biological degradation offers an environmentally friendly and straightforward method for treating HTLAP. It relies on microbial activity to consume organic compounds in HTLAP and convert them into value-added products, such as methane, hydrogen, lipid-rich cell mass, and other valuable chemicals. Unlike thermochemical methods, biological degradation is performed at milder temperatures of 25-40°C but generally requires a longer treatment time [20]. Various biological methods, such as microalgal cultivation, AD, and aerobic fermentation have been evaluated for HTLAP treatment.

One of the major challenges in the biological treatment of HTLAP is the high concentration of potential inhibitors, such as aromatic, furanic, and N-heterocyclic compounds, which can inhibit microbial activity. To mitigate the inhibition, HTLAP is often diluted with

freshwater before microbial cultivation to reduce the inhibitor concentration to acceptable levels. However, dilution also reduces the concentration of nutrients in HTLAP and incurs the cost of additional water. Consequently, this is expected to lower the yields of value-added products and negatively impact the overall economic feasibility of the process. To address this issue, various pretreatment methods can be applied to remove these inhibitors. The different pretreatment methods are discussed in section 2.2.2.

Another challenge in the biological treatment of HTLAP is its high variability depending on the HTL operating conditions and the types of biomass feedstocks used, as detailed in section 2.1.2. The relationship between the HTL operating conditions, feedstock type, and chemical composition of HTLAP is not yet fully understood, making it difficult to standardize a biological treatment method that ensures the effectiveness across different types of HTLAP. sections 2.2.3 to 2.2.5 review existing studies on HTLAP biodegradation, highlighting the advantages and disadvantages of various biological treatment methods. It is important to note that directly comparing HTLAP biodegradation results across different studies is challenging due to variations in HTL biomass feedstock and operating conditions used to produce HTLAP. Additionally, the dosage of HTLAP varied among studies, as indicated by differences in starting COD or TOC concentrations used for biological treatment. Therefore, this chapter only compares biodegradation results for HTLAP with similar starting COD or TOC levels that are produced from comparable feedstocks and operating conditions.

2.2.2 Pretreatment of hydrothermal liquefaction aqueous phase

Oxidation, adsorption, and precipitation have been examined as pretreatment methods for improving the biodegradability of HTLAP. Oxidation can be conducted through ozonation, H₂O₂, UV, chlorine, and Fenton reaction. These advanced oxidation processes (AOPs) have

traditionally been used for removing micro pollutants in wastewater treatment and have recently gained increasing attention for applications in treating recalcitrant wastewater with high organic matter content [40]. AOPs generally have a smaller footprint, making it easier to integrate them with other treatment processes [41]. The oxidation process generates reactive oxygen species (e.g., hydroxyl ions, alkoxyl radical, and superoxide anion radical) that can easily oxidize other compounds. Among them, hydroxyl is deemed the most reactive, being able to attack numerous contaminants [40]. Hydroxyl ions react non-specifically, initiating a series of oxidation reactions and eventually degrade organic matter into CO₂ and H₂O [42].

Ozonation is a highly attractive oxidation method due to its strong oxidation potential and the fact that it primarily produces oxygen as a byproduct, without generating secondary pollutants [6]. It is generally accepted that an alkaline pH is more favorable for ozone treatment than an acidic pH since higher pH encourages hydroxyl radical formation [43,44]. Ozonation has been applied to textile wastewater treatment in pilot scale and demonstrated promising results of more than 94% phenolic compounds removal and approximately 5-fold increase in biochemical oxygen demand (BOD), suggesting that the wastewater became more biodegradable after ozone treatment [43]. This study also demonstrated differential removal of phenolic compounds. Specifically, phenols, quinones, hydroquinones, phthalic, muconic, fumaric, and maleic intermediates were completely degraded after ozone treatment for 60 min. In contrast, 2-methyl-5-s-propyl-phenol and 4-hydroxyoctahydronaphthalen-1(2H)-one remained intact [43].

Other than phenolic compounds, ozonation can also convert N-heterocyclic compounds and furanic compounds [45,46]. Yang et al. examined the effect of ozonation on HTLAP of sludge and found that the oxidation process effectively lowered the relative peak area of phenolic compounds from 9.2% to 0% and the relative peak area of N-heterocyclic compounds

from 30.6% to 24.6% [44]. However, in comparison to phenolic compounds, N-heterocycles were only minimally converted, even after ozonation for 130 min. Most N-heterocycles exhibited relatively low removal rates, ranging from 16% to 23% [44]. With ozonation treatment, this study increased the 5-day biochemical oxygen demand (BOD₅) to COD ratio from 0.31 to 0.41, indicating that the ozone treatment effectively improved the biodegradability of HTLAP [44].

One drawback of ozonation for HTLAP pretreatment is the need for a high ozone dosage. This requirement arises from the non-selectivity of this method and the high concentrations of organic compounds present in HTLAP [44,47], which can significantly increase the cost of HTLAP treatment. Additionally, the oxidation mechanism of HTLAP is not well understood, making it challenging to develop a well-controlled oxidation process. In some cases, ozone oxidation has been shown to increase the toxicity of treated waste streams [47,48]. Further studies are needed to develop a better controlled oxidation process for HTLAP pretreatment and to justify the increased cost associated with incorporating ozone treatment.

Adsorption is another pretreatment method that can selectively remove inhibitors from HTLAP. Potential adsorbents include activated carbon, zeolite, and biochar, all of which are characterized by their high porosity and effective pollutant adsorption capacity. Compared to activated carbon, zeolite, biochar, and hydrochar are more economical to obtain. Li et al. found that zeolite achieved maximum adsorption of total ammonia nitrogen (TAN) (i.e., free ammonia nitrogen + ammonium nitrogen) at 39°C, and 0.44 mm of zeolite size with the zeolite dose of 0.36 g/mL [49]. At this optimal condition, zeolite removed 8.6 g/L of TAN, which accounts for 84% of the TAN present in untreated HTLAP, and approximately 50% of the N-heterocyclic compounds, such as pyrazine and its derivatives [49]. On the other hand, zeolite adsorption left most other organic compounds largely intact, as evidenced by only slight reduction in COD and

TOC values, both of which were decreased by less than 7% [49]. In this study, the zeolite pretreatment increased the methane yield of AD by 32-117% for HTLAP with COD concentrations of 2-7 g/L, indicating the positive effect of zeolite adsorption on AD of HTLAP [49].

Similarly, biochar, hydrochar, and granulated carbon were also shown to remove TAN, phenolics and/or N-heterocycles from HTLAP, leading to improved biodegradability of HTLAP [50-54]. However, the effectiveness of different adsorbents for HTLAP pretreatment may differ greatly. It has been shown that at the same dosage, granular activated carbon removed over 50% of COD from HTLAP whereas biochar and hydrochar only removed approximately 10% of COD [51]. In addition, the affinity of adsorbents to various HTLAP components appeared to significantly differ from each other. For example, granular activated carbon and biochar were shown to mainly remove phenolic compounds and leave most of the ammonia nitrogen and volatile fatty acids intact [51,54]. In contrast, zeolite were shown to remove the majority of ammonia nitrogen and N-heterocycles [49,54]. Therefore, it could be possible to select the types of adsorbents for HTLAP pretreatment based on the characteristics of targeted HTLAP.

Although adsorption can greatly improve the biodegradability of HTLAP by removing the inhibitory components, the pretreatment itself only served as a way to isolate the components. The spent adsorbents still need to be regenerated for reuse, which could increase the cost of HTLAP treatment [6].

Precipitation is another method to pretreat HTLAP. HTLAP may contain high concentrations of certain components, such as ammonium and phenolic compounds, both of which can be selectively precipitated. For HTLAP derived from protein feedstock, it generally contains high concentrations of ammonium that is known to adversely affect microbial

degradation. Specifically, TAN concentrations of above 1700 mg/L is known to greatly inhibit high-rate AD [55]. $\text{NH}_4^+\text{-N}$ was also shown to inhibit the formation and metabolic activity of aerobic biofilm at concentration above 25 mg/L [56]. The excess ammonium in HTLAP can be removed by precipitation in the form of magnesium ammonium phosphate hexahydrate ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$, struvite) when the stoichiometric ration of $\text{Mg}^{2+}:\text{NH}_4^+:\text{PO}_4^{3-}$ ions are at 1:1:1 and at an alkaline pH [57]. The stoichiometric ratio can be achieved by adding Mg^{2+} and PO_4^{3-} containing salts such as $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and KH_2PO_4 when ammonium is in excess. The precipitated struvite can be used as a slow-release fertilizer while allowing the recovery of NH_4^+ and PO_4^{3-} from HTLAP. Wang et al. found that struvite precipitation removed approximately 80% of ammonia in HTLAP and improved the COD removal efficiency of HTLAP and the CH_4 production in AD by more than threefold, indicating the promising potential of using struvite precipitation pretreatment for ammonium removal from HTLAP [54].

Apart from struvite precipitation, ammonia nitrogen can be removed through air stripping, ion exchange and adsorption, membrane separation, and chlorination, all of which are commonly used for ammonia nitrogen removal on industrial scale [58]. Among these methods, air stripping is more suitable for wastewater with high TAN and can remove 90% of ammonia nitrogen [58]. The removed ammonia nitrogen by air stripping can also be recycled for other applications. The method of air stripping can be performed easily and is of low cost. It is done in an alkaline environment to shift the chemical equilibrium of ammonia to ammonium towards ammonia formation (Eq. 2.2). With the aid of air passing through the system, the gas-liquid equilibrium allows the ammonia in wastewater to be transferred to the air. Increasing temperature of the system can further improve the removal efficiency of the method.



Air stripping can potentially remove volatile compounds along with ammonia, especially those with a low water solubility and a high vapor pressure. Although air stripping did not seem to remove any acetic acid and furfural in a detoxification study, care should still be taken when performing air stripping to evaluate whether any volatile compounds are also removed together with ammonia [59]. It would be worth assessing air stripping as a pretreatment method for removing ammonia nitrogen from HTLAP, especially given the high ammonia concentrations found in some HTLAP (> 10 g/L). Unlike struvite precipitation, air stripping does not require the addition of Mg^{2+} and PO_4^{3-} salts for ammonia removal, which could provide a competitive advantage for its adoption as an HTLAP pretreatment method.

Overliming represents a precipitation method that can potentially be applied to remove the phenolic compounds in HTLAP. Overliming is a alkali pretreatment method with $Ca(OH)_2$ that has been commonly used for detoxification of lignocellulosic biomass hydrolysates to improve its fermentation performance [60]. The pretreatment is generally conducted at alkaline pH of 9-12 and temperature of 50-90°C for 1-6 h [61]. After the alkali treatment, the feedstock is adjusted back to a lower pH suitable for fermentation with either sulfuric acid or hydrochloric acid. The choice of acid used for neutralization did not show any negative effect on the removal of fermentation inhibitors [62]. Compared with other alkali treatment methods, overliming has been shown to remove more fermentation inhibitors, such as phenolic compounds and furan derivatives, making it one of the most effective detoxification methods [61-63]. The mechanisms of improved fermentability with overliming pretreatment are complex and cannot be explained solely by the removal of inhibitors because the pretreatment method has also been shown to transform the inhibitors to other compounds that can potentially be easier biodegraded [62,64]. The overall removal efficiency of overliming on phenolic and furfural compounds ranges from

35% to 68% and from 46% to 76%, respectively [65,66]. It has been reported that the removal efficiency of different phenolic and furfural compounds by overliming pretreatment can differ significantly [66]. Specifically, the study reported that cinnamic acid can be removed by 75% by using overliming, whereas vanillic acid can only be removed by 1% [66]. Similarly, overliming was shown to remove around 80% of furfural and 5-methyl furfural, but lower percentage of hydroxymethylfurfural (65%) [66]. With higher pH and longer treatment time (e.g., pH 12 for 170 h), overliming was shown to convert some types of sugars into lactic acids while leaving others intact [67,68]. Therefore, overliming should be conducted carefully at appropriate pH and duration to avoid loss of fermentable sugars.

Overliming pretreatment of lignocellulose hydrolyzates improved ethanol productivity of *Saccharomyces cerevisiae* and *Candida shehatae* NCIM 3501 by 5-30 times and 1.5 times, respectively [62,63,65]. Similarly, overliming was shown to reduce the inhibitory effects of pyrolytic sugar syrup, allowing 10-time higher inclusion ratio of the feedstock for ethanol fermentation with *E. coli* KO11+ Igk [68]. Overliming of acetic acid-rich aqueous phase from pyrolysis of lignocellulosic biomass removed larger fractions of acetol and phenolics compared to KOH and NaOH treatment and improved the tolerance level of microalgae *Chlamydomonas reinhardtii* to the bio-oil fraction 55 times [69]. Likewise, overliming of the pyrolysis aqueous phase was also demonstrated to improve the biogas production during AD up to 32-fold compared to the non-treated control and was able to increase the microbial tolerance to the aqueous phase from 5% to 14% [70]. To summarize, overliming appeared to be a promising method for the treatment of lignocellulosic biomass-originated feedstocks. However, the level of fermentation improvement greatly varies depending on the types of feedstocks and fermenting

cultures. It is therefore of interest to evaluate the impacts of overliming on the fermentability of HTLAP, especially those generated from the HTL of lignocellulosic biomass.

2.2.3 Algae cultivation

Using HTLAP as a nutrient source for algae cultivation is an attractive option for its treatment. Algae consumes CO₂ in the presence of light energy and can produce a wide range of value-added products, such as carotenoids, carbohydrates, lipids, and nutraceuticals [71]. Therefore, algae-based technology offers dual benefits of greenhouse gas reduction in the atmosphere and production of valuable bioproducts. However, the cultivation of algal biomass requires substantial amounts of nutrients. Specifically, to produce each ton of microalgal biomass, approximately 2 tons of CO₂, 0.1 ton of nitrogen, and 0.01 ton of phosphorus are needed [72]. It is expensive and energy intensive to produce the needed nutrients for algae cultivation, not to mention that both nitrogen and phosphorus are also required for agricultural production. Therefore, it is essential to utilize waste streams as nutrient sources for algae cultivation to reduce the cost and make the process more sustainable [72]. HTLAP represents one of the waste streams that can potentially be used for algae cultivation.

Numerous studies have explored the feasibility of using HTLAP as a medium for algae cultivation. This approach generates biomass that can subsequently be used as feedstock for additional HTL processes. In most studies, HTLAP requires heavy dilution, typically around 100×, before it can be used as an algae cultivation medium due to the presence of growth inhibitors [6]. Biller et al. investigated the potential of recycling the aqueous phase derived from the HTL of *Chlorella vulgaris*, *Scenedesmus dimorphous*, *Spirulina platensis*, and *Chlorogloeopsis fritschii* biomass for culturing the corresponding algae [73]. They found that apart from *Chlorella*, which showed slight growth in 50× diluted HTLAP, all other algae

exhibited no growth at this dilution level. The lack of growth observed in this study when cultivating algae in heavily diluted HTLAP suggested strong inhibitory effects of HTLAP on algae. The need for such heavy dilution implies the addition of a significant amount of clean water for algae cultivation using HTLAP, raising concerns about the practicality and efficiency by using algae for HTLAP treatment.

Algae cultivation was effective at removing the nitrogen and phosphorus in HTLAP. In most studies, over 50% of ammonia and phosphorus in HTLAP can be removed by algae cultivation [6]. *Chlorella* is the most studied algae strain for HTLAP treatment, likely due to its ability to accumulate a large amount of lipids and its robust metabolic capacities that allows it to thrive in autotrophic, mixotrophic, and/or heterotrophic cultivation [6,74]. López Barreiro et al. cultivated *C. vulgaris* and *S. almeriensis* in 392× diluted HTLAP (starting TOC < 0.04 g/L) and found that the TOC was removed by approximately 25-42% by the algae cultivation [75]. Pham et al. grew *C. protothecoides* in the HTLAP with up to 4.2 g/L COD and found that COD was removed by approximately 20% [76]. Similarly, Yang et al. found that *C. vulgaris* demonstrated poor growth of only 40 mg/L·d in HTLAP at 8 g/L COD and were unable to grow at higher HTLAP dosages [44]. However, the inhibitory effect of HTLAP was reduced by AD in the study, demonstrating that the use of anaerobic digestate of HTLAP improved the growth of *C. vulgaris* by more than 3 folds and the algae cultivation removed 20% COD from the digestate [44]. Overall, previous studies on algae for HTLAP treatment suggested the need to include additional methods to further remove the organic matter in HTLAP. In addition, since algae were prone to inhibitors in HTLAP, incorporation of proper pretreatment methods would be needed to detoxify HTLAP and reduce the need for heavy dilution.

2.2.4 Anaerobic digestion

AD is a cost-effective biological technology that has been implemented for industrial production of electricity, heat, and biogas [6]. AD is a complicated biochemical process, consisting of four main stages, i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis, as illustrated in Figure 2.5 [77]. During these stages, microbial consortia work together to break down complex biopolymers into biogas and digestate in the absence of free oxygen. A variety of feedstocks can be degraded in AD, such as manure, crop residues, agro-processing residues, sewage sludge, and food waste. HTLAP has also been evaluated as a feedstock for AD. Similar to algae, the microbial consortia of AD were also greatly inhibited by HTLAP. Generally, AD was conducted with 10-50× diluted HTLAP, corresponding to approximately 1-5 g/L COD loading, to avoid severe inhibition by HTLAP [6].

Zheng et al. conducted an anaerobic toxicity assay on HTLAP and found that HTLAP started to exhibit a sign of toxicity at volumetric inclusion of above 5%, which corresponded to approximately 0.4 g/L HTLAP-derived COD [78], resulting in a 15% inhibition [78]. Increasing the HTLAP inclusion ratio to 6% and 13% further increased the inhibition to 50% and 80%, respectively, while nearly complete inhibition occurred at an inclusion ratio above 24% [78]. The study also reported that AD of HTLAP required 90 and 130 days of incubation at 6% and 12% inclusion ratios, respectively, which indicated the recalcitrancy and toxicity of HTLAP compared to other waste streams (e.g., manure and food waste) that required shorter incubation time of 20-30 days [78]. Similarly, Li et al. noted a lag phase of 9 to 12 days in AD of HTLAP at starting COD concentrations of 2-7 g/L [49]. Bueno et al. reported a higher tolerance of AD to HTLAP, noting that complete inhibition of methane production occurred at an HTLAP dosage of 16 g/L COD [79]. This increased tolerance may be attributed to the lower HTL reaction temperature of

260°C used in their study to produce HTLAP, compared to the 300°C employed in the other two studies [49,78,79]. It has been reported that HTLAP produced at higher HTL temperatures is less biodegradable in AD. This is likely due to the more intense decomposition of the biomass feedstock at higher temperatures, which leads to higher concentrations of hydrophobic and aromatic compounds in HTLAP, making it less biodegradable [24].

The HTL temperature also appeared to affect the COD removal by AD from the corresponding HTLAP. Approximately 20-55% COD can be removed by AD from HTLAP at reaction temperature above 300°C [49,78,79]. At lower HTL temperatures of 170-260°C, the COD removal by AD was improved to approximately 70% [24,80]. To improve the biodegradability of HTLAP and reduce its toxicity in AD, pretreatment methods such as oxidation with H₂O₂ and ozone, and adsorption with zeolite and activated carbon can be applied as described in section 2.2.2. Pretreated HTLAP can be included at higher dosage (up to 20 g/L COD) for AD without causing severe inhibition [47]. In addition, pretreated HTLAP demonstrated a higher COD removal rate of 44-93% in AD compared to untreated HTLAP and resulted in an improved methane yield by 13% to 300% [47,49,78,81,82]. Therefore, pretreatment appears to be necessary for AD of HTLAP with high recalcitrance. Without pretreatment, AD of HTLAP experiences a significant inhibition, requiring heavy dilution and extended incubation time. Additionally, this process is characterized by low biogas yields and reduced COD removal.

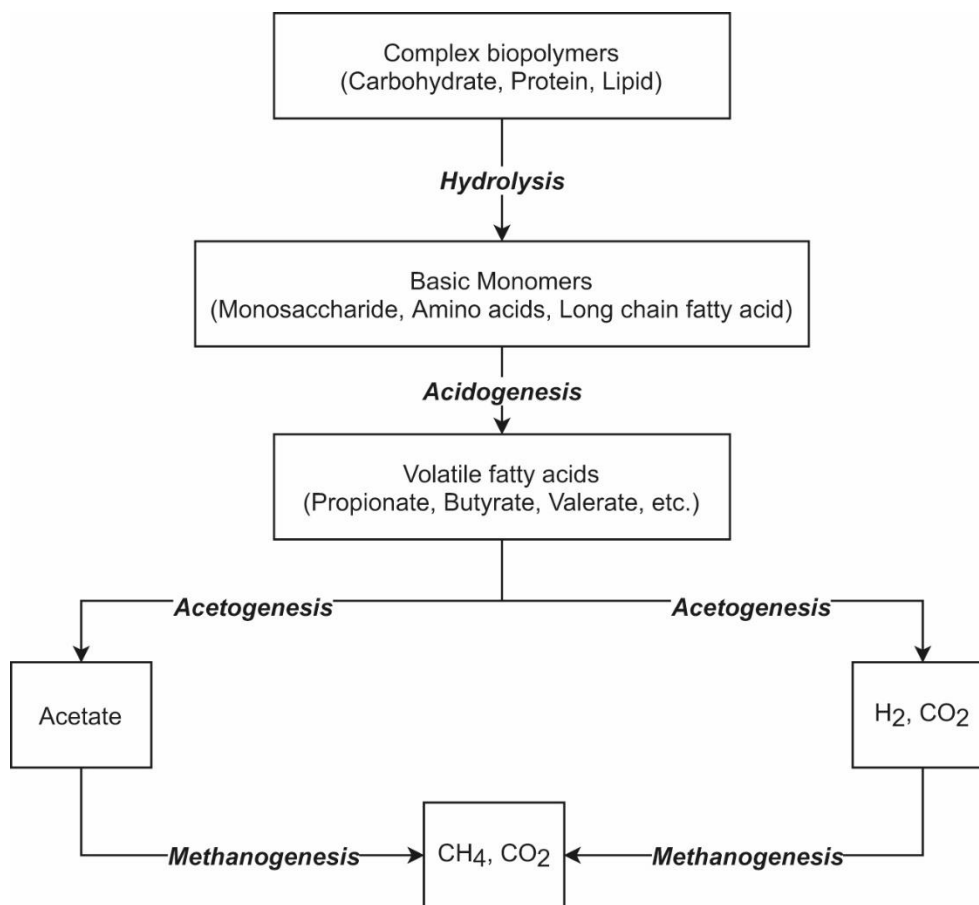


Figure 2.5 AD biomass decomposition stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) and key compounds [83].

2.2.5 Aerobic treatment

Aerobic microorganisms can metabolize a wider range of molecules compared to anaerobic microorganisms due to their effective aerobic respiration processes. Several studies examined the cultivation of aerobic microorganisms in HTLAP. Compared to algae cultivation and AD, aerobic cultivation demonstrated greater tolerance to HTLAP, thus requiring less dilution (<20× dilution) [84-87].

Biological treatment of HTLAP with secondary sludge inoculum in a lab-scale aerobic reactor achieved 67-95% COD reduction in 18 h [84]. The microbial consortium in secondary sludge were able to tolerate 0.8-1.4 g/L initial COD loading and possibly even higher HTLAP

dosages, as the dilution used in the study was primarily to prevent excessive foaming [84]. Similarly, Basar et al. compared four scenarios of HTLAP biological treatments: aerobic treatment with secondary sludge, mesophilic AD, thermophilic AD, and dark fermentation [88]. They found that aerobic treatment experienced the least inhibition from HTLAP [88]. Specifically, no acute inhibition was observed with aerobic treatment of HTLAP derived from 290-360°C. In contrast, both mesophilic and thermophilic AD experienced inhibition when treating HTLAP generated under 350°C and 360°C, and dark fermentation was completely inhibited across all HTLAP samples [88]. Although aerobic treatment with secondary sludge is a fast and effective method for HTLAP treatment, it has several drawbacks, including high energy consumption due to aeration, greenhouse gas emissions, and increased secondary sludge production the requires downstream treatment and disposal.

Aerobic treatment of HTLAP can also be carried out using either pure or co-cultures of selected microorganisms. When applied in aerobic fermentation, defined cultures can potentially recover nutrients from the aqueous phase as biomass, lipids, and valuable chemical compounds. Strains such as *Yarrowia lipolytica*, *Rhodococcus opacus*, *Rhodococcus jostii*, *Escherichia coli*, and *Pseudomonas putida* have been tested in the aerobic fermentation of HTLAP. These strains were found to be able to tolerate less than 10× diluted HTLAP while achieving a reduction of 30-90% in COD or TOC, depending on the type of HTLAP and the duration of cultivation [85–87].

Bacteria generally require less biodegradation time than fungi. *P. putida* is one of the well-studied bacterial species for bioremediation and is known to have robust and versatile metabolism that allow the bacterium to thrive in contaminated environments with limited nutrients [89]. *P. putida* is also a non-pathogenic chassis organism with a well-studied genome that allows it to be metabolically engineered to have favorable traits for industrial applications

[89,90]. Various *P. putida* strains are known to degrade N-heterocyclic compounds via pyrrolidine pathway [91]. *P. putida* strains isolated from waste streams with high phenolic concentrations have been shown to degrade phenol and its derivatives [92,93]. Therefore, it is of value to examine the biodegradation performance of *P. putida* in HTLAP that generally has high concentrations of these compounds.

Rhodococcus strains, such as *R. opacus* PD630 and *R. jostii* RHA1 are known to transform lignin into lipids [94,95]. These oleaginous bacteria can utilize lignin and lignin monomers such as 4-hydroxybenzoic and vanillic acid and accumulate more than 20% wt lipids [94]. Some *Rhodococcus* strain can also degrade N-heterocyclic compounds via pyridine catalytic pathway [91]. He et al. examined *R. opacus* PD630, *R. jostii* RHA1, and *R. jostii* RHA1 VanA- for biodegradation of HTLAP from algae and pine wood feedstocks [85]. In the study, the *R. jostii* RHA1 strain and its mutant showed comparable COD removal efficiency of 92-94% on day 11 of cultivation. The two strains degraded a wide variety of N&O heterocyclic and phenolic compounds in the HTLAP while accumulating 9-35% wt lipids, suggesting the promising potential of *Rhodococcus* strains for HTLAP fermentation [85].

Compared to bacteria, fungi have received considerably less attention for contaminant removal in biological wastewater treatment [96]. However, filamentous fungi, such as *Aspergillus niger*, *Aspergillus. oryzae*, *Phanerochaete chrysosporium*, and *Trametes versicolor* are known for their abilities to degrade a range of problematic organic compounds, including antibiotics and pharmaceuticals that are resistant to bacterial degradation. This is due to the non-specific nature of their fungal enzymes, such as laccase and peroxidase [97]. In addition, fungi can survive and thrive under challenging conditions, including high inhibitor concentration and other environmental stress [98]. Previous studies have shown that *Penicillium corylophilum* and

A. niger were able to dominate 95-98% of the microbial community in sludge treatment processes when the inoculum size exceeded 10% [99]. Moreover, filamentous fungi could form spherical pellets during cultivation, facilitating the separation of fungal biomass from the waste stream [100,101]. To the best of our knowledge, filamentous fungi have not been studied for HTLAP treatment.

The oleaginous yeast *Y. lipolytica* was capable of growing in 5× diluted HTLAP when supplemented with corn stover hydrolysate, which was rich in glucose and xylose, along with other nutrients [87]. During this process, the yeast produced value-added compounds, including polymer precursors, lipids, and organic acids [87]. Other oleaginous yeasts, including isolates closely related to *Rhodotorula mucilaginosa*, have been employed to treat olive mill wastewater which is known for its high phenolic compound toxicity [102,103]. These yeasts have been effective in producing lipids and biosurfactants as value-added products during the treatment process, suggesting their potential for use in HTLAP fermentation [102,103].

2.3 Summary

HTL is a promising technology for the valorization and disposal of biomass feedstocks. However, one by-product of HTL, HTLAP, contains a significant portion of biomass nutrients and poses potential hazards, necessitating proper treatment before discharge to the environment. The development of effective treatment technologies for HTLAP is crucial for enabling an economically viable HTL for biofuel production.

Biological treatment of HTLAP is both simple and environmentally friendly. Three primary categories of biological treatment methods (i.e., algae cultivation, AD, and aerobic treatment) have been explored to degrade the organic matter in HTLAP while potentially

generating value-added products. Each of these methods has its own set of advantages and disadvantages.

Aerobic treatment with secondary sludge is rapid and effective and suffers less inhibition from HTLAP compared to algae cultivation and AD. However, it does not produce value-added products and generates additional sludge biomass that must be disposed of. On the other hand, aerobic fermentation using pure or co-cultures of selected microorganisms can produce value-added products and tolerate less-diluted HTLAP, potentially improving the process economy. Given that aerobic fermentation has been less studied compared to algae cultivation and AD, exploring its potential for HTLAP treatment presents an interesting avenue for research.

References

- [1] A.R.K. Gollakota, N. Kishore, S. Gu, A review on hydrothermal liquefaction of biomass, *Renewable and Sustainable Energy Reviews* 81 (2018) 1378–1392.
<https://doi.org/10.1016/j.rser.2017.05.178>.
- [2] L. Cao, C. Zhang, H. Chen, D.C.W. Tsang, G. Luo, S. Zhang, J. Chen, Hydrothermal liquefaction of agricultural and forestry wastes: state-of-the-art review and future prospects, *Bioresource Technology* 245 (2017) 1184–1193. <https://doi.org/10.1016/j.biortech.2017.08.196>.
- [3] A. Mathanker, S. Das, D. Pudasainee, M. Khan, A. Kumar, R. Gupta, A Review of Hydrothermal Liquefaction of Biomass for Biofuels Production with a Special Focus on the Effect of Process Parameters, Co-Solvents, and Extraction Solvents, *Energies* 14 (2021) 4916.
<https://doi.org/10.3390/en14164916>.
- [4] W.-T. Chen, Y. Zhang, J. Zhang, G. Yu, L.C. Schideman, P. Zhang, M. Minarick, Hydrothermal liquefaction of mixed-culture algal biomass from wastewater treatment system

into bio-crude oil, *Bioresource Technology* 152 (2014) 130–139.

<https://doi.org/10.1016/j.biortech.2013.10.111>.

[5] A.A. Peterson, F. Vogel, R.P. Lachance, M. Fröling, M.J. Antal, Jr., J.W. Tester,

Thermochemical biofuel production in hydrothermal media: A review of sub- and supercritical water technologies, *Energy Environ. Sci.* 1 (2008) 32. <https://doi.org/10.1039/b810100k>.

[6] J. Watson, T. Wang, B. Si, W.-T. Chen, A. Aierzhati, Y. Zhang, Valorization of hydrothermal liquefaction aqueous phase: pathways towards commercial viability, *Progress in Energy and Combustion Science* 77 (2020) 100819. <https://doi.org/10.1016/j.pecs.2019.100819>.

[7] J. Lu, J. Zhang, Z. Zhu, Y. Zhang, Y. Zhao, R. Li, J. Watson, B. Li, Z. Liu, Simultaneous production of biocrude oil and recovery of nutrients and metals from human feces via hydrothermal liquefaction, *Energy Conversion and Management* 134 (2017) 340–346.

<https://doi.org/10.1016/j.enconman.2016.12.052>.

[8] Z. Liu, A. Quek, S. Kent Hoekman, R. Balasubramanian, Production of solid biochar fuel from waste biomass by hydrothermal carbonization, *Fuel* 103 (2013) 943–949.

<https://doi.org/10.1016/j.fuel.2012.07.069>.

[9] I. Bargmann, M.C. Rillig, A. Kruse, J. Greef, M. Kücke, Effects of hydrochar application on the dynamics of soluble nitrogen in soils and on plant availability, *Z. Pflanzenernähr. Bodenk.* 177 (2014) 48–58. <https://doi.org/10.1002/jpln.201300069>.

[10] L. Yang, L. Nazari, Z. Yuan, K. Corscadden, C. (Charles) Xu, Q. (Sophia) He, Hydrothermal liquefaction of spent coffee grounds in water medium for bio-oil production, *Biomass and Bioenergy* 86 (2016) 191–198. <https://doi.org/10.1016/j.biombioe.2016.02.005>.

[11] L. Ding, B. Zou, Y. Li, H. Liu, Z. Wang, C. Zhao, Y. Su, Y. Guo, The production of hydrochar-based hierarchical porous carbons for use as electrochemical supercapacitor electrode

- materials, *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 423 (2013) 104–111. <https://doi.org/10.1016/j.colsurfa.2013.02.003>.
- [12] R. Shakya, J. Whelen, S. Adhikari, R. Mahadevan, S. Neupane, Effect of temperature and Na₂CO₃ catalyst on hydrothermal liquefaction of algae, *Algal Research* 12 (2015) 80–90. <https://doi.org/10.1016/j.algal.2015.08.006>.
- [13] Y. Li, S. Leow, A.C. Fedders, B.K. Sharma, J.S. Guest, T.J. Strathmann, Quantitative multiphase model for hydrothermal liquefaction of algal biomass, *Green Chem.* 19 (2017) 1163–1174. <https://doi.org/10.1039/C6GC03294J>.
- [14] M.N. Nabi, M.M. Rahman, M.A. Islam, F.M. Hossain, P. Brooks, W.N. Rowlands, J. Tulloch, Z.D. Ristovski, R.J. Brown, Fuel characterisation, engine performance, combustion and exhaust emissions with a new renewable Licella biofuel, *Energy Conversion and Management* 96 (2015) 588–598. <https://doi.org/10.1016/j.enconman.2015.02.085>.
- [15] J.A. Ramirez, R.J. Brown, T.J. Rainey, A Review of Hydrothermal Liquefaction Bio-Crude Properties and Prospects for Upgrading to Transportation Fuels, *Energies* 8 (2015) 6765–6794. <https://doi.org/10.3390/en8076765>.
- [16] B. Kegl, Influence of biodiesel on engine combustion and emission characteristics, *Applied Energy* 88 (2011) 1803–1812. <https://doi.org/10.1016/j.apenergy.2010.12.007>.
- [17] C. Tian, B. Li, Z. Liu, Y. Zhang, H. Lu, Hydrothermal liquefaction for algal biorefinery: A critical review, *Renewable and Sustainable Energy Reviews* 38 (2014) 933–950. <https://doi.org/10.1016/j.rser.2014.07.030>.
- [18] I.A. Basar, H. Liu, C. Eskicioglu, Incorporating hydrothermal liquefaction into wastewater treatment – Part III: Aqueous phase characterization and evaluation of on-site

treatment, *Chemical Engineering Journal* 467 (2023) 143422.

<https://doi.org/10.1016/j.cej.2023.143422>.

[19] H. Shahbeik, H. Kazemi Shariat Panahi, M. Dehhaghi, G.J. Guillemin, A. Fallahi, H. Hosseinzadeh-Bandbafha, H. Amiri, M. Rehan, D. Raikwar, H. Latine, B. Pandalone, B. Khoshnevisan, C. Sonne, L. Vaccaro, A.-S. Nizami, V.K. Gupta, S.S. Lam, J. Pan, R. Luque, B. Sels, W. Peng, M. Tabatabaei, M. Aghbashlo, Biomass to biofuels using hydrothermal liquefaction: A comprehensive review, *Renewable and Sustainable Energy Reviews* 189 (2024) 113976. <https://doi.org/10.1016/j.rser.2023.113976>.

[20] Y. Gu, X. Zhang, B. Deal, L. Han, Biological systems for treatment and valorization of wastewater generated from hydrothermal liquefaction of biomass and systems thinking: A review, *Bioresour Technol* 278 (2019) 329–345. <https://doi.org/10.1016/j.biortech.2019.01.127>.

[21] M. Liu, J. Wang, I. Umeda, Z. Wang, S. Kumar, Y. Zheng, Harnessing filamentous fungi and fungal-bacterial co-culture for biological treatment and valorization of hydrothermal liquefaction aqueous phase from corn stover, *Bioresource Technology* 409 (2024) 131240. <https://doi.org/10.1016/j.biortech.2024.131240>.

[22] I.A. Basar, H. Liu, C. Eskicioglu, Incorporating hydrothermal liquefaction into wastewater treatment – Part III: Aqueous phase characterization and evaluation of on-site treatment, *Chemical Engineering Journal* 467 (2023) 143422. <https://doi.org/10.1016/j.cej.2023.143422>.

[23] H. Chen, J. Wan, K. Chen, G. Luo, J. Fan, J. Clark, S. Zhang, Biogas production from hydrothermal liquefaction wastewater (HTLWW): Focusing on the microbial communities as revealed by high-throughput sequencing of full-length 16S rRNA genes, *Water Research* 106 (2016) 98–107. <https://doi.org/10.1016/j.watres.2016.09.052>.

- [24] S. Hao, S. Ren, N. Zhou, H. Chen, M. Usman, C. He, Q. Shi, G. Luo, S. Zhang, Molecular composition of hydrothermal liquefaction wastewater from sewage sludge and its transformation during anaerobic digestion, *Journal of Hazardous Materials* 383 (2020) 121163. <https://doi.org/10.1016/j.jhazmat.2019.121163>.
- [25] H. Chen, C. Zhang, Y. Rao, Y. Jing, G. Luo, S. Zhang, Methane potentials of wastewater generated from hydrothermal liquefaction of rice straw: focusing on the wastewater characteristics and microbial community compositions, *Biotechnol Biofuels* 10 (2017) 140. <https://doi.org/10.1186/s13068-017-0830-0>.
- [26] B. Maddi, E. Panisko, T. Wietsma, T. Lemmon, M. Swita, K. Albrecht, D. Howe, Quantitative Characterization of Aqueous Byproducts from Hydrothermal Liquefaction of Municipal Wastes, Food Industry Wastes, and Biomass Grown on Waste, *ACS Sustainable Chem. Eng.* 5 (2017) 2205–2214. <https://doi.org/10.1021/acssuschemeng.6b02367>.
- [27] P. SundarRajan, K.P. Gopinath, J. Arun, K. GracePavithra, A. Adithya Joseph, S. Manasa, Insights into valuing the aqueous phase derived from hydrothermal liquefaction, *Renewable and Sustainable Energy Reviews* 144 (2021) 111019. <https://doi.org/10.1016/j.rser.2021.111019>.
- [28] E.A. Ramos-Tercero, A. Bertucco, D.W.F. (Wim) Brilman, Process Water Recycle in Hydrothermal Liquefaction of Microalgae To Enhance Bio-oil Yield, *Energy Fuels* 29 (2015) 2422–2430. <https://doi.org/10.1021/ef502773w>.
- [29] Y. Hu, S. Feng, Z. Yuan, C. (Charles) Xu, A. Bassi, Investigation of aqueous phase recycling for improving bio-crude oil yield in hydrothermal liquefaction of algae, *Bioresource Technology* 239 (2017) 151–159. <https://doi.org/10.1016/j.biortech.2017.05.033>.

- [30] P. Biller, R.B. Madsen, M. Klemmer, J. Becker, B.B. Iversen, M. Glasius, Effect of hydrothermal liquefaction aqueous phase recycling on bio-crude yields and composition, *Bioresource Technology* 220 (2016) 190–199. <https://doi.org/10.1016/j.biortech.2016.08.053>.
- [31] M. Damizia, M.P. Bracciale, S. Mousavi, L. Tai, P. De Filippis, B. de Caprariis, Red mud as hydrogen producer in hydrothermal liquefaction of pinewood: Minimization of process wastes by recycling the water and hydrochar phases, *Renewable Energy* 232 (2024) 121139. <https://doi.org/10.1016/j.renene.2024.121139>.
- [32] R. Cherad, J.A. Onwudili, P. Biller, P.T. Williams, A.B. Ross, Hydrogen production from the catalytic supercritical water gasification of process water generated from hydrothermal liquefaction of microalgae, *Fuel* 166 (2016) 24–28. <https://doi.org/10.1016/j.fuel.2015.10.088>.
- [33] P. Escot Bocanegra, C. Reverte, C. Aymonier, A. Loppinet-Serani, M.M. Barsan, I.S. Butler, J.A. Kozinski, I. Gökalp, Gasification study of winery waste using a hydrothermal diamond anvil cell, *The Journal of Supercritical Fluids* 53 (2010) 72–81. <https://doi.org/10.1016/j.supflu.2010.02.015>.
- [34] E. Kıpçak, O.Ö. Söğüt, M. Akgün, Hydrothermal gasification of olive mill wastewater as a biomass source in supercritical water, *The Journal of Supercritical Fluids* 57 (2011) 50–57. <https://doi.org/10.1016/j.supflu.2011.02.006>.
- [35] M.B. García Jarana, J. Sánchez-Oneto, J.R. Portela, E. Nebot Sanz, E.J. Martínez de la Ossa, Supercritical water gasification of industrial organic wastes, *The Journal of Supercritical Fluids* 46 (2008) 329–334. <https://doi.org/10.1016/j.supflu.2008.03.002>.
- [36] P.-G. Duan, S.-K. Yang, Y.-P. Xu, F. Wang, D. Zhao, Y.-J. Weng, X.-L. Shi, Integration of hydrothermal liquefaction and supercritical water gasification for improvement of energy

recovery from algal biomass, *Energy* 155 (2018) 734–745.

<https://doi.org/10.1016/j.energy.2018.05.044>.

[37] E. Tito, G. Zoppi, G. Pipitone, E. Miliotti, A.D. Fraia, A.M. Rizzo, R. Pirone, D. Chiaramonti, S. Bensaid, Conceptual design and techno-economic assessment of coupled hydrothermal liquefaction and aqueous phase reforming of lignocellulosic residues, *Journal of Environmental Chemical Engineering* 11 (2023) 109076.

<https://doi.org/10.1016/j.jece.2022.109076>.

[38] Y. Jiang, L. Ou, L. Snowden-Swan, H. Cai, S. Li, K. Ramasamy, A. Schmidt, H. Wang, D.M. Santosa, M.V. Olarte, M. Guo, M.R. Thorson, Aqueous-phase product treatment and monetization options of wet waste hydrothermal liquefaction: Comprehensive techno-economic and life-cycle GHG emission assessment unveiling research opportunities, *Bioresource Technology* 397 (2024) 130504. <https://doi.org/10.1016/j.biortech.2024.130504>.

[39] G. Pipitone, G. Zoppi, S. Bocchini, A.M. Rizzo, D. Chiaramonti, R. Pirone, S. Bensaid, Aqueous phase reforming of the residual waters derived from lignin-rich hydrothermal liquefaction: investigation of representative organic compounds and actual biorefinery streams, *Catalysis Today* 345 (2020) 237–250. <https://doi.org/10.1016/j.cattod.2019.09.040>.

[40] A. Saravanan, V.C. Deivayanai, P.S. Kumar, G. Rangasamy, R.V. Hemavathy, T. Harshana, N. Gayathri, K. Alagumalai, A detailed review on advanced oxidation process in treatment of wastewater: Mechanism, challenges and future outlook, *Chemosphere* 308 (2022) 136524. <https://doi.org/10.1016/j.chemosphere.2022.136524>.

[41] J.A. Garrido-Cardenas, B. Esteban-García, A. Agüera, J.A. Sánchez-Pérez, F. Manzano-Agugliaro, Wastewater Treatment by Advanced Oxidation Process and Their Worldwide

Research Trends, *Int J Environ Res Public Health* 17 (2020) 170.

<https://doi.org/10.3390/ijerph17010170>.

[42] V.K. Saharan, D.V. Pinjari, P.R. Gogate, A.B. Pandit, Chapter 3 - Advanced Oxidation Technologies for Wastewater Treatment: An Overview, in: V.V. Ranade, V.M. Bhandari (Eds.), *Industrial Wastewater Treatment, Recycling and Reuse*, Butterworth-Heinemann, Oxford, 2014: pp. 141–191. <https://doi.org/10.1016/B978-0-08-099968-5.00003-9>.

[43] C.A. Somensi, E.L. Simionatto, S.L. Bertoli, A. Wisniewski, C.M. Radetski, Use of ozone in a pilot-scale plant for textile wastewater pre-treatment: Physico-chemical efficiency, degradation by-products identification and environmental toxicity of treated wastewater, *Journal of Hazardous Materials* 175 (2010) 235–240. <https://doi.org/10.1016/j.jhazmat.2009.09.154>.

[44] L. Yang, B. Si, M.A. Martins, J. Watson, H. Chu, Y. Zhang, X. Tan, X. Zhou, Y. Zhang, Improve the biodegradability of post-hydrothermal liquefaction wastewater with ozone: conversion of phenols and N-heterocyclic compounds, *Water Science and Technology* 2017 (2018) 248–255. <https://doi.org/10.2166/wst.2018.108>.

[45] R. Andreozzi, A. Insola, V. Caprio, M.G. D'Amore, Ozonation of pyridine in aqueous solution: Mechanistic and kinetic aspects, *Water Research* 25 (1991) 655–659. [https://doi.org/10.1016/0043-1354\(91\)90040-W](https://doi.org/10.1016/0043-1354(91)90040-W).

[46] M. Leili, G. Moussavi, K. Naddafi, Degradation and mineralization of furfural in aqueous solutions using heterogeneous catalytic ozonation, *Desalination and Water Treatment* 51 (2013) 6789–6797. <https://doi.org/10.1080/19443994.2013.792135>.

[47] B. Si, L. Yang, X. Zhou, J. Watson, G. Tommaso, W.-T. Chen, Q. Liao, N. Duan, Z. Liu, Y. Zhang, Anaerobic conversion of the hydrothermal liquefaction aqueous phase: fate of

organics and intensification with granule activated carbon/ozone pretreatment, *Green Chem.* 21 (2019) 1305–1318. <https://doi.org/10.1039/C8GC02907E>.

[48] S.M. de A.G.U. de Souza, K.A.S. Bonilla, A.A.U. de Souza, Removal of COD and color from hydrolyzed textile azo dye by combined ozonation and biological treatment, *Journal of Hazardous Materials* 179 (2010) 35–42. <https://doi.org/10.1016/j.jhazmat.2010.02.053>.

[49] R. Li, D. Liu, Y. Zhang, J. Zhou, Y.F. Tsang, Z. Liu, N. Duan, Y. Zhang, Improved methane production and energy recovery of post-hydrothermal liquefaction waste water via integration of zeolite adsorption and anaerobic digestion, *Science of The Total Environment* 651 (2019) 61–69. <https://doi.org/10.1016/j.scitotenv.2018.09.175>.

[50] K. Aktas, H. Liu, I.A. Basar, C. Eskicioglu, Adsorption enhanced biological treatment of hydrothermal liquefaction aqueous phase derived from municipal sludge, *Bioresource Technology* 407 (2024) 131093. <https://doi.org/10.1016/j.biortech.2024.131093>.

[51] K. Aktas, H. Liu, C. Eskicioglu, Treatment of aqueous phase from hydrothermal liquefaction of municipal sludge by adsorption: Comparison of biochar, hydrochar, and granular activated carbon, *Journal of Environmental Management* 356 (2024) 120619. <https://doi.org/10.1016/j.jenvman.2024.120619>.

[52] F. Marrakchi, S. Sohail Toor, A. Haaning Nielsen, T. Helmer Pedersen, L. Aistrup Rosendahl, Bio-crude oils production from wheat stem under subcritical water conditions and batch adsorption of post-hydrothermal liquefaction aqueous phase onto activated hydrochars, *Chemical Engineering Journal* 452 (2023) 139293. <https://doi.org/10.1016/j.cej.2022.139293>.

[53] L. Yang, B. Si, X. Tan, H. Chu, X. Zhou, Y. Zhang, Y. Zhang, F. Zhao, Integrated anaerobic digestion and algae cultivation for energy recovery and nutrient supply from post-

hydrothermal liquefaction wastewater, *Bioresource Technology* 266 (2018) 349–356.

<https://doi.org/10.1016/j.biortech.2018.06.083>.

[54] P. Wang, Y. Sakhno, S. Adhikari, H. Peng, D. Jaisi, T. Soneye, B. Higgins, Q. Wang, Effect of ammonia removal and biochar detoxification on anaerobic digestion of aqueous phase from municipal sludge hydrothermal liquefaction, *Bioresource Technology* 326 (2021) 124730. <https://doi.org/10.1016/j.biortech.2021.124730>.

[55] O. Yenigün, B. Demirel, Ammonia inhibition in anaerobic digestion: A review, *Process Biochemistry* 48 (2013) 901–911. <https://doi.org/10.1016/j.procbio.2013.04.012>.

[56] X. Weng, Z. Mao, H.-M. Fu, Y.-P. Chen, J.-S. Guo, F. Fang, X.-W. Xu, P. Yan, Biofilm formation during wastewater treatment: Motility and physiological response of aerobic denitrifying bacteria under ammonia stress based on surface plasmon resonance imaging, *Bioresource Technology* 361 (2022) 127712. <https://doi.org/10.1016/j.biortech.2022.127712>.

[57] S.R. Shanmugam, S. Adhikari, R. Shakya, Nutrient removal and energy production from aqueous phase of bio-oil generated via hydrothermal liquefaction of algae, *Bioresource Technology* 230 (2017) 43–48. <https://doi.org/10.1016/j.biortech.2017.01.031>.

[58] S. Xiang, Y. Liu, G. Zhang, R. Ruan, Y. Wang, X. Wu, H. Zheng, Q. Zhang, L. Cao, New progress of ammonia recovery during ammonia nitrogen removal from various wastewaters, *World J Microbiol Biotechnol* 36 (2020) 144. <https://doi.org/10.1007/s11274-020-02921-3>.

[59] H. Wang, D. Livingston, R. Srinivasan, Q. Li, P. Steele, F. Yu, Detoxification and Fermentation of Pyrolytic Sugar for Ethanol Production, *Appl Biochem Biotechnol* 168 (2012) 1568–1583. <https://doi.org/10.1007/s12010-012-9879-1>.

- [60] E. Palmqvist, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification, *Bioresource Technology* 74 (2000) 17–24. [https://doi.org/10.1016/S0960-8524\(99\)00160-1](https://doi.org/10.1016/S0960-8524(99)00160-1).
- [61] I. Haq, Y. Arshad, A. Nawaz, M. Aftab, A. Rehman, H. Mukhtar, Z. Mansoor, Q. Syed, Removal of phenolic compounds through overliming for enhanced saccharification of wheat straw, *J of Chemical Tech & Biotech* 93 (2018) 3011–3017. <https://doi.org/10.1002/jctb.5659>.
- [62] † Per Persson, † Jessica Andersson, † Lo Gorton, ‡ Simona Larsson, ‡ and Nils-Olof Nilvebrant, § Leif J. Jönsson*, Effect of Different Forms of Alkali Treatment on Specific Fermentation Inhibitors and on the Fermentability of Lignocellulose Hydrolysates for Production of Fuel Ethanol, ACS Publications (2002). <https://doi.org/10.1021/jf025565o>.
- [63] S. Larsson, A. Reimann, N.-O. Nilvebrant, L.J. Jönsson, Comparison of different methods for the detoxification of lignocellulose hydrolyzates of spruce, *Appl Biochem Biotechnol* 77 (1999) 91–103. <https://doi.org/10.1385/ABAB:77:1-3:91>.
- [64] X. Zhao, L. Jarboe, Z. Wen, Utilization of pyrolytic substrate by microalga *Chlamydomonas reinhardtii*: cell membrane property change as a response of the substrate toxicity, *Appl Microbiol Biotechnol* 100 (2016) 4241–4251. <https://doi.org/10.1007/s00253-016-7439-2>.
- [65] A.K. Chandel, R.K. Kapoor, A. Singh, R.C. Kuhad, Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501, *Bioresource Technology* 98 (2007) 1947–1950. <https://doi.org/10.1016/j.biortech.2006.07.047>.
- [66] Y. Zhang, C. Xia, M. Lu, M. Tu, Effect of overliming and activated carbon detoxification on inhibitors removal and butanol fermentation of poplar prehydrolysates, *Biotechnol Biofuels* 11 (2018) 178. <https://doi.org/10.1186/s13068-018-1182-0>.

- [67] R. Millati, C. Niklasson, M.J. Taherzadeh, Effect of pH, time and temperature of overliming on detoxification of dilute-acid hydrolyzates for fermentation by *Saccharomyces cerevisiae*, *Process Biochemistry* 38 (2002) 515–522. [https://doi.org/10.1016/S0032-9592\(02\)00176-0](https://doi.org/10.1016/S0032-9592(02)00176-0).
- [68] Z. Chi, M. Rover, E. Jun, M. Deaton, P. Johnston, R.C. Brown, Z. Wen, L.R. Jarboe, Overliming detoxification of pyrolytic sugar syrup for direct fermentation of levoglucosan to ethanol, *Bioresource Technology* 150 (2013) 220–227. <https://doi.org/10.1016/j.biortech.2013.09.138>.
- [69] X. Zhao, K. Davis, R. Brown, L. Jarboe, Z. Wen, Alkaline treatment for detoxification of acetic acid-rich pyrolytic bio-oil for microalgae fermentation: Effects of alkaline species and the detoxification mechanisms, *Biomass and Bioenergy* 80 (2015) 203–212. <https://doi.org/10.1016/j.biombioe.2015.05.007>.
- [70] H. Zhou, R.C. Brown, Z. Wen, Anaerobic digestion of aqueous phase from pyrolysis of biomass: Reducing toxicity and improving microbial tolerance, *Bioresource Technology* 292 (2019) 121976. <https://doi.org/10.1016/j.biortech.2019.121976>.
- [71] K. Chandrasekhar, T. Raj, S.V. Ramanaiah, G. Kumar, J.R. Banu, S. Varjani, P. Sharma, A. Pandey, S. Kumar, S.-H. Kim, Algae biorefinery: A promising approach to promote microalgae industry and waste utilization, *Journal of Biotechnology* 345 (2022) 1–16. <https://doi.org/10.1016/j.jbiotec.2021.12.008>.
- [72] F.G. Ación Fernández, J.M. Fernández Sevilla, E. Molina Grima, Chapter 21 - Costs analysis of microalgae production, in: A. Pandey, J.-S. Chang, C.R. Soccol, D.-J. Lee, Y. Chisti (Eds.), *Biofuels from Algae* (Second Edition), Elsevier, 2019: pp. 551–566. <https://doi.org/10.1016/B978-0-444-64192-2.00021-4>.

- [73] P. Biller, A.B. Ross, S.C. Skill, A. Lea-Langton, B. Balasundaram, C. Hall, R. Riley, C.A. Llewellyn, Nutrient recycling of aqueous phase for microalgae cultivation from the hydrothermal liquefaction process, *Algal Research* 1 (2012) 70–76. <https://doi.org/10.1016/j.algal.2012.02.002>.
- [74] N. Arora, G.P. Philippidis, Insights into the physiology of *Chlorella vulgaris* cultivated in sweet sorghum bagasse hydrolysate for sustainable algal biomass and lipid production, *Sci Rep* 11 (2021) 6779. <https://doi.org/10.1038/s41598-021-86372-2>.
- [75] D. López Barreiro, M. Bauer, U. Hornung, C. Posten, A. Kruse, W. Prins, Cultivation of microalgae with recovered nutrients after hydrothermal liquefaction, *Algal Research* 9 (2015) 99–106. <https://doi.org/10.1016/j.algal.2015.03.007>.
- [76] M. Pham, L. Schideman, J. Scott, N. Rajagopalan, M.J. Plewa, Chemical and Biological Characterization of Wastewater Generated from Hydrothermal Liquefaction of *Spirulina*, *Environ. Sci. Technol.* 47 (2013) 2131–2138. <https://doi.org/10.1021/es304532c>.
- [77] A. Rahimieh, M. Nosrati, A review on biochemistry, microbiology and thermodynamic aspects of propionate: The key intermediate in the anaerobic digestion and wastewater treatment, *Desalination and Water Treatment* 317 (2024) 100191. <https://doi.org/10.1016/j.dwt.2024.100191>.
- [78] M. Zheng, L.C. Schideman, G. Tommaso, W.-T. Chen, Y. Zhou, K. Nair, W. Qian, Y. Zhang, K. Wang, Anaerobic digestion of wastewater generated from the hydrothermal liquefaction of *Spirulina*: Toxicity assessment and minimization, *Energy Conversion and Management* 141 (2017) 420–428. <https://doi.org/10.1016/j.enconman.2016.10.034>.
- [79] B. Egerland Bueno, L. Américo Soares, D. Quispe-Arpasi, I. Kimiko Sakamoto, Y. Zhang, M.B. Amancio Varesche, R. Ribeiro, G. Tommaso, Anaerobic digestion of aqueous

phase from hydrothermal liquefaction of *Spirulina* using biostimulated sludge, *Bioresource Technology* 312 (2020) 123552. <https://doi.org/10.1016/j.biortech.2020.123552>.

[80] B. Si, J. Li, Z. Zhu, M. Shen, J. Lu, N. Duan, Y. Zhang, Q. Liao, Y. Huang, Z. Liu, Inhibitors degradation and microbial response during continuous anaerobic conversion of hydrothermal liquefaction wastewater, *Science of The Total Environment* 630 (2018) 1124–1132. <https://doi.org/10.1016/j.scitotenv.2018.02.310>.

[81] D. Quispe-Arpasi, R. de Souza, M. Stablein, Z. Liu, N. Duan, H. Lu, Y. Zhang, A.L. de Oliveira, R. Ribeiro, G. Tommaso, Anaerobic and photocatalytic treatments of post-hydrothermal liquefaction wastewater using H₂O₂, *Bioresource Technology Reports* 3 (2018) 247–255. <https://doi.org/10.1016/j.biteb.2018.08.003>.

[82] S.R. Shanmugam, S. Adhikari, Z. Wang, R. Shakya, Treatment of aqueous phase of bio-oil by granular activated carbon and evaluation of biogas production, *Bioresource Technology* 223 (2017) 115–120. <https://doi.org/10.1016/j.biortech.2016.10.008>.

[83] M.M. Uddin, M.M. Wright, Anaerobic digestion fundamentals, challenges, and technological advances, *Physical Sciences Reviews* 8 (2023) 2819–2837. <https://doi.org/10.1515/psr-2021-0068>.

[84] Y. Kulikova, S. Klementev, A. Sirotkin, I. Mokrushin, M. Bassyouni, Y. Elhenawy, M.A. El-Hadek, O. Babich, Aqueous Phase from Hydrothermal Liquefaction: Composition and Toxicity Assessment, *Water* 15 (2023) 1681. <https://doi.org/10.3390/w15091681>.

[85] Y. He, X. Li, X. Xue, M.S. Swita, A.J. Schmidt, B. Yang, Biological conversion of the aqueous wastes from hydrothermal liquefaction of algae and pine wood by *Rhodococci*, *Bioresource Technology* 224 (2017) 457–464. <https://doi.org/10.1016/j.biortech.2016.10.059>.

- [86] M. Nelson, L. Zhu, A. Thiel, Y. Wu, M. Guan, J. Minty, H.Y. Wang, X.N. Lin, Microbial utilization of aqueous co-products from hydrothermal liquefaction of microalgae *Nannochloropsis oculata*, *Bioresource Technology* 136 (2013) 522–528. <https://doi.org/10.1016/j.biortech.2013.03.074>.
- [87] L.T. Cordova, B.C. Lad, S.A. Ali, A.J. Schmidt, J.M. Billing, K. Pomraning, B. Hofstad, M.S. Swita, J.R. Collett, H.S. Alper, Valorizing a hydrothermal liquefaction aqueous phase through co-production of chemicals and lipids using the oleaginous yeast *Yarrowia lipolytica*, *Bioresource Technology* 313 (2020) 123639. <https://doi.org/10.1016/j.biortech.2020.123639>.
- [88] I.A. Basar, A. Stokes, C. Eskicioglu, Evaluation of on-site biological treatment options for hydrothermal liquefaction aqueous phase derived from sludge in municipal wastewater treatment plants, *Water Research* 252 (2024) 121206. <https://doi.org/10.1016/j.watres.2024.121206>.
- [89] P.I. Nikel, V. de Lorenzo, *Pseudomonas putida* as a functional chassis for industrial biocatalysis: From native biochemistry to trans-metabolism, *Metabolic Engineering* 50 (2018) 142–155. <https://doi.org/10.1016/j.ymben.2018.05.005>.
- [90] W.R. Henson, A.W. Meyers, L.N. Jayakody, A. DeCapite, B.A. Black, W.E. Michener, C.W. Johnson, G.T. Beckham, Biological upgrading of pyrolysis-derived wastewater: Engineering *Pseudomonas putida* for alkylphenol, furfural, and acetone catabolism and (methyl)muconic acid production, *Metabolic Engineering* 68 (2021) 14–25. <https://doi.org/10.1016/j.ymben.2021.08.007>.
- [91] Y. Mu, Q. Chen, R.E. Parales, Z. Lu, Q. Hong, J. He, J. Qiu, J. Jiang, Bacterial catabolism of nicotine: Catabolic strains, pathways and modules, *Environmental Research* 183 (2020) 109258. <https://doi.org/10.1016/j.envres.2020.109258>.

- [92] C.F. Feist, G.D. Hegeman, Phenol and Benzoate Metabolism by *Pseudomonas putida*: Regulation of Tangential Pathways, *J Bacteriol* 100 (1969) 869–877.
<https://doi.org/10.1128/jb.100.2.869-877.1969>.
- [93] R.-S. Juang, K.-J. Tseng, Experimental investigation of bio-removal of toxic organic pollutants from highly saline solutions in a triphasic system, *Journal of Hazardous Materials* 178 (2010) 706–712. <https://doi.org/10.1016/j.jhazmat.2010.01.145>.
- [94] M. Kosa, A.J. Ragauskas, Bioconversion of lignin model compounds with oleaginous *Rhodococci*, *Appl Microbiol Biotechnol* 93 (2012) 891–900. <https://doi.org/10.1007/s00253-011-3743-z>.
- [95] M. Ahmad, C.R. Taylor, D. Pink, K. Burton, D. Eastwood, G.D. Bending, T.D.H. Bugg, Development of novel assays for lignin degradation: comparative analysis of bacterial and fungal lignin degraders, *Mol. BioSyst.* 6 (2010) 815–821. <https://doi.org/10.1039/B908966G>.
- [96] B.B. Negi, C. Das, Mycoremediation of wastewater, challenges, and current status: A review, *Bioresource Technology Reports* 22 (2023) 101409.
<https://doi.org/10.1016/j.biteb.2023.101409>.
- [97] M. Naghdi, M. Taheran, S.K. Brar, A. Kermanshahi-pour, M. Verma, R.Y. Surampalli, Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes, *Environmental Pollution* 234 (2018) 190–213.
<https://doi.org/10.1016/j.envpol.2017.11.060>.
- [98] T.T. More, S. Yan, R.D. Tyagi, R.Y. Surampalli, Potential use of filamentous fungi for wastewater sludge treatment, *Bioresource Technology* 101 (2010) 7691–7700.
<https://doi.org/10.1016/j.biortech.2010.05.033>.

- [99] S. Mannan, A. Fakhru'l-Razi, M.Z. Alam, Use of fungi to improve bioconversion of activated sludge, *Water Research* 39 (2005) 2935–2943.
<https://doi.org/10.1016/j.watres.2005.04.074>.
- [100] L. Veiter, V. Rajamanickam, C. Herwig, The filamentous fungal pellet—relationship between morphology and productivity, *Appl Microbiol Biotechnol* 102 (2018) 2997–3006.
<https://doi.org/10.1007/s00253-018-8818-7>.
- [101] J. Zhang, J. Zhang, The filamentous fungal pellet and forces driving its formation, *Critical Reviews in Biotechnology* 36 (2016) 1066–1077.
<https://doi.org/10.3109/07388551.2015.1084262>.
- [102] L. Derguine-Mecheri, S. Kebbouche-Gana, D. Djenane, Biosurfactant production from newly isolated *Rhodotorula* sp.YBR and its great potential in enhanced removal of hydrocarbons from contaminated soils, *World J Microbiol Biotechnol* 37 (2021) 18.
<https://doi.org/10.1007/s11274-020-02983-3>.
- [103] T.M. Jiru, D. Abate, N. Kiggundu, C. Pohl, M. Groenewald, Oleaginous yeasts from Ethiopia, *AMB Expr* 6 (2016) 78. <https://doi.org/10.1186/s13568-016-0242-8>.

Chapter 3 - Comparative Exploration of Biological Treatment of Hydrothermal Liquefaction Wastewater

Paper title:

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

Published in:

Journal of Environmental Management, Volume 349, 1 January 2024, Pages 119527-119538

Authors' names:

Meicen Liu¹, Chandan Mahata², Zhiwu Wang², Sandeep Kumar³, Yi Zheng¹

Authors' affiliations:

1. Department of Grain Science and Industry, Kansas State University, 1980 Kimball Avenue,
Manhattan, KS 66506, USA
2. Department of Biological Systems Engineering, Virginia Tech, 1230 Washington St. SW,
Blacksburg, VA, 24061, USA
3. Department of Civil and Environmental Engineering, Old Dominion University, Norfolk, VA
23529, USA

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.

3.1 Introduction

Hydrothermal liquefaction (HTL) is a thermochemical conversion process conducted at 250-370°C and 4-22 MPa [1]. 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 [2]. 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 [3,4]. 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) [4]. 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 [2].

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 [5–9]. 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 [10,11]. 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) [12]. $\text{NH}_4^+\text{-N}$ was also shown to inhibit the formation and metabolic activity of aerobic biofilm at concentration above 25 mg/L [13]. 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 [2]. 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 [2]. As shown in Appendix Table A.1, 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 [6,9,14,15]. 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 (Appendix Table A.1) [9,16,17]. 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 (Appendix Table A.1) [18,19]. 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 [20].

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) [21]. In addition, fungi can survive and thrive under adverse conditions with high inhibitor concentrations and environmental challenges [22]. 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% [23]. In addition, filamentous fungi can form spherical pellets during cultivation, making it easier to separate the fungal biomass from the waste stream [24,25]. 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 (Appendix Table A.1). 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.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 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 [26]. 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.

3.2.1.2 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 [27,28]. 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, Appendix Figure A.1).

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 OD₆₀₀ 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 OD₆₀₀ 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 (Appendix Figure A.2). 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 (Appendix Figure A.2, a, b). Since Mad did not produce any spores, 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 (Appendix Figure A.2c). The large inocula were prepared with the 3-day-old fungal biomass established in potato dextrose broth (Becton Dickinson GmbH, Heidelberg, Germany) (Appendix Figure A.2, d-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.

3.2.1.3 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 [29]. 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

NH₄Cl, 10 g KH₂PO₄, 2.5 g CaCl₂·2H₂O, 5 g MgSO₄·7H₂O, and 5 g FeSO₄·7H₂O, respectively.

All the concentrated stocks were sterilized by filtration through a 0.22-μm syringe filter and stored at 4°C until used.

3.2.2 Cultivation of microorganisms in sludge HTLWW

3.2.2.1 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 3.2.1.3. 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.

3.2.2.2 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 3.2.2.1. 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).

3.2.2.3 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 3.2.2.1. Once the large fungal inocula were inoculated, flasks were allowed to equilibrate for 2 h before a sample of time “0” was taken to exclude any unspecific adsorption of wastewater components by the fungal biomass. Liquid samples were taken every 3 days for COD determination until the COD change was negligible (day 12). Once the COD stabilized, 85% of the liquid volume was replaced with freshly diluted HTLWW with the same composition as that initially used in this test. This stepwise wastewater feeding was to evaluate continuous performance of the fungal inocula.

3.2.3 Analytical methods

3.2.3.1 Microbial growth analysis

Bacterial growth during screening experiments was measured as OD_{600} , whereas fungal growth was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as absorbance at 560 nm [30–33]. MTT reacts with metabolically active cells to

develop a purple color that can be solubilized and quantified on a spectrophotometer. Intensity of the purple color is therefore directly related to the concentration of active cells (Appendix Figure A.3). Since DCM of fungus cannot be reliably determined for screening experiments conducted in microplates due to the small volume, MTT assay instead of optical density was used. MTT assay was performed immediately at the end of incubation when the tested filamentous fungi can grow into loose pellets consisting of intertwined hyphae, whereas the yeast cells settled down at the bottom of the microplate. In both cases, about three fourths of the supernatant in the microplate can be carefully replaced with potato dextrose broth while leaving the fungal biomass largely intact. Then, MTT solution (5 mg MTT solubilized in 1 mL of saline phosphate buffer solution) was added at 50 μ L MTT solution per mL of liquid in the 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- μ m filter paper and measuring the cell dry weight using oven drying at 105°C for overnight.

3.2.3.2 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 Appendix Table A.2. To quantify N-heterocyclic compounds, 1 mL of sample was adjusted to pH 2-4 with 20 μ L of 1 M sulfuric acid and extracted twice with dichloromethane. The dichloromethane extract of 1 μ L 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 μ L of 1 M sulfuric acid, and 30 μ L 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 N₂ 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 μ L 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 μ m 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 μ L with the column and RID temperatures at 60 and 45°C, respectively. Mobile phase was 5 mM H₂SO₄ 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).

3.2.3.3 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 [34]. 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 3.2.1.2. Upon inoculation of microbial biomass into HTLWW, the reactor was sealed and subjected to continuous nitrogen purging for 4 h, as shown in Appendix Figure A.4. 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.

3.2.4 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 ($\alpha = 0.05$ [35]). JMP Pro 16 software (SAS Institute, Cary, NC, USA) was used for all the statistical analyses.

3.3. Results and Discussion

3.3.1 Characteristics of Sludge HTLWW

Table 3.1 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 [35]. Since sewage sludge has high S content, the element likely migrates to HTLWW in the form of sulfate [36,37]. 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 [2]. 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 [38]. The low TP value of HTLWW is probably due to the phosphorus precipitation conducted after the HTL process.

Table 3.1 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 3.2 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 [39]. 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 (Appendix Table A.3). The major compounds identified in HTLWW were similar to those in the literature for HTLWW generated from sewage sludge [7,38,40]. 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 [7]. 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 3.2 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

^aData are presented as mean ± standard deviation of triplicate measurements.

3.3.2 Cultivation of microbes in HTLWW under screened conditions

3.3.2.1 Effect of dilution factor on microbial growth

The growth of bacteria and fungi in HTLWW diluted with commercial culture media is illustrated in Figure 3.1a 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 (Figure 3.1a). 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

(Figure 3.1b). Based on the results, HTLWW was diluted 10X for bacterial cultivation and 20X for fungal cultivation in the rest of the study.

3.3.2.2 Effect of pH on microbial growth

Figure 3.1c 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 3.2.1.3. 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 [41,42]. 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.

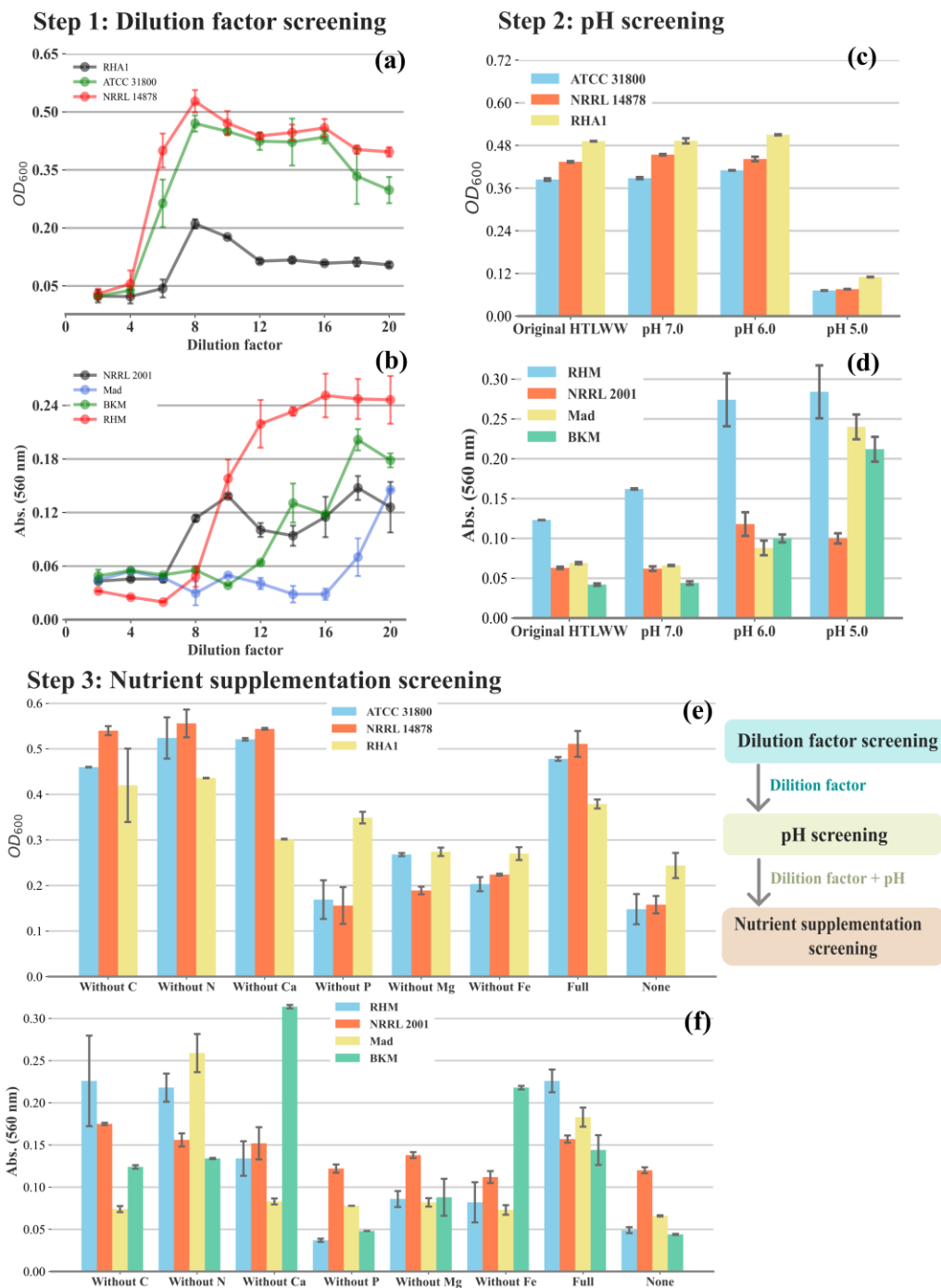


Figure 3.1 Growth of the tested microbes under screened HTLWW dilution factor (a, b), pH (c, d), and nutrient supplementation (e, f). Bacterial growth was quantified by measuring OD₆₀₀ on day 3 of the incubation. Fungal growth was quantified using MTT assay as the absorbance (Abs.)

at 560 nm on day 4 of the incubation. All the screening experiments were performed in duplicates.

3.3.2.3 Effect of nutrient supplementation on microbial growth

Nutrient supplementation showed different impact on the growth of microbial strains in HTLWW (Figure 3.1e 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 [43,44]. 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 3.1). 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 oculata*, where glucose supplementation was required for the growth of the yeast in HTLWW [19]. 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 3.3) for cultivation of the selected microbes in HTLWW in this study.

Table 3.3 Dilution factor, pH, and nutrient supplementation for microbial cultivation in HTLWW

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

3.3.3 Growth and COD removal with batch microbial cultivation

Table 3.4 lists the removal efficiency of quantifiable (on GC-MS) organic acids during cultivation of the tested microbes under conditions listed in Table 3.3, 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 OD₆₀₀ reached maximum on day 2 (Figure 3.2a). Similarly, the growth of *R. jostii* RHA1 mostly happened on the first 3 days but showed some fluctuation afterwards (Figure 3.2b). The removal efficiency of organic acids by RHA1 remained low throughout the 15-day cultivation period (14-19%, Table 3.4). 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 3.4). 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 3.4).

Table 3.4 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
ATCC 31800	Day 1	Day 2	Day 3
	74.5±2.0	82.5±0.7	82.7±1.7
	83.4±0.5	85.7±0.6	85.3±1.0
NRRL 14878	Day 6	Day12	Day 15
	14.2±4.1	17.1±1.9	19.1±2.6

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

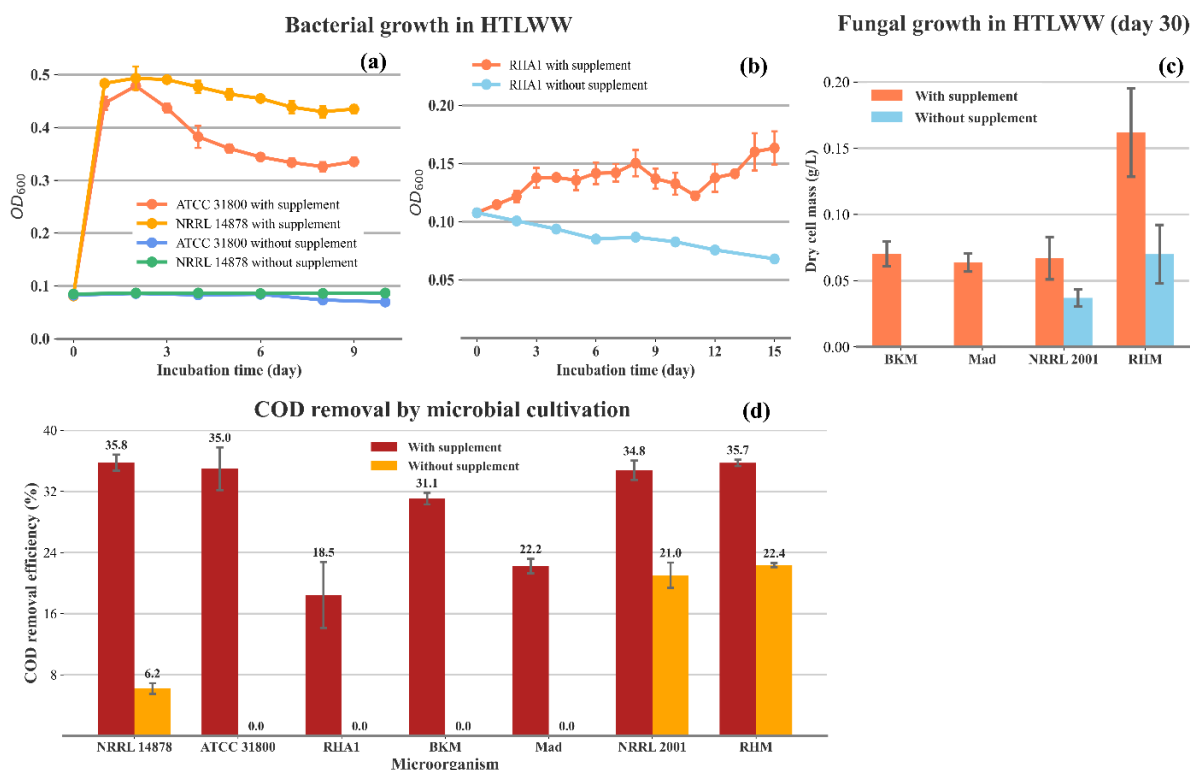


Figure 3.2 Growth of the tested microbes in HTLWW with and without nutrient supplementation (a-c) and wastewater COD removal efficiency after the microbial cultivation (d). If the COD values at the end of cultivation are not significantly different from the untreated HTLWW as determined

by Tukey-Kramer HSD test at $\alpha=0.05$, 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 Figure 3.2a-c. In general, supplementing nutrients significantly increased the growth of microbes, which confirmed the findings of screening experiments (section 3.3.2). 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 3.3. As shown in Figure 3.2d, significantly higher COD removal efficiencies were achieved at the end of HTLWW biodegradation with nutrient supplementation as compared to those without additional nutrients ($p<0.05$, Appendix Table A.4). 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 ($p<0.05$, Appendix Table A.4). 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 ($p<0.05$, Appendix Table A.4). 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 (Appendix Table A.1), this COD removal efficiency is still too low and additional treatment steps (e.g., adsorption and ozone oxidation) may be needed.

3.3.4 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 [45,46]. 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 (Appendix Figure A.2). 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.

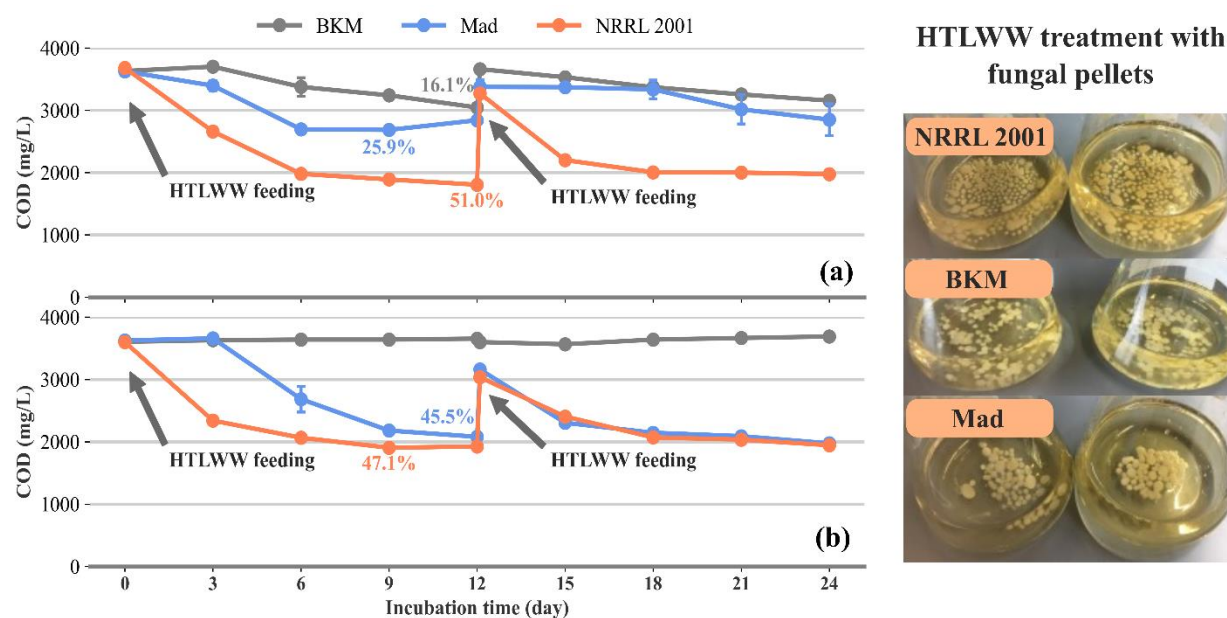


Figure 3.3 COD variation of HTLWW during treatment with large inocula of BKM, Mad, and NRRL 2001 with (a) and without (b) nutrients supplementation (n = 2). The highest COD removal efficiency prior to feeding additional HTLWW on day 12 was labeled.

Figure 3.3 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 treatments were comparable to that on day 9. Afterwards, additional HTLWW was fed as described in section 3.2.2.3 on day 12, and COD was monitored for another 12 days. For Mad and NRRL 2001, large inocula for HTLWW treatment significantly increased the COD removal efficiency compared with that of small inocula even without nutrient supplementation ($p < 0.05$, Appendix Table A.5). In addition, the higher COD removal efficiency was achieved in 12 days of incubation, which was much shorter than the 30-day incubation time needed for small inocula (Table 3.4 and Appendix Table A.5). The result suggested that nutrient supplementation was no longer necessary when large inocula of Mad and NRRL 2001 were used for HTLWW treatment, since the two strains achieved similar or higher COD removal efficiency without nutrient supplementation compared with the nutrient addition (Appendix Table A.5).

Specifically, the highest COD removal efficiency by Mad with and without supplementing nutrients were 25.9% and 45.5%, respectively (Figure 3.3a and b). For NRRL 2001, the highest COD removal efficiency with and without nutrients were 51% and 47.1%, respectively. After replacing 85% of the liquid volume in flask with freshly diluted HTLWW on day 12, the large inocula of Mad and NRRL 2001 continuously reduced COD of HTLWW without any nutrient supplementation (Figure 3.3b). On day 24, the COD of HTLWW treated with Mad and NRRL 2001 were comparable to that on day 12 prior to feeding fresh HTLWW. Therefore, it appears that that large inocula of Mad and NRRL 2001 can be continuously used for HTLWW treatment without the need of additional nutrient addition. On the contrary, BKM large inocula only achieved a COD removal efficiency of 16.1% with nutrient supplementation (Figure 3.3a), which was significantly lower than the 31.3% COD removal when small inocula was used ($p < 0.05$,

Appendix Table A.5). Without nutrient supplementation, BKM was not able to remove COD at all (Figure 3.3b). The lower COD removal efficiency by BKM large inocula compared with the small inocula could be due to the shorter incubation time or different metabolic states of the small and large fungal inocula [47]. It is well-accepted that the macromorphological structure of production fungal strains greatly affects the yield of bioprocess [24,47]. However, no simple relationship has been identified that could describe the connection between the morphology of filamentous fungi and their productivity [24]. In agreement with this, our study also suggested that the biodegradation effectiveness of fungal spores and pellets when cultivated in HTLWW is strain dependent.

The different performance of small and large fungal inocula during HTLWW treatment with and without nutrient supplementation suggested that the nutrient requirement for small and large fungal inocula are different. Specifically, sporulation and hyphae growth of the small fungal inocula may require higher levels of nutrients, such as P, Mg, Ca, and Fe, which were deficient in HTLWW (Table 3.1). Therefore, supplementation of these nutrients greatly benefited the HTLWW treatment performance of the small fungal inocula. However, the growth of small inocula in HTLWW were too slow for effective treatment of HTLWW even with nutrient supplementation, resulting in lower COD removal efficiencies after prolonged incubation (Figure 3.2d). On the other hand, the large fungal inocula was well developed fungal biomass that may not require the presence of P, Mg, Ca, and Fe at high concentrations. Therefore, the large inocula can be continuously used for HTLWW treatment without nutrient supplementation. In addition, since the growth of bacterial and yeast strains tested in this study were shown to heavily rely on the addition of P, Mg, and Fe, it will be less likely for them to outcompete the large inocula of Mad and NRRL 2001 when these nutrients are not supplemented, suggesting that large fungal

inocula may not be prone to contamination. Further research is needed to study the behavior of large 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 [48]. 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 Appendix Table A.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 3.5 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)

3.3.6 Biodegradation of chemical compounds

Besides COD removal, the removal of different chemical compounds in HTLWW by the tested microbes was also monitored (Figure 3.4). 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 Figure 3.4 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 3.2, Figure 3.4a). Valeric acid, 2-methylbutyric acid, 4-methylvaleric acid, and butyric acid were also organic acids of high concentration in HTLWW

(Table 3.2). 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-methylbutyric acid, 4-methylvaleric acid, and butyric acid (Figure 3.4a). Benzenepropanoic acid and phloretic acid are the two major phenolic compounds in HTLWW (Table 3.2). As shown in Figure 3.4a, 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 [49,50]. However, in this study, N-heterocyclic compounds were removed poorly by all the tested microbes, with removal efficiency less than 30% in most cases (Figure 3.4a). This could be due to the high concentration of these compounds in HTLWW (Table 3.2) 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 [16]. Therefore, it is of importance to develop microorganisms and treatment processes that can better remove N-heterocycles and/or remove these compounds with physico-chemical methods such as zeolite adsorption [6] and ozone treatment [51].

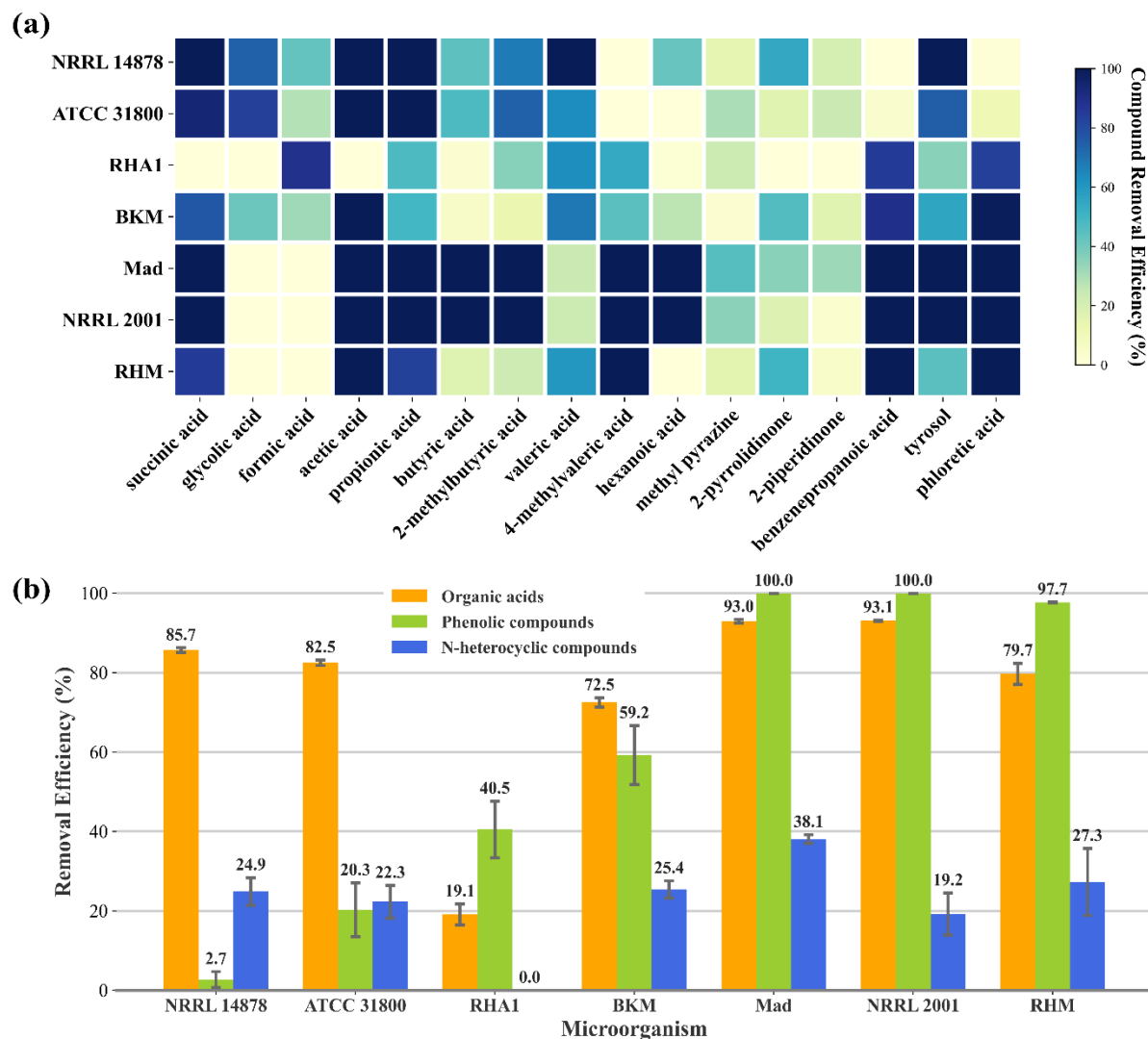


Figure 3.4 The removal efficiencies of individual compounds (a) and compounds in the categories of organic acids, phenolic compounds, and N-heterocyclic compounds (b) in HTLWW through biodegradation with different microbes (n=3 for all microbes, except for Mad and NRRL 2001 that have n=2).

The removal efficiencies of compounds under total organic acid, phenolic, and N-heterocycle categories were shown in Figure 3.4b. *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 isolated from textile wastewater and is known to degrade phenol [52,53]. In accordance with the literature, ATCC 31800 were able to degrade phenol present in HTLWW (Appendix Table A.3) [52,53]. In addition, ATCC 31800 also effectively removed other phenolic compounds, including tyrosol, *p*-cresol, and benzyl alcohol. NRRL 14878 is an isolate from municipal sewage sludge (Maryland, U.S.). The compound removal profile of this strain was similar to ATCC 31800 (Figure 3.4a), except that the strain was able to remove hexanoic acid and benzenecetic acid but not *p*-cresol and benzyl alcohol (Figure 3.4a, Appendix Table A.3). Our results suggested that *P. putida* strains ATCC 31800 and NRRL 14878 can only degrade a small fraction of the phenolic compounds in HTLWW. The bacterial strain RHA1 has been used for degrading HTLWW from algae and pine wood, during which the strain removed over 90% of COD and most of the organic compounds [18]. In addition, genetic and biochemical studies have demonstrated that RHA1 can degrade a variety of phenolic compounds including biphenyls, aromatic acids, and lignin [54]. However, when cultivated in HTLWW, RHA1 removed only 19.1% of organic acids, 40.5% of phenolic compounds, undetectable percentage of N-heterocyclic compounds, and 18.5% of COD (Figure 3.2d and 4b). The significantly lower COD removal efficiency in our study by RHA1 as compared with the previous study suggested that some inhibitors (e.g., TAN) might be present in HTLWW that greatly reduced the performance of RHA1. Therefore, in the next section, we examined the effect of TAN removal from HTLWW on its biological treatment with RHA1.

Compared with the bacterial strains, fungal strains were better at removing phenolic compounds, with BKM having the lowest removal efficiency, 59.2%, and all other fungal strains having removal efficiencies above 97% (Figure 3.4b). In addition, all the fungal strain effectively

removed phenylpropanamide, which failed to be removed by the bacterial strains (Appendix Table A.3). The better degradation of phenolic compounds by fungal strains is likely due to the non-specificity of fungal enzymes which allow the microbes to target a wider variety of compounds [21]. Among the tested fungal strains, RHM is closely related to *Rhodotorula mucilaginosa*. Yeast isolates identified with close relationship with *R. mucilaginosa* have been used to treat olive mill wastewater that is high in phenolic compound toxicity and were known to produce lipids and biosurfactants as value-added products [55,56]. Both NRRL 2001 and Mad large inocula were able to remove over 93% of the organic acids and all the phenolic compounds quantified in this study, making them the most effective strains for treatment of HTLWW in this study (Figure 3.4b). *A. niger* NRRL 2001 had been used for production of citric acid from agro-industrial waste [57]. In addition, the strain was better than other organic acid-producing *A. niger* strains (NRRL 3122 and NRRL 567) for bioleaching of loblolly pine, suggesting that NRRL 2001 is less dependent on nutritious substrates for growth and can better tolerate inhibitory phenolic compounds [58]. White-rot fungi (e.g., *T. versicolor* and *P. chrysosporium*) are known to degrade a wide range of pollutants, such as PAHs, pharmaceutical waste, and synthetic dyes [59]. Between the two white-rot fungi examined in this study, BKM had stronger tolerance to HTLWW than Mad (Figure 3.1b). However, the strain may require supplementation of Mg and P to achieve satisfactory bioremediation, especially if the feedstock is deficient in them (Figure 3.2d and 3). On the other hand, when established fungal pellets (large inocula) of Mad were used for treatment of HTLWW, the fungal biomass can effectively and continuously degrade a variety of organic acids and phenolic compounds without the need of supplementary nutrients (Figure 3.3 and Figure 3.4). Similarly, immobilized biomass of *T. versicolor* on sorghum grains have

been used for treatment of wastewater rich in humic acids and was able to continuously remove color for four batches [60].

3.3.7 Effect of TAN removal on biological treatment of HTLWW

TAN is a well-known inhibitor of AD and was shown to inhibit the formation and metabolism of aerobic biofilm [12,13]. Ammonium salts inhibited the growth of some fungi but stimulated the growth of others [61,62]. In this study, TAN removal was conducted by stripping the HTLWW at pH 12 and 50°C overnight until TAN of HTLWW was reduced to undetectable level (Table 3.6). The TAN stripping was also shown to remove volatile organic and inorganic contaminants, which led to COD reduction [62,63]. In this study, the COD of HTLWW was reduced by 20.1% along with the TAN removal (Table 3.6).

Table 3.6 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

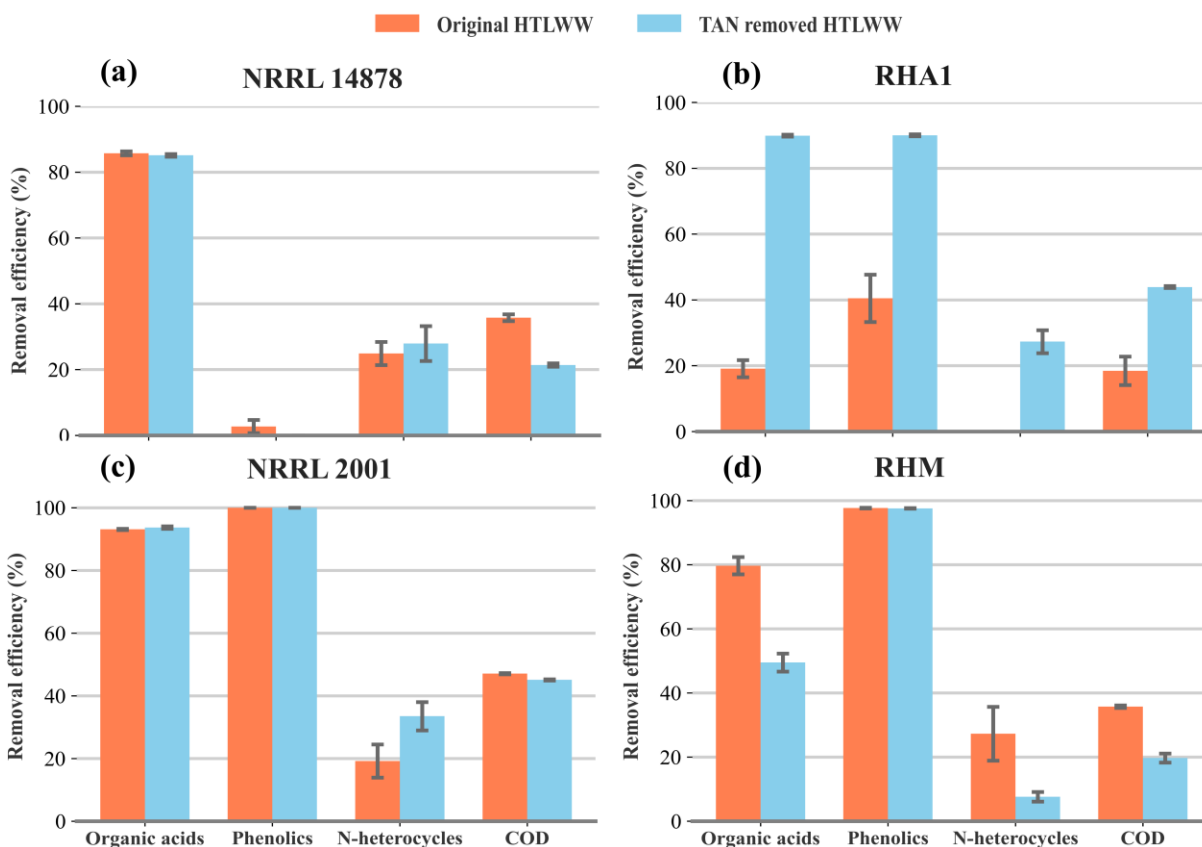


Figure 3.5 Removal efficiencies of organic acids, phenolic compounds, N-heterocyclic compounds, and COD by NRRL 14878 (a), RHA1 (b), NRRL 2001 (c), and RHM (d) when cultivated in original and TAN removed HTLWW. n=3 for organic acids, N-heterocycles, and COD removal data for NRRL 14878, RHA1, and RHM, and n=2 for all other data.

The organic compound and COD removal efficiencies upon biological treatment of HTLWW with and without TAN removal are shown in Figure 3.5. TAN stripping increased the removal efficiency of N-heterocyclic compounds by NRRL 14878 and NRRL 2001 but did not improve the removal efficiency of organic acids and phenolic compounds (Figure 3.5, a and c). Specifically, TAN stripping increased the removal of 2-pyrrolidinone for both strains and increased the removal of methyl pyrazine and 2-piperidione for NRRL 2001 only (Appendix Figure A.5). TAN stripping improved the treatment performance of RHA1 greatly, with the

removal efficiencies of organic acids, phenolics, and N-heterocycles, and COD being increased by 4.7, 2.2, 9.1, and 2.4 folds, respectively (Figure 3.5b). With the TAN removal, RHA1 was able to completely degrade acetic acid, 2-methylbutyric acid, hexanoic acid, and methyl pyrazine, which were poorly degraded by the strain in the original HTLWW (Appendix Figure A.5). Therefore, TAN appeared to be a strong inhibitor of RHA1 when the concentration is high. On the contrary, TAN removal hampered the biodegradation of HTLWW by RHM. Final dry cell mass of the strain was reduced by half, and COD removal efficiency decreased from 35.7% to 19.7% (Figure 3.5d). Although RHM still degraded over 97% of phenolic compounds in HTLWW with TAN removal, the strain removed less organic acids and N-heterocycles. Our result suggested that RHM might lack the metabolic pathway for effective degradation and utilization of N-heterocyclic compounds since it appeared to require the presence of TAN as nitrogen source.

3.4 Conclusions

A comparative study was conducted on the biodegradation of sludge HTLWW with three bacterial strains and four fungal strains. COD removal during biological treatment was greatly improved through pH adjustment and nutrient supplementation of P, Mg, Ca, and Fe in a strain dependent manner. Bacterial strains were able to tolerate 10X dilution of HTLWW without pH adjustment and required shorter time for biodegradation. Among bacterial strains, *P. putida* ATCC 31800 and NRRL 14878 demonstrated rapid growth in HTLWW and removed 35% of COD. Biodegradation by *R. jostii* RHA1 was greatly inhibited by the high TAN concentration in sludge HTLWW. TAN stripping improved the COD removal efficiency of the strain by 2.4 folds to 44% COD removal. HTLWW treatment with fungal strains required 20X dilution, pH adjustment to 5, and longer treatment time of 12-30 days. However, fungal strains were shown to

degrade a wider range of compounds, especially phenolic compounds which can be degraded for 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.

Acknowledgements

This work was supported by the National Science Foundation [Grant number: 2001593]. The authors thank Dr. Xavier Fonoll from Great Lakes Water Authority (GLWA) for providing the sludge HTLWW used throughout this study. We also thank Dr. Lindsay D. Eltis (Departments of Microbiology and Biochemistry, The University of British Columbia, Vancouver, Canada) for offering us *R. jostii* RHA1. We highly appreciate the support of the U.S. Department of Agriculture Forest Service and Agricultural Research Service Culture Collection (Northern Regional Research Laboratory, U.S. Department of Agriculture) that provided us the fungal and bacterial strains for this study.

References

- [1] A.R.K. Gollakota, N. Kishore, S. Gu, A review on hydrothermal liquefaction of biomass, *Renew. Sustain. Energy Rev.* 81 (2018) 1378–1392. <https://doi.org/10.1016/j.rser.2017.05.178>.
- [2] J. Watson, T. Wang, B. Si, W.-T. Chen, A. Aierzhati, Y. Zhang, Valorization of hydrothermal liquefaction aqueous phase: pathways towards commercial viability, *Prog. Energy Combust. Sci.* 77 (2020) 100819. <https://doi.org/10.1016/j.pecs.2019.100819>.

- [3] M. Pham, L. Schideman, B.K. Sharma, Y. Zhang, W.-T. Chen, Effects of hydrothermal liquefaction on the fate of bioactive contaminants in manure and algal feedstocks, *Bioresour. Technol.* 149 (2013) 126–135. <https://doi.org/10.1016/j.biortech.2013.08.131>.
- [4] Y. Gu, X. Zhang, B. Deal, L. Han, Biological systems for treatment and valorization of wastewater generated from hydrothermal liquefaction of biomass and systems thinking: A review, *Bioresour. Technol.* 278 (2019) 329–345. <https://doi.org/10.1016/j.biortech.2019.01.127>.
- [5] W.-T. Chen, Y. Zhang, J. Zhang, G. Yu, L.C. Schideman, P. Zhang, M. Minarick, Hydrothermal liquefaction of mixed-culture algal biomass from wastewater treatment system into bio-crude oil, *Bioresour. Technol.* 152 (2014) 130–139. <https://doi.org/10.1016/j.biortech.2013.10.111>.
- [6] R. Li, D. Liu, Y. Zhang, J. Zhou, Y.F. Tsang, Z. Liu, N. Duan, Y. Zhang, Improved methane production and energy recovery of post-hydrothermal liquefaction waste water via integration of zeolite adsorption and anaerobic digestion, *Sci. Total Environ.* 651 (2019) 61–69. <https://doi.org/10.1016/j.scitotenv.2018.09.175>.
- [7] B. Maddi, E. Panisko, T. Wietsma, T. Lemmon, M. Swita, K. Albrecht, D. Howe, Quantitative Characterization of Aqueous Byproducts from Hydrothermal Liquefaction of Municipal Wastes, Food Industry Wastes, and Biomass Grown on Waste, *ACS Sustain. Chem. Eng.* 5 (2017) 2205–2214. <https://doi.org/10.1021/acssuschemeng.6b02367>.
- [8] M. Usman, H. Chen, K. Chen, S. Ren, J.H. Clark, J. Fan, G. Luo, S. Zhang, Characterization and utilization of aqueous products from hydrothermal conversion of biomass for bio-oil and hydro-char production: a review, *Green Chem.* 21 (2019) 1553–1572. <https://doi.org/10.1039/C8GC03957G>.

- [9] L. Yang, B. Si, X. Tan, H. Chu, X. Zhou, Y. Zhang, Y. Zhang, F. Zhao, Integrated anaerobic digestion and algae cultivation for energy recovery and nutrient supply from post-hydrothermal liquefaction wastewater, *Bioresour. Technol.* 266 (2018) 349–356. <https://doi.org/10.1016/j.biortech.2018.06.083>.
- [10] U. Ekpo, A.B. Ross, M.A. Camargo-Valero, P.T. Williams, A comparison of product yields and inorganic content in process streams following thermal hydrolysis and hydrothermal processing of microalgae, manure and digestate, *Bioresour. Technol.* 200 (2016) 951–960. <https://doi.org/10.1016/j.biortech.2015.11.018>.
- [11] G. Yu, Y. Zhang, B. Guo, T. Funk, L. Schideman, Nutrient Flows and Quality of Bio-crude Oil Produced via Catalytic Hydrothermal Liquefaction of Low-Lipid Microalgae, *BioEnergy Res.* 7 (2014) 1317–1328. <https://doi.org/10.1007/s12155-014-9471-3>.
- [12] O. Yenigün, B. Demirel, Ammonia inhibition in anaerobic digestion: A review, *Process Biochem.* 48 (2013) 901–911. <https://doi.org/10.1016/j.procbio.2013.04.012>.
- [13] X. Weng, Z. Mao, H.-M. Fu, Y.-P. Chen, J.-S. Guo, F. Fang, X.-W. Xu, P. Yan, Biofilm formation during wastewater treatment: Motility and physiological response of aerobic denitrifying bacteria under ammonia stress based on surface plasmon resonance imaging, *Bioresour. Technol.* 361 (2022) 127712. <https://doi.org/10.1016/j.biortech.2022.127712>.
- [14] P. Wang, Y. Sakhno, S. Adhikari, H. Peng, D. Jaisi, T. Soneye, B. Higgins, Q. Wang, Effect of ammonia removal and biochar detoxification on anaerobic digestion of aqueous phase from municipal sludge hydrothermal liquefaction, *Bioresour. Technol.* 326 (2021) 124730. <https://doi.org/10.1016/j.biortech.2021.124730>.
- [15] M. Zheng, L.C. Schideman, G. Tommaso, W.-T. Chen, Y. Zhou, K. Nair, W. Qian, Y. Zhang, K. Wang, Anaerobic digestion of wastewater generated from the hydrothermal

- liquefaction of *Spirulina*: Toxicity assessment and minimization, *Energy Convers. Manag.* 141 (2017) 420–428. <https://doi.org/10.1016/j.enconman.2016.10.034>.
- [16] M. Pham, L. Schideman, J. Scott, N. Rajagopalan, M.J. Plewa, Chemical and Biological Characterization of Wastewater Generated from Hydrothermal Liquefaction of *Spirulina*, *Environ. Sci. Technol.* 47 (2013) 2131–2138. <https://doi.org/10.1021/es304532c>.
- [17] Y. Zhou, L. Schideman, G. Yu, Y. Zhang, A synergistic combination of algal wastewater treatment and hydrothermal biofuel production maximized by nutrient and carbon recycling, *Energy Environ. Sci.* 6 (2013) 3765. <https://doi.org/10.1039/c3ee24241b>.
- [18] Y. He, X. Li, X. Xue, M.S. Swita, A.J. Schmidt, B. Yang, Biological conversion of the aqueous wastes from hydrothermal liquefaction of algae and pine wood by *Rhodococci*, *Bioresour. Technol.* 224 (2017) 457–464. <https://doi.org/10.1016/j.biortech.2016.10.059>.
- [19] M. Nelson, L. Zhu, A. Thiel, Y. Wu, M. Guan, J. Minty, H.Y. Wang, X.N. Lin, Microbial utilization of aqueous co-products from hydrothermal liquefaction of microalgae *Nannochloropsis oculata*, *Bioresour. Technol.* 136 (2013) 522–528. <https://doi.org/10.1016/j.biortech.2013.03.074>.
- [20] L.T. Cordova, B.C. Lad, S.A. Ali, A.J. Schmidt, J.M. Billing, K. Pomraning, B. Hofstad, M.S. Swita, J.R. Collett, H.S. Alper, Valorizing a hydrothermal liquefaction aqueous phase through co-production of chemicals and lipids using the oleaginous yeast *Yarrowia lipolytica*, *Bioresour. Technol.* 313 (2020) 123639. <https://doi.org/10.1016/j.biortech.2020.123639>.
- [21] M. Naghdi, M. Taheran, S.K. Brar, A. Kermanshahi-pour, M. Verma, R.Y. Surampalli, Removal of pharmaceutical compounds in water and wastewater using fungal oxidoreductase enzymes, *Environ. Pollut.* 234 (2018) 190–213. <https://doi.org/10.1016/j.envpol.2017.11.060>.

- [22] T.T. More, S. Yan, R.D. Tyagi, R.Y. Surampalli, Potential use of filamentous fungi for wastewater sludge treatment, *Bioresour. Technol.* 101 (2010) 7691–7700.
<https://doi.org/10.1016/j.biortech.2010.05.033>.
- [23] S. Mannan, A. Fakhru'l-Razi, M.Z. Alam, Use of fungi to improve bioconversion of activated sludge, *Water Res.* 39 (2005) 2935–2943. <https://doi.org/10.1016/j.watres.2005.04.074>.
- [24] L. Veiter, V. Rajamanickam, C. Herwig, The filamentous fungal pellet—relationship between morphology and productivity, *Appl. Microbiol. Biotechnol.* 102 (2018) 2997–3006.
<https://doi.org/10.1007/s00253-018-8818-7>.
- [25] J. Zhang, J. Zhang, The filamentous fungal pellet and forces driving its formation, *Crit. Rev. Biotechnol.* 36 (2016) 1066–1077. <https://doi.org/10.3109/07388551.2015.1084262>.
- [26] D. Cronin, A.J. Schmidt, J. Billing, T.R. Hart, S.P. Fox, X. Fonoll, J. Norton, M.R. Thorson, Comparative Study on the Continuous Flow Hydrothermal Liquefaction of Various Wet-Waste Feedstock Types, *ACS Sustain. Chem. Eng.* 10 (2022) 1256–1266.
<https://doi.org/10.1021/acssuschemeng.1c07214>.
- [27] M. Johnson, I. Zaretskaya, Y. Raytselis, Y. Merezuk, S. McGinnis, T.L. Madden, NCBI BLAST: a better web interface, *Nucleic Acids Res.* 36 (2008) W5–W9.
<https://doi.org/10.1093/nar/gkn201>.
- [28] E.W. Sayers, E.E. Bolton, J.R. Brister, K. Canese, J. Chan, D.C. Comeau, R. Connor, K. Funk, C. Kelly, S. Kim, T. Madej, A. Marchler-Bauer, C. Lanczycki, S. Lathrop, Z. Lu, F. Thibaud-Nissen, T. Murphy, L. Phan, Y. Skripchenko, T. Tse, J. Wang, R. Williams, B.W. Trawick, K.D. Pruitt, S.T. Sherry, Database resources of the national center for biotechnology information, *Nucleic Acids Res.* 50 (2022) D20–D26. <https://doi.org/10.1093/nar/gkab1112>.

- [29] M. Ijadpanahsaravi, M. Punt, H.A.B. Wösten, W.R. Teertstra, Minimal nutrient requirements for induction of germination of *Aspergillus niger* conidia, *Fungal Biol.* 125 (2021) 231–238. <https://doi.org/10.1016/j.funbio.2020.11.004>.
- [30] L. Benov, Effect of growth media on the MTT colorimetric assay in bacteria, *PLOS ONE* 14 (2019) e0219713. <https://doi.org/10.1371/journal.pone.0219713>.
- [31] Z. Cui, M. Montanaro, A. Gerace, J.R. Schott, B. Markham, Requirement sensitivity studies for a future Landsat sensor, in: *Earth Obs. Syst. XX*, SPIE, 2015: pp. 204–210. <https://doi.org/10.1117/12.2188482>.
- [32] Z. Cui, J.P. Kerekes, Potential of Red Edge Spectral Bands in Future Landsat Satellites on Agroecosystem Canopy Green Leaf Area Index Retrieval, *Remote Sens.* 10 (2018) 1458. <https://doi.org/10.3390/rs10091458>.
- [33] H. Liu, J. Wang, J. Zhao, S. Lu, J. Wang, W. Jiang, Z. Ma, L. Zhou, Isoquinoline Alkaloids from *Macleaya cordata* Active against Plant Microbial Pathogens, *Nat. Prod. Commun.* 4 (2009) 1934578X0900401. <https://doi.org/10.1177/1934578X0900401120>.
- [34] X. Ji, M. Zhu, M. Li, N. Wang, M. Li, L. Song, R. Shan, Adsorption and Degradation of Organics in Wastewater on Municipal Sludge, *ACS Omega* 8 (2023) 33349–33357. <https://doi.org/10.1021/acsomega.3c02765>.
- [35] J. Lu, J. Watson, Z. Liu, Y. Wu, Elemental migration and transformation during hydrothermal liquefaction of biomass, *J. Hazard. Mater.* 423 (2022) 126961. <https://doi.org/10.1016/j.jhazmat.2021.126961>.
- [36] R. Li, D. Liu, Y. Zhang, G. Tommaso, B. Si, Z. Liu, N. Duan, Enhanced anaerobic digestion of post-hydrothermal liquefaction wastewater: Bio-methane production, carbon

distribution and microbial metabolism, *Sci. Total Environ.* 837 (2022) 155659.

<https://doi.org/10.1016/j.scitotenv.2022.155659>.

[37] A.A. Shah, S.S. Toor, F. Conti, A.H. Nielsen, L.A. Rosendahl, Hydrothermal liquefaction of high ash containing sewage sludge at sub and supercritical conditions, *Biomass Bioenergy* 135 (2020) 105504. <https://doi.org/10.1016/j.biombioe.2020.105504>.

[38] I.A. Basar, H. Liu, C. Eskicioglu, Incorporating hydrothermal liquefaction into wastewater treatment – Part III: Aqueous phase characterization and evaluation of on-site treatment, *Chem. Eng. J.* 467 (2023) 143422. <https://doi.org/10.1016/j.cej.2023.143422>.

[39] M. Gong, S. Nanda, M.J. Romero, W. Zhu, J.A. Kozinski, Subcritical and supercritical water gasification of humic acid as a model compound of humic substances in sewage sludge, *J. Supercrit. Fluids* 119 (2017) 130–138. <https://doi.org/10.1016/j.supflu.2016.08.018>.

[40] K. Kapusta, Effect of ultrasound pretreatment of municipal sewage sludge on characteristics of bio-oil from hydrothermal liquefaction process, *Waste Manag.* 78 (2018) 183–190. <https://doi.org/10.1016/j.wasman.2018.05.043>.

[41] J. Rousk, P.C. Brookes, E. Bååth, Investigating the mechanisms for the opposing pH relationships of fungal and bacterial growth in soil, *Soil Biol. Biochem.* 42 (2010) 926–934. <https://doi.org/10.1016/j.soilbio.2010.02.009>.

[42] S. Sankaran, S.K. Khanal, N. Jasti, B. Jin, A.L. Pometto, J.H. Van Leeuwen, Use of Filamentous Fungi for Wastewater Treatment and Production of High Value Fungal Byproducts: A Review, *Crit. Rev. Environ. Sci. Technol.* 40 (2010) 400–449. <https://doi.org/10.1080/10643380802278943>.

[43] M. Sandy, A. Butler, Microbial Iron Acquisition: Marine and Terrestrial Siderophores, *Chem. Rev.* 109 (2009) 4580–4595. <https://doi.org/10.1021/cr9002787>.

- [44] G.M. Walker, The Roles of Magnesium in Biotechnology, Crit. Rev. Biotechnol. 14 (1994) 311–354. <https://doi.org/10.3109/07388559409063643>.
- [45] L.H. Grimm, S. Kelly, J. Hengstler, A. Göbel, R. Krull, D.C. Hempel, Kinetic studies on the aggregation of *Aspergillus niger* conidia: Conidial Aggregation, Biotechnol. Bioeng. 87 (2004) 213–218. <https://doi.org/10.1002/bit.20130>.
- [46] C. Wittmann, R. Krull, eds., Biosystems Engineering II, Springer Berlin Heidelberg, Berlin, Heidelberg, 2010. <https://doi.org/10.1007/978-3-642-13866-9>.
- [47] M. Tegelaar, D. Aerts, W.R. Teertstra, H.A.B. Wösten, Spatial induction of genes encoding secreted proteins in micro-colonies of *Aspergillus niger*, Sci. Rep. 10 (2020) 1536. <https://doi.org/10.1038/s41598-020-58535-0>.
- [48] Z. An, X. Zhang, Y. Zheng, Z.-W. Wang, Aerobic granulation of single culture protist, Process Biochem. 110 (2021) 163–167. <https://doi.org/10.1016/j.procbio.2021.08.014>.
- [49] K.V. Padoley, S.N. Mudliar, R.A. Pandey, Heterocyclic nitrogenous pollutants in the environment and their treatment options – An overview, Bioresour. Technol. 99 (2008) 4029–4043. <https://doi.org/10.1016/j.biortech.2007.01.047>.
- [50] R.K. Singh, R. Tripathi, A. Ranjan, A.K. Srivastava, Chapter 9 - Fungi as potential candidates for bioremediation, in: P. Singh, A. Kumar, A. Borthakur (Eds.), Abat. Environ. Pollut., Elsevier, 2020: pp. 177–191. <https://doi.org/10.1016/B978-0-12-818095-2.00009-6>.
- [51] L. Yang, B. Si, M.A. Martins, J. Watson, H. Chu, Y. Zhang, X. Tan, X. Zhou, Y. Zhang, Improve the biodegradability of post-hydrothermal liquefaction wastewater with ozone: conversion of phenols and N-heterocyclic compounds, Water Sci. Technol. 2017 (2018) 248–255. <https://doi.org/10.2166/wst.2018.108>.

- [52] G. Annadurai, R.-S. Juang, D.-J. Lee, Microbiological degradation of phenol using mixed liquors of *Pseudomonas putida* and activated sludge, *Waste Manag.* 22 (2002) 703–710. [https://doi.org/10.1016/S0956-053X\(02\)00050-8](https://doi.org/10.1016/S0956-053X(02)00050-8).
- [53] R.-S. Juang, K.-J. Tseng, Experimental investigation of bio-removal of toxic organic pollutants from highly saline solutions in a triphasic system, *J. Hazard. Mater.* 178 (2010) 706–712. <https://doi.org/10.1016/j.jhazmat.2010.01.145>.
- [54] K.C. Yam, S. Okamoto, J.N. Roberts, L.D. Eltis, Adventures in *Rhodococcus* — from steroids to explosives This article is based on a presentation by Dr. Lindsay Eltis at the 60th Annual Meeting of the Canadian Society of Microbiologists in Hamilton, Ontario, 14 June 2010. Dr. Eltis was the recipient of the 2010 Norgen Biotek Corporation / CSM Award, an annual award sponsored by Norgen Biotek and the Canadian Society of Microbiologists intended to recognize outstanding scientific work in microbiology by a Canadian researcher., *Can. J. Microbiol.* 57 (2011) 155–168. <https://doi.org/10.1139/W10-115>.
- [55] L. Derguine-Mecheri, S. Kebbouche-Gana, D. Djenane, Biosurfactant production from newly isolated *Rhodotorula* sp.YBR and its great potential in enhanced removal of hydrocarbons from contaminated soils, *World J. Microbiol. Biotechnol.* 37 (2021) 18. <https://doi.org/10.1007/s11274-020-02983-3>.
- [56] T.M. Jiru, D. Abate, N. Kiggundu, C. Pohl, M. Groenewald, Oleaginous yeasts from Ethiopia, *AMB Express* 6 (2016) 78. <https://doi.org/10.1186/s13568-016-0242-8>.
- [57] G.S. Dhillon, S.K. Brar, S. Kaur, M. Verma, Screening of agro-industrial wastes for citric acid bioproduction by *Aspergillus niger* NRRL 2001 through solid state fermentation, *J. Sci. Food Agric.* 93 (2013) 1560–1567. <https://doi.org/10.1002/jsfa.5920>.

- [58] M. Liu, J. Hunter, J. Shi, Y. Zheng, Bioleaching to produce clean loblolly pine for thermochemical conversion, *Fuel* 344 (2023) 128148.
<https://doi.org/10.1016/j.fuel.2023.128148>.
- [59] D. Gao, L. Du, J. Yang, W.-M. Wu, H. Liang, A critical review of the application of white rot fungus to environmental pollution control, *Crit. Rev. Biotechnol.* 30 (2010) 70–77.
<https://doi.org/10.3109/07388550903427272>.
- [60] M. Zahmatkesh, H. Spanjers, J.B. van Lier, A novel approach for application of white rot fungi in wastewater treatment under non-sterile conditions: immobilization of fungi on sorghum, *Environ. Technol.* 39 (2018) 2030–2040. <https://doi.org/10.1080/09593330.2017.1347718>.
- [61] D.A. DePasquale, T.J. Montville, Mechanism by which ammonium bicarbonate and ammonium sulfate inhibit mycotoxigenic fungi, *Appl. Environ. Microbiol.* 56 (1990) 3711–3717. <https://doi.org/10.1128/aem.56.12.3711-3717.1990>.
- [62] K. Veverka, J. Štolcová, P. Růžek, Sensitivity of fungi to urea, ammonium nitrate and their quimolar solution UAN, *Plant Prot. Sci.* 43 (2007) 157–164. <https://doi.org/10.17221/2239-PPS>.
- [63] M. Poveda, Q. Yuan, The Effectiveness of Pretreatment Methods on COD and Ammonia Removal from Landfill Leachate, *Int. J. Environ. Sci. Dev.* 7 (2016) 257–262.
<https://doi.org/10.7763/IJESD.2016.V7.780>.

Chapter 4 - Harnessing Fungal-Bacterial Co-culture for Treatment and Valorization of Hydrothermal Liquefaction Aqueous Phase

Paper title:

Harnessing filamentous fungi and fungal-bacterial co-culture for biological treatment and valorization of hydrothermal liquefaction aqueous phase from corn stover

Published in:

Bioresource Technology, Volume 409, October 2024, Pages 131240-131250

Authors' names:

Meicen Liu¹, Jiefu Wang², Isamu Umeda³, Zhiwu Wang², Sandeep Kumar³, Yi Zheng¹

Authors' affiliations:

1. Department of Grain Science and Industry, Kansas State University, 1980 Kimball Avenue, Manhattan, KS 66506, USA
2. Department of Biological Systems Engineering, Virginia Tech, 1230 Washington St. SW, Blacksburg, VA, 24061, USA
3. Department of Civil and Environmental Engineering, Old Dominion University, Norfolk, VA 23529, USA

Abstract

To promote the sustainability of hydrothermal liquefaction (HTL) for biofuel production, fungal fermentation was investigated to treat HTL aqueous phase (HTLAP) from corn stover. The most promising fungus, *Aspergillus niger* demonstrated superior tolerance to HTLAP and capability to produce oxalic acid as a value-added product. The fungal-bacterial co-culture of *A. niger* and *Rhodococcus jostii* was beneficial at low COD (chemical oxygen demand) loading of 3800 mg/L in HTLAP, achieving 69% COD removal while producing 0.5 g/L oxalic acid and 11% lipid content in microbial biomass. However, higher COD loading of 4500, 6040, and 7800 mg/L significantly inhibited *R. jostii*, but promoted *A. niger* growth with increased oxalic acid production while COD removal remained similar (58-65%). Additionally, most total organic carbon (TOC) in HTLAP was transformed into oxalic acid, representing 46-56% of the consumed TOC. These findings highlighted the potential of fungi for bio-upcycling of HTLAP into value-added products.

4.1 Introduction

Crop residue represents one of the most abundant feedstocks of renewable energy [1]. Among them, corn stover has been extensively studied and demonstrated great potential to be used for biofuel production. Hydrothermal liquefaction (HTL) is a type of thermochemical conversion method that is generally conducted at 250-370°C and 4-22 MPa in the presence of water serving as a reactant and catalyst [1]. HTL bypasses the need for a time-consuming and energy-intensive biomass drying step, as required in pyrolysis and gasification. Consequently, it has been receiving increasing attention over the past few years. Numerous studies have examined the HTL of corn stover under various reaction conditions, aiming to improve the yield and quality of biocrude [2,3].

Yet, hydrothermal liquefaction aqueous phase (HTLAP) poses a challenge as an emerging hazardous waste and a source of significant energy loss, necessitating the development of effective methods for treatment and valorization to facilitate the commercial viability of HTL [2]. Approximately 45% of organic carbon and 80% of other nutrients (e.g., nitrogen and phosphorous) of the feedstock end up in the HTLAP [2,4]. Different methods have been developed for recovering energy and economic output from HTLAP, including recycling, catalytic gasification, aqueous phase reforming, microalgae cultivation, anaerobic digestion, etc. [3,5,6], of which catalytic gasification and anaerobic digestion (AD) have been the most studied methods [3]. Recently, aqueous phase reforming (APR) has emerged to be a promising technology for valorizing HTLAP given its milder reaction conditions compared with gasification and its production of H₂ (used for HTL biocrude upgrading) [6,7]. However, each of these existing methods has its own unique benefits and drawbacks. Gasification and APR both require high temperature (220-400°C) and may suffer from significant catalyst deactivation depending on HTLAP composition [7,8]. On the other hand, the presence of inhibitors in HTLAP leads to long incubation time and heavy dilution requirement (100- to 1000-fold dilution) for AD treatment and microalgae cultivation [3,9].

Aerobic fermentation is a widely used method for industrial wastewater valorization [10,11]. However, it is less studied for HTLAP, even though existing literature suggested that aerobic fermentation could tolerate much more concentrated HTLAP than AD and microalgae cultivation. Bacteria of the *Rhodococcus* genus are known to be able to convert lignin to lipids [12]. He et al. examined *R. opacus* PD630, *R. jostii* RHA1, and *R. jostii* RHA1 VanA for biodegradation of HTLAP from algae and pine wood [13] and found that *R. jostii* RHA1 and its mutant removed 92-94% chemical oxygen demand (COD) on day 11 of cultivation in undiluted

HTLAP. In addition, the two strains degraded a wide variety of N&O heterocyclic and phenolic compounds in HTLAP while accumulating 9-35 wt% lipids, making their fermentation a promising option for valorizing HTLAP. Cordova et al. cultivated an oleaginous yeast species *Yarrowia lipolytica* in 5-fold diluted HTLAP with the supplementation of glucose- and xylose-rich corn stover hydrolysate and other nutrients to produce lipid and valuable chemicals [14]. Recently, a comparative study was conducted on the fermentation of HTLAP with various strains of bacteria (*Pseudomonas putida* and *R. jostii*) and fungi (*Rhodotorula sp.*, *A. niger*, *P. chrysosporium*, and *T. versicolor*), of which the filamentous fungi *A. niger* and *T. versicolor* demonstrated great potential for HTLAP fermentation [15]. Unlike the bacterial strains, *A. niger* and *T. versicolor* were more tolerant of the HTLAP which lacks key minerals, such as P, Fe, and Mg for microbial growth. In addition, the two fungi degraded a wider variety of compounds, including those present in trace concentrations, and achieved higher COD removal than other tested microbes. Due to the filamentous nature of the fungi, their separation from HTLAP after cultivation is easier, contributing to the reduction of operation cost (Sankaran et al., 2010).

To the best of current knowledge, little work has been done on filamentous fungus fermentation for the valorization of HTLAP. Therefore, this research aims to achieve three objectives: 1) compare organic compound and COD removal by three fungi, identifying the strains and fermentation conditions with the highest COD removal and value-added product yield; 2) investigate the synergistic effect of *R. jostii* and fungi in HTLAP fermentation; 3) assess the COD removal and value-added product profile of *A. niger* and *R. jostii* co-culture when subjected to increased HTLAP loadings in a sequential batch fermentation configuration. This study reports for the first time the potential of filamentous fungi for HTLAP upcycling to promote HTL of lignocellulosic biomass for bioenergy production.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Hydrothermal liquefaction aqueous phase of corn stover

HTL process was conducted in a Parr reactor using corn stover with a particle size of 10 mesh. During HTL, 40 g of corn stover (dry weight) was mixed with 200 mL of deionized water, and the reactor temperature was ramped up from room temperature to 350°C at a rate of approximately 5°C per minute. Once it reached 350°C, the reactor was maintained at this temperature for 30 minutes, after which the reaction was immediately quenched by introducing tap water into the reactor through a cooling coil. The solid and liquid products were separated by filtration through a filter paper with a pore size of 40 µm. Dichloromethane was added to the liquid fraction to extract bio-oil, and the remaining aqueous phase was the HTLAP used in this study.

The pH of HTLAP was adjusted to 4, 5, 6, and 7 using 6 M HCl followed by sterilization via filtration through a 0.22-µm syringe filter before being used for microbial cultivation. Diluting pH-adjusted HTLAP with sterile ultrapure water did not significantly alter the pH of the resulting diluted solution. All microbial cultivation in this study was conducted under sterile conditions to prevent potential result interference due to contamination. All chemicals used in the study were purchased from Fisher Scientific (Hampton, NH, USA) unless noted, otherwise.

4.2.1.2 Microorganisms

A. niger NRRL 2001 was 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) and *P. chrysosporium* BKM-F-1767 were generously provided by Dr. Daniel L. Lindner (U.S. Department of Agriculture Forest

Service). The bacterium *R. jostii* strain RHA1 was kindly provided by Dr. Lindsay D. Eltis (Departments of Microbiology and Biochemistry, University of British Columbia, Vancouver, Canada). *R. jostii* was propagated on tryptic soy agar, while the three fungal strains were cultured on potato dextrose agar. Throughout the study, all microorganisms underwent monthly subculturing at 25°C and were stored as agar-plate stock cultures in a refrigerator. To prepare *R. jostii* inoculum, cells were harvested from a 2-day-old agar plate, washed three times with ultrapure water, and then utilized to generate cell suspension with an OD₆₀₀ (optical density at $\lambda=600$ nm) of 1.0. Inocula of fungi (*A. niger*, *T. versicolor*, and *P. chrysosporium*) were prepared by cultivating the spores (*A. niger* and *P. chrysosporium*) or hyphae (*T. versicolor*) of the fungi in potato dextrose broth to form spherical pellets of around 1 mm diameter. The pellets of desired size were harvested and subjected to thorough washing for five times with ultrapure water before being used as inocula. All media used for maintaining seed cultures and preparing inocula were sourced from Becton Dickinson GmbH, Heidelberg, Germany. All microbial cultivations with liquid media in this study were conducted at 25°C and 150 rpm in a humidified incubator (MaxQ 4450, Thermo Fisher Scientific, Waltham, MA, USA)

4.2.1.3 Supplementary nutrient stocks

Stock solutions of supplementary nutrients used in the study were prepared as described previously [15]. The stock solutions of nitrogen source (N), phosphorus source (P), magnesium source (Mg), calcium source (Ca), and iron source (Fe) were added into diluted HTLAP to achieve final concentrations of 0.4 g/L NH₄Cl, 0.1 g/L KH₂PO₄, 50 mg/L MgSO₄·7H₂O, 25 mg/L CaCl₂·2H₂O, and 5 mg/L FeSO₄·7H₂O, respectively.

4.2.2 Aerobic fermentation of corn stover hydrothermal liquefaction aqueous phase

4.2.2.1 Optimization of fungal fermentation conditions

A series of screening experiments were conducted in 24-well microplates as described previously to determine the appropriate pH, nutrient supplementation, and HTLAP dilution factor for cultivation of the fungal strains [15]. On the last day of incubation, cell activity in the microplates was determined with an established MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay where absorbance (abs.) of the purple color measured at 560 nm is directly related to the amount of metabolically active microbial biomass [15]. All experiments in this study were conducted in duplicate unless specified, otherwise.

4.2.2.2 Fungal cultivation followed by *R. jostii* inoculation

The pH, nutrient supplementation, and HTLAP dilution factor determined in section 4.2.2.1 were used to conduct batch cultivation of *A. niger*, *T. versicolor*, and *P. chrysosporium* with HTLAP being the sole COD source. Each 100-mL Erlenmeyer flask was charged with 30 mL of diluted HTLAP (pH6) and nutrient supplement, and inoculated with fungal pellets with 1-mm diameter to achieve target fungal biomass loadings of approximately 50 mg of *T. versicolor*, or *P. chrysosporium* (dry cell mass) per gram of COD, and 25 mg *A. niger* (dry cell mass) per gram of COD. For instance, fungal pellets were inoculated into 15-fold diluted HTLAP (with around 2 g/L of COD) at a rate of 1 pellet per mL of the diluted HTLAP. Given that the dry weight of *T. versicolor*, and *P. chrysosporium* pellets was around 0.1 mg each and *A. niger* pellet was approximately 0.05 mg each, the aforementioned fungal biomass loading was used. Flasks were incubated at 25°C and 150 rpm for 12 days with the fungal pellets. Immediately after the 12-day incubation, the fungal biomass in each flask was removed through centrifugation, leaving only the supernatant. Then, the supernatant was inoculated with 2% (v/v) *R. jostii* cell suspension (OD₆₀₀ = 1.0) prepared in section 4.2.1.2 to start the bacterial fermentation stage with *R. jostii*. The flasks were incubated for another 6 days. Throughout the sequential fungal and bacterial

fermentation stages, an aliquot of 1.5-mL homogeneous sample was taken every two days from each flask and centrifuged to obtain supernatant for COD measurement and HPLC analysis to monitor the variation of organic acid and phenolic compound concentrations throughout the fermentation process.

4.2.2.3 Co-culture of *A. niger* with *R. jostii*

An initial assessment of *A. niger* and *R. jostii* co-culture was carried out using 7.5× diluted HTLAP, supplemented with N and Fe as detailed in section 4.2.2.1. The co-culture was run in parallel with *A. niger* pure culture, each in separate 100-mL Erlenmeyer flasks containing 30 mL of diluted HTLAP adjusted to pH 6. *A. niger* pellets were inoculated on day 0, following the procedure outlined in section 4.2.2.2, for both treatments. On day 2 of incubation, *R. jostii* cell suspension (OD₆₀₀=1.0) was added at a concentration of 3% (v/v) to the flasks in the co-culture treatment to initiate the fungal-bacterial co-culture. Flasks were incubated for 12 days from day 0 onwards, during which a 0.5-mL sample was taken every 2 days for COD analysis.

Sequential batch fermentation with *A. niger* and *R. jostii* co-culture was conducted, where the co-culture was subjected to increasing concentrations of HTLAP. Specifically, the sequential batch fermentation began with 8 flasks, each containing 30 mL of 7.5× diluted HTLAP and inoculated with *A. niger* pellets, followed by *R. jostii* cells as described above. The flasks were incubated for 12 days, after which the supernatant in the flasks was removed by centrifugation at 5000 rpm, leaving only the microbial cells. Then, 30 mL of 6× diluted HTLAP was freshly prepared and added to each flask containing the microbial cells. Similarly, fermentation in 6× diluted HTLAP was conducted for 12 days, after which the resulting microbial cells were used for the 12-day fermentation of 4.75× diluted HTLAP, and lastly for the fermentation of 3.75× diluted HTLAP. At the end of each cycle of 12-day fermentation, the microbial cells from 2

flasks were harvested to determine the dry weight and fatty acid profile. All the diluted HTLAP used for the sequential batch fermentation was supplemented with N and Fe as described in section 4.2.2.1. During the fermentation, a 0.5-mL sample was taken from each flask every 2 days for COD measurement.

4.2.3 Analytical methods

4.2.3.1 Chemical analysis

Chemical compound analysis of HTLAP was primarily performed on a high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (7.8×300 mm, Bio-Rad Laboratories, CA, USA) and a refractive index detector (RID) as described previously [15]. A five-point standard curve was generated and used to quantify each compound. Around 68% (w/w) of the organic carbon in HTLAP can be quantified with the HPLC. The concentrations of oxalic acid produced by *A. niger* were also determined using the HPLC method. A gas chromatography-mass spectrometry (GC-MS) (Agilent GC 6890 coupled with a MS 5973, Agilent Technologies, CA, USA) equipped with a 30-meter long Zebron ZB-1701 column (0.25 mm inner diameter and 0.25 μ m film thickness) (Phenomenex, CA, USA) was used in conjunction with HPLC to conduct semi-quantitative compound analyses on fermentation samples. The samples were derivatized with MSTFA + 1% TMCS (TS-48915, Thermo Fisher Scientific, MA, USA) and analyzed on the GC-MS as described previously [15]. The inlet, MS source, and MS quadrupole analyzer temperatures were set at 250, 230, and 150°C, respectively. Spectrum acquisition was set at scan mode to cover mass range between 50 and 650. Chemical compounds were identified by GC-MS with a match score above 90.

Total organic carbon (TOC), COD, total nitrogen (TN), and total phosphorus (TP) were measured using test kits (Hach Company, IA, USA). The inorganic elements, including K, Ca, S,

Mn, Mg, and Fe were measured on an inductively coupled plasma optical emission spectroscopy (ICP-OES) (Agilent 5800, Agilent Technologies, CA, USA). The pH was measured using a pH meter (Accumet AE150, Fisher Scientific, NH, USA).

4.2.3.2 Microbial cell analysis

The harvested bacterial and fungal biomass was washed five times with ultrapure water by centrifugation and freeze-dried, followed by weighing for cell dry mass. To exclude the weight of calcium oxalate precipitated during fermentation, the microbial cells were washed with 2 M HCl overnight to dissolve the precipitate and yield oxalic acid [17]. The concentration of oxalic acid in the wash solution was determined using HPLC, as described in section 4.2.3.1. The molar concentration of oxalic acid in the wash solution was found to closely mirror the calcium concentration in HTLAP, suggesting their precipitation as calcium oxalate in the harvested microbial cells. Therefore, the calcium concentration in HTLAP was used to calculate the weight of calcium oxalate to be excluded from the harvested cell mass.

To prepare cell mass samples for fatty acid profile analysis, 10 μ L tridecanoic acid methyl ester (C_{13} FAME) internal standard (20 mg/mL in hexane), 1 mL chloroform:methanol (2:1, v/v), and 1.5 mL HCl:methanol (5 mL HCl 12 M + 95 mL methanol) were added to freeze-dried cell mass. The mixture was incubated at 85°C for 1.5 h to convert all lipids into fatty acid methyl esters (FAME), which were later extracted with 2 mL hexane for 1 h. The FAME were quantified on a GC (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a RESTEK Rt-2560 capillary column (100 m $\times$ 0.25 mm, RESTEK, Bellefonte, PA), as described previously [18]. The identity of FAME was also confirmed by using a GC-MS with a match score above 90. The total lipid fraction of dry cell mass is determined using Eq. (4.1), in accordance with the NREL protocol [19]

$$\% \text{ Total lipid} = \sum_{i=4}^{24} M_i \times \frac{M_{13 \text{ (added)}}}{M_{13}} \div M_{\text{Dry cell}} \times 100 \quad (4.1)$$

M_i : the weight of C_i FAME measured by GC (μg)

M_{13} : the weight of C_{13} FAME (internal standard) measured by GC (μg)

$M_{13 \text{ (added)}}$: the weight of C_{13} FAME (internal standard) added to each sample (200 μg)

$M_{\text{Dry cell}}$: the sample dry cell mass

4.3 Results and Discussion

4.3.1 Characteristics of corn stover hydrothermal liquefaction aqueous phase

Table 4.1 shows the characteristics of the corn stover HTLAP used in this study. Potassium (1246 ppm) and calcium (580 ppm) are the major inorganic elements in the HTLAP, whereas sulfur (10.2 ppm), manganese (4.9 ppm), and magnesium (3.5 ppm) are present at lesser concentrations (Table 4.1a). Iron was found to be below the detection limit of 1 ppm. These observations are in line with previous studies. Lignocellulosic biomass, such as corn stover is rich in potassium and calcium, which are solubilized into the aqueous phase during HTL reaction [20]. Panisko et al. also reported potassium and calcium as the major inorganic elements in HTLAP, in the ranges of 400-1400 ppm and 1.2-41 ppm, respectively [21]. The high calcium concentration observed in the HTLAP of this study could be attributed to the absence of a buffering agent (Na_2CO_3). Addition of Na_2CO_3 increases the pH of during HTL reaction, which is known to reduce the leaching of calcium to HTLAP. The high calcium concentration could explain the low TP concentration in HTLAP, since phosphorous is known to precipitate by excess calcium ions [20]. As shown in Table 4.1b, a moderate concentration of TOC (10 g/L) and COD (29 g/L) of the HTLAP were also measured. TN was 0.12 g/L, giving a high C/N ratio

of 85:1. This is expected for HTLAP from corn stover due to the high carbohydrate to protein ratio of the feedstock and is responsible for low pH (3.9) of HTLAP [3].

Table 4.1 Inorganic element concentrations (a), water quality parameters (b), and organic compound concentrations (c) of HTLAP^a

(a)		(b)		(c)		
Inorganic element	Concentration (ppm)	Water quality parameter	Concentration	Organic compound	Concentration (g/L)	% TOC
K	1246±95	TOC	10.2±0.3 (g/L)	Acetic acid	10.82±0.08	42.4
Ca	510±38	COD	29.4±1.0 (g/L)	Glycolic acid	2.68±0.00	8.3
S	10.2±0.6	TN	0.12±0.00 (g/L)	Propionic acid	0.84±0.14	4
Mn	4.9±0.4	TP	< 1 (ppm)	Formic acid	0.92±0.09	2.4
Mg	3.5±0.3	C/N ratio	85:1	Lactic acid	0.47±0.01	1.8
Fe	< 2	pH	3.93±0.00	Succinic acid	0.46±0.00	1.8
				Catechol	0.64±0.00	4.1
				4-Methylcatechol	0.28±0.01	1.9
				Hydroquinone	0.18±0.01	1.2

^a Concentrations are presented as mean ± standard deviation of duplicate measurements.

Table 4.1c shows the concentrations of major organic compounds in HTLAP. Acetic acid (10.8 g/L) was the most abundant organic acid in HTLAP, representing 42% of the TOC value, followed by glycolic acid (2.7 g/L), formic acid (0.9 g/L), propionic acid (0.8 g/L), lactic acid (0.47 g/L), and succinic acid (0.46 g/L). Acetic acid is associated with the hydrolysis of carbohydrates, cellulose, and hemicellulose during HTL and was also reported to be the predominate compound in HTLAP of sewage sludge [22], distiller's dried grains with solubles [23], algae [24], and lignocellulosic biomass [25]. Panisko et al. reported an acetic acid concentration of 7-10 g/L in HTLAP of corn stover [21], while they found glycolic acid at higher

concentration of 14-18 g/L in HTLAP than that reported in this study (2.7 g/L). This difference could potentially be attributed to the higher pH of the HTL reaction conducted in their study, which was buffered to above 4 with Na₂CO₃.

As shown in Table 4.1c, phenolic compounds were measured to have lower concentrations in HTLAP compared to organic acids. Specifically, catechol had the highest concentration of 0.6 g/L, followed by 4-methylcatechol (0.3 g/L) and hydroquinone (0.2 g/L). Catechol and hydroquinone have been identified in corn stover HTLAP in previous studies but was not quantified in their studies [8,21]. These results showed that catechol was the major phenolic compound in HTLAP of corn stover, representing 4% of COD. This finding was confirmed in the GC-MS chromatograph of HTLAP, showing catechol as a predominant peak (Appendix Figure B.1). The GC-MS chromatograph also confirmed most compounds in Table 4.1c, except for acetic acid, formic acid, and propionic acid which were unable to be detected by the GC-MS method due to their volatile nature. Additional compounds, such as levulinic acid and other substituted phenols were also identified (Appendix Figure B.1) in HTLAP but were not quantified in this study due to their lower concentrations.

4.3.2 Optimization of fungal fermentation conditions in hydrothermal liquefaction aqueous phase

Figure 4.1a-c show the cell activity of *A. niger*, *T. versicolor*, and *P. chrysosporium* under various dilution, pH, and nutrient supplementation conditions in HTLAP. Among the fungi tested, *A. niger* exhibited the highest tolerance to HTLAP, demonstrating significant cell activity starting at an 8× dilution of HTLAP (Figure 4.1a). *T. versicolor*, and *P. chrysosporium* were more inhibited by HTLAP and didn't have significant cell activity until 16× and 20× dilution of HTLAP, respectively (Figure 4.1a). The original pH (pH 4) of HTLAP greatly inhibited fungal

cell activity, as evidenced by the noticeably lower bars depicted in Figure 4.1b at pH 4.

Increasing pH to 5 and 6 alleviated the inhibition of HTLAP, whereas further increase of pH to 7 and 8 made HTLAP less favorable for fungal cell activity (Figure 4.1b). At a pH lower than 4.76 (pKa value of acetic acid), high concentration of acetic acid is mainly in its undissociated form in HTLAP, which is known to have increased toxicity on microorganisms [26].

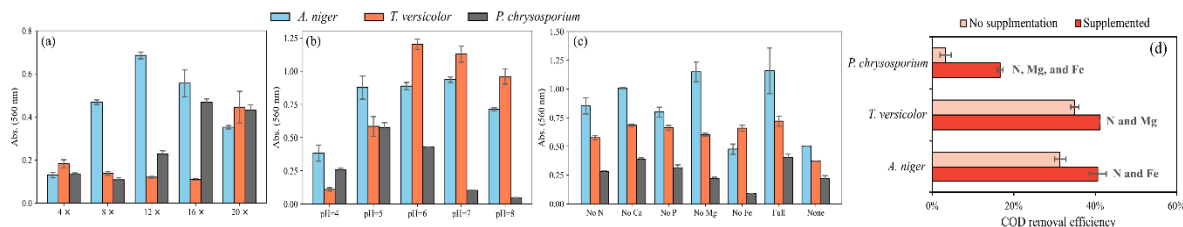


Figure 4.1 Cell activity of the tested fungi under screened HTLAP dilution fold (a), pH (b), and nutrient supplementation (c), and COD removal efficiencies by the three fungi on day 12 of fermentation in HTLAP with and without nutrient supplementation (d). In (c), “Full” denotes the supplementation of all nutrient sources including N, Ca, P, Mg, and Fe. “None” indicates the absence of any nutrient supplementation. The remaining treatments represent the supplementation of all nutrient sources except for one (e.g., “No N” indicates supplementation of all nutrients except N). Each bar represents the average and standard deviation of duplicate analyses. In (d), the initial COD and pH were 1900 mg/L and 6.0, respectively, for all three fungi. The nutrient supplementation for each fungus is labeled in the figure.

Fe, Mg, P, and N were the major nutrients that require supplementation for at least one of the fungi, as shown by the lower cell activity in HTLAP without supplementation (Figure 4.1c). This result suggested that proper nutrient supplementation in fungal treatment of HTLAP is necessary to support the optimal fungal cell activity. Phosphorous is a non-renewable resource that may subject to shortage and cost increase due to its uneven distribution and increasing

scarcity [27]. In addition, phosphorus supplementation may encourage bacterial growth, making the fungal fermentation system more susceptible to contamination [15]. Therefore, the decision was made not to supplement P for HTLAP fermentation, despite its positive impact on fungal cell activity.

Figure 4.1d shows the COD removal efficiencies by the three fungi on day 12 of fermentation in HTLAP. The initial COD loading, pH, and nutrient supplementation were determined based on screening results described in Figure 4.1a-c. Nutrient supplementation improved COD removal efficiency from 31% to 41% for *A. niger*, from 35% to 41% for *T. versicolor*, and from 3.3% to 17% for *P. chrysosporium*, further confirming the importance of N, Fe, and/or Mg supplement for HTLAP fermentation with fungi.

4.3.3 Effect of *R. jostii* inoculation following fungal fermentation

Due to the low COD removal achieved by fungal pure cultures, this study further examined the degradation profile of major compounds in HTLAP during fermentation, as illustrated in Figure 4.2. Even with nutrient supplementation, it was found that organic acids such as acetic acid (Figure 4.2a), glycolic acid (Figure 4.2b), malic acid (Figure 4.2c), and succinic acid (Figure 4.2d) persisted in the medium for at least one of the fungal strains after 12-days of fermentation. This was especially notable for glycolic acid and succinic acid, which account for 8% and 2% of TOC in HTLAP, respectively. None of the three fungal strains were shown to consume glycolic acid and succinic acid (Figure 4.2b and d). In contrast to organic acids, all fungal strains effectively degraded catechol (Figure 4.2e) and hydroquinone (Figure 4.2f). *T. versicolor* and *A. niger* completely removed catechol and hydroquinone by day 4 of incubation, regardless of nutrient supplementation, whereas *P. chrysosporium* required longer time (6-10 days) for the degradation.

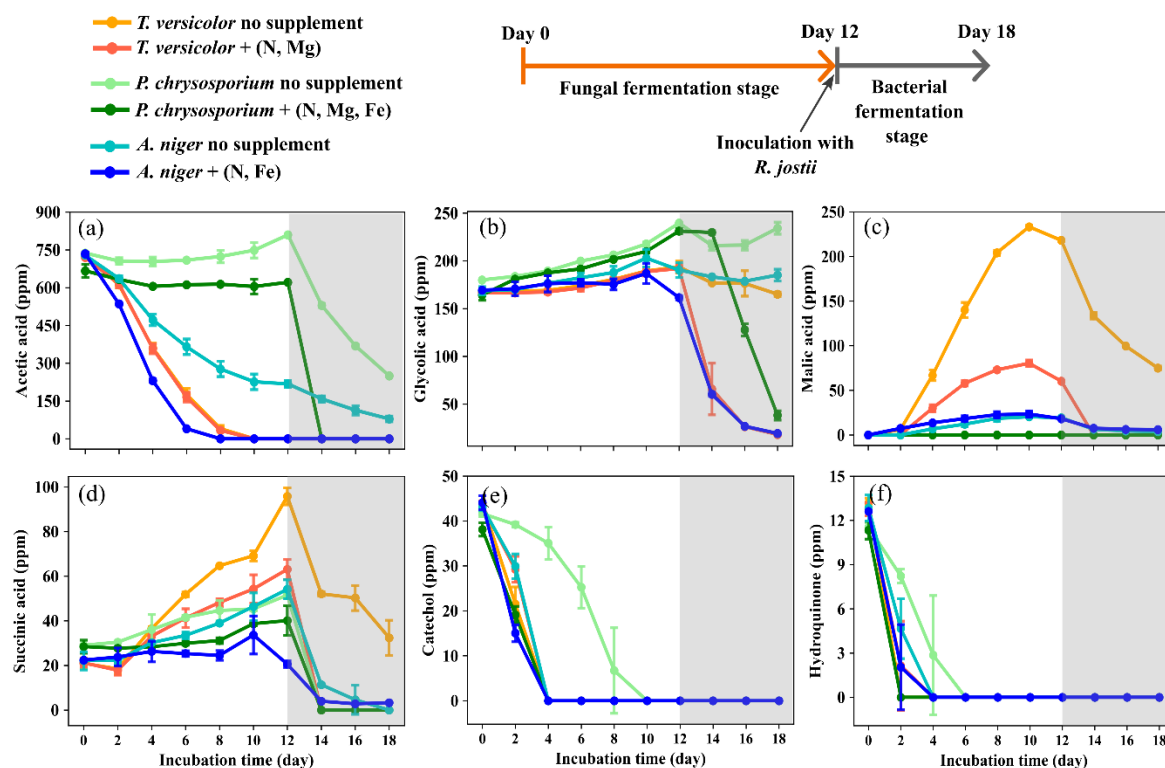


Figure 4.2 Concentrations of acetic acid (a), glycolic acid (b), malic acid (c), succinic acid (d), catechol (e), and hydroquinone (f) during fungal fermentation stage (day 0-12) and bacterial fermentation stage (day 12-18; shaded in gray) in HTLAP (n=2) with or without nutrient supplementation. HTLAP was the only COD source in the media. Each data point represents the average and standard deviation of duplicate measurements.

To address the excess organic acids left behind by fungal fermentation, the bacterium *R. jostii* was inoculated to the system immediately following the 12-day fungal fermentation stage (Figure 4.2). The compound degradation during the bacterial fermentation stage (days 12-18) is illustrated in Figure 4.2. Overall, *R. jostii* was effective at removing all the organic acid residues left by fungi, with removal efficiency notably enhanced by nutrient supplementation. Except for glycolic acid left behind by *P. chrysosporium* fermentation, all other organic acids were

completely removed during the bacterial fermentation stage with nutrient supplementation (Figure 4.2a-d).

Overall, these results suggested that inoculating the bacterium *R. jostii* complements fungal fermentation of HTLAP by removing the organic acids left behind by the fungi. Additionally, the results highlight the importance of Fe, Mg, and N supplementation for improved HTLAP fermentation with both fungi and bacteria. This is in accordance with the previous findings on biodegradation of sludge HTLAP, where the supplementation of essential nutrients in HTLAP such as Fe, Mg, and P also led to over 6-fold and 1.5-fold increase of COD removal for bacterial and fungal strains, respectively [15].

4.3.4 Sequential batch fermentation with *A. niger* and *R. jostii* co-culture

Given the high tolerance of *A. niger* to HTLAP, as depicted in Figure 4.1a, and recognizing the synergistic effect observed between *A. niger* and *R. jostii* (Figure 4.2), the potential of employing *A. niger* and *R. jostii* co-culture for HTLAP fermentation was investigated. Initially a preliminary fermentation assessment was conducted with an initial COD loading of 3800 mg/L, the results of which are illustrated in Appendix Figure B.2. The co-culture system achieved a higher COD removal of 63% compared to 46% achieved with single culture of *A. niger* (Appendix Figure B.2), which is consistent with the findings in section 4.3.3, indicating that *R. jostii* can remove residual organic acids left by fungi. These results justified the implementation of the *A. niger* and *R. jostii* co-culture for HTLAP fermentation.

Following the preliminary investigation, a sequential batch fermentation with *A. niger* and *R. jostii* was developed to evaluate the performance of the fungal-bacterial co-culture under increasing HTLAP loading. Conducting fermentation with higher HTLAP loading is desired as it reduces the need of water dilution and facilitates higher accumulation of fermentation products.

The profile of COD during fermentation is depicted in Figure 4.3a. The rising HTLAP loading is evident from the increasing COD at the beginning of each cycle of fermentation (Cycle 1: 3765 mg/L; Cycle 2: 4500 mg/L; Cycle 3: 6040 mg/L; and Cycle 4: 7835 mg/L). At the end of each 12-day cycle of fermentation, the co-culture consistently removed 59-65% of COD (Figure 4.3a), indicating that the co-culture maintained its COD removal effectiveness throughout the sequential batch fermentation despite exposure to increasing HTLAP loading. Major organic compounds shown in Table 4.1 were completely removed by the co-culture as evidenced by the HPLC chromatograms before and after fermentation (Figure 4.3b). The peak-area reduction of 13 unquantified compounds following co-culture fermentation was also shown to exceed 99%, except for methylsuccinic acid and pentanedioic acid which were not degraded by the co-culture (Appendix Table B.1).

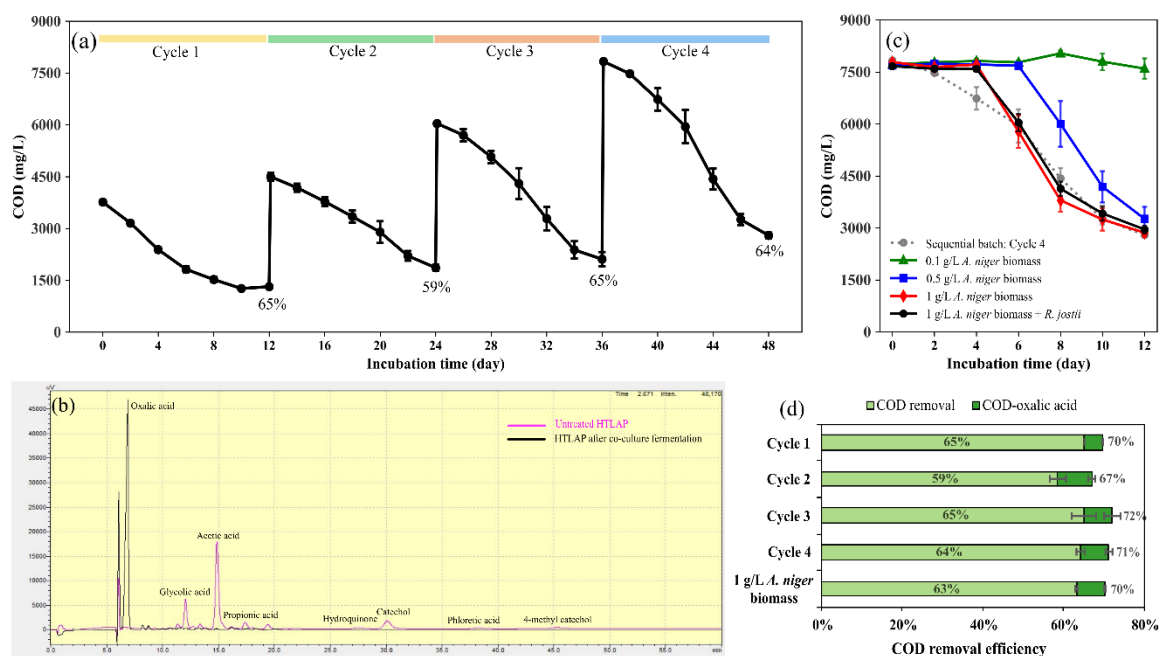


Figure 4.3 COD profile during sequential batch fermentation with *A. niger* and *R. jostii* co-culture when subjecting to increasing initial COD loading (cycle 1: 3765 mg/L; cycle 2: 4500 mg/L; cycle 3: 6040 mg/L; and cycle 4: 7835 mg/L) from HTLAP (a), HPLC chromatogram of

HTLAP before (pink line) and after (black line) fermentation with *A. niger* and *R. jostii* co-culture (peaks of major compounds were labeled in the graph) (b), COD profile during batch cultivation of *A. niger* with different initial fungal mass concentration (dry weight basis) of 0.1 (green triangle), 0.5 (blue square), and 1 g/L (red diamond), as well as during co-culture of *A. niger* at 1 g/L initial cell mass concentration and *R. jostii* (black circle) (c), and COD removals under various fermentation conditions with and without consideration of COD contributed from oxalic acid production (d). COD variation during the last cycle of sequential batch cultivation in (a) is overlaid with those in (c) as a dotted gray line. The COD removal at the end of each cycle of sequential batch fermentation is labeled in (a). The COD removal before and after excluding oxalic acid production is labeled in (d). Both N and Fe were supplemented for all cultivations. Each data point represents the average and standard deviation of duplicate measurements.

The microbial cells were reused throughout the sequential batch fermentation, resulting in a continuous increase in their mass during the process. By the end of cycle 4 fermentation (day 48), the dry cell mass increased to approximately 1 g/L. To investigate the influence of the increasing *A. niger* mass on HTLAP fermentation, *A. niger* was inoculated at mass concentrations of 0.1, 0.5, and 1 g/L for fermentation with an initial COD of approximately 7800 mg/L. Figure 4.3c depicts the COD variation during fermentation with different *A. niger* mass inocula, which was also compared with the COD variation during cycle 4 of the sequential batch fermentation, represented by a dotted gray line. As illustrated in Figure 4.3c, the fungal mass loading had a significant impact on COD removal during fermentation. On day 8, the removal efficiencies for inocula of 0.1, 0.5, and 1 g/L were 0%, 22%, and 51%, respectively (Figure 4.3c). Notably, the COD removal by 1 g/L *A. niger* closely mirrored that of the co-culture

containing 1 g/L *A. niger* plus *R. jostii* (Figure 4.3c), indicating that 1 g/L *A. niger* inoculum alone can effectively remove COD during HTLAP fermentation at the higher HTLAP loading of 7800 mg/L COD, regardless of co-culture with *R. jostii*. Further discussion on the absence of impact from *R. jostii* co-culture can be found in section 4.3.5.2.

The fermentation with 1 g/L *A. niger* achieved comparable COD removal to cycle 4 of the sequential batch cultivation by day 12 (Figure 4.3c). This suggests that the accumulation of microbial mass during the sequential batch fermentation is responsible for maintaining consistent COD removal under increasing HTLAP loading.

During HTLAP fermentation, oxalic acid produced remained in the fermentation broth and thus contributed to the final COD measurement. Figure 4.3d presents the COD removal efficiencies with and without accounting for oxalic acid-derived COD. Excluding oxalic acid-originated COD led to an increase in COD removal efficiency by *A. niger* culture fermentation from 59-65% to 67-72%. Considering the recalcitrancy of HTLAP, which typically exhibits 20-50% COD removal efficiency through aerobic fermentation, microalga cultivation, or anaerobic digestion [2,3,15], the achieved COD removal in this study suggests promising potential for utilizing *A. niger* in HTLAP fermentation.

4.3.5 Value-added product profiles during hydrothermal liquefaction aqueous phase fermentation

4.3.5.1 Oxalic acid production

A. niger was found to produce oxalic acid during HTLAP fermentation (Figure 4.4). Oxalic acid is a naturally occurring organic acid with a wide range of applications across diverse sectors including pharmaceuticals, environmental protection, crop production, chemical industries, and textiles [28]. It serves as a platform chemical, metal chelator, and/or sterilization

agent. The global oxalic acid market is projected to expand at a compound annual growth rate of 4.9%, reaching US\$ 1.55 billion by 2033 [28]. Currently, the primary method for producing oxalic acid involves chemical synthesis using fossil resources [29]. Using microbial fermentation to produce oxalic acid from industrial waste without competing with food supplies would improve process sustainability and promote circular economy. A recent review article on this subject highlighted the use of food processing by-products (e.g., fruit peels, nut shells, whey permeate, and molasses) and agricultural waste (e.g., various straws, corn stover, and rice husk) for oxalic acid production [28]. This study suggested that HTLAP is a promising feedstock for oxalic acid production without competing with human food supply.

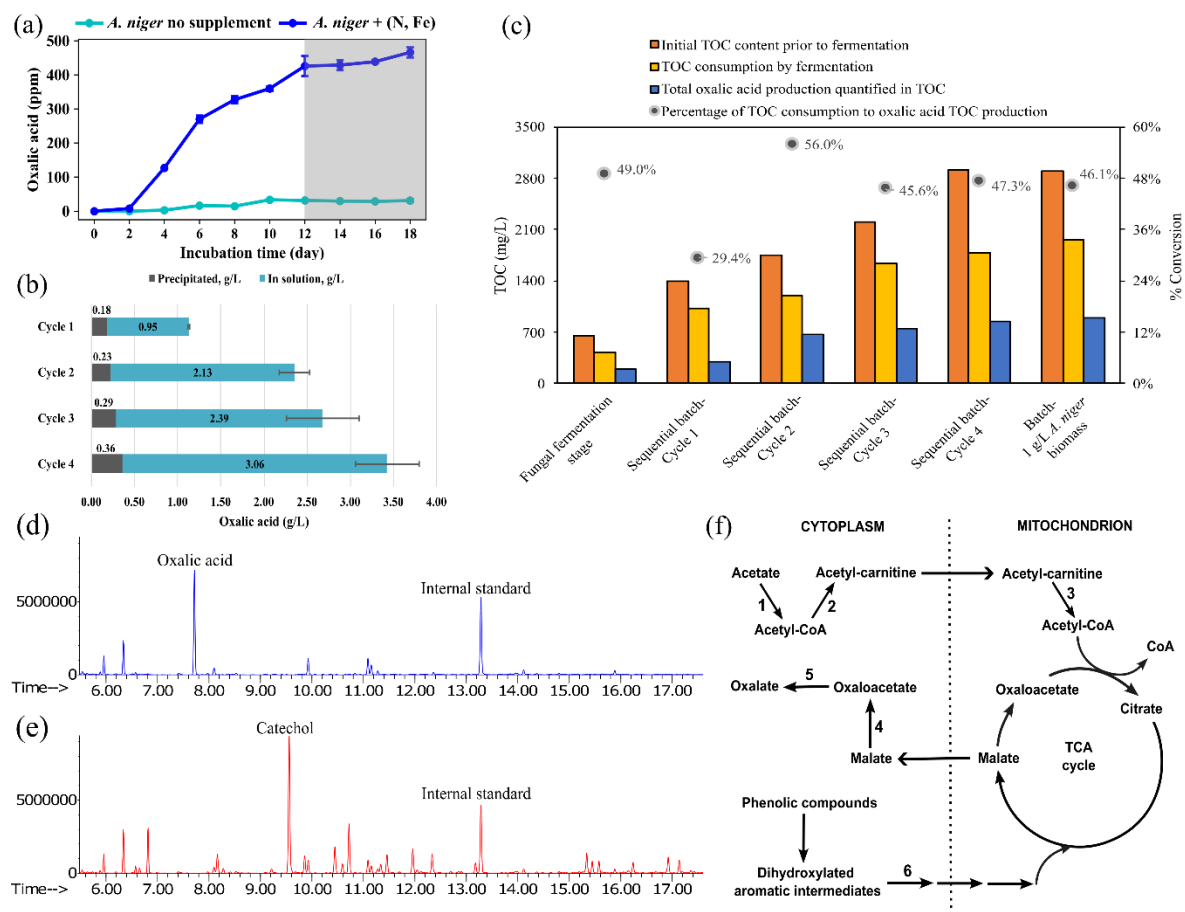


Figure 4.4 Variation of oxalic acid concentration during HTLAP fermentation with and without N and Fe supplementations (a), oxalic acid concentrations at the end of different cycles of sequential batch fermentation (b), and percentage of HTLAP-originated TOC conversion to oxalic acid and the corresponding TOC concentration at the end of fungal fermentation stage, sequential batch fermentation cycles 1 to 4, and batch fermentation with 1 g/L *A. niger* biomass (c). Oxalic acid precipitated due to the presence of calcium in HTLAP is represented by gray bars while oxalic acid in solution is depicted by blue bars in (b). Predominant oxalic acid production was illustrated in the GC-MS chromatogram of post-fermentation HTLAP (d) compared to untreated HTLAP (e). Relevant metabolic pathways contributing to oxalic acid production in HTLAP fermentation with *A. niger* are outlined in (f). Key enzymes of the

pathways are numbered as follows: 1. cytosolic acetyl-CoA synthetase (ACS); 2. cytosolic carnitine acetyltransferase; 3. mitochondrial carnitine acetyltransferase; 4. malate dehydrogenase (Mdh); 5. oxaloacetate acetyl hydrolase (OahA); and 6. intradiol ring cleavage dioxygenases.

As shown in Figure 4.4a, over a 15-fold increase in oxalic acid production was achieved when N and Fe were supplemented into HTLAP compared to no supplementation. Previous studies indicated the necessity of nutrients, such as P and N for oxalic acid production by *A. niger* [30]. Although nitrogen supplementation was also important for oxalic acid synthesis by *A. niger* in this research, phosphorus supplementation was found unnecessary given negligible phosphorous in HTLAP (<1 ppm). *A. niger* is able to solubilize phosphorus from rock deposits and wheat straw through secretion of organic acids such as oxalic acid and citric acid [30,31]. Therefore, this fungus may produce more oxalic acid in response to low phosphorus level, possibly as a strategy to access insoluble phosphorus [30]. However, further research is needed to determine the precise phosphorus concentration required for optimal oxalic acid production.

While Fe supplementation has not been directly associated with oxalic acid production in *A. niger*, it plays a crucial role in biological processes by serving as a cofactor for essential enzymes. For example, intradiol ring cleavage dioxygenases, a group of enzymes involved in the critical ring-opening step of phenolic compounds degradation in *A. niger*, were found to require ferric iron at their active site [32]. Therefore, Fe supplementation is important for HTLAP fermentation with *A. niger*, as it may facilitate the activity of enzymes responsible for breaking down abundant phenolic compounds present in HTLAP. Furthermore, it is noteworthy from Figure 4.4a that the bacterial fermentation stage with *R. jostii* (highlighted in gray in the figure) following the fungal fermentation stage with *A. niger* did not reduce the oxalic acid

concentration. This observation suggests that *R. jostii* does not utilize oxalic acid but instead targets other organic acids present in HTLAP (Figure 4.2).

Figure 4.4b presents the oxalic acid concentration at the end of each cycle of sequential batch fermentation. Across cycle 1 to 4, the produced oxalic acid concentrations were 1.13, 2.36, 2.68, and 3.42 g/L, respectively. Over 84-90% of the oxalic acid was dissolved in the fermentation broth, while the remainder precipitated due to the presence of calcium in HTLAP. This is supported by the closely matching molar concentration of precipitated oxalic acid (determined as described in section 4.2.3.2) and the calcium concentration in HTLAP. Figure 4.4c illustrates the amount and percentage of consumed TOC to oxalic acid conversion during fermentation with *A. niger* at different settings. Notably, except for cycle 1 of the sequential batch fermentation, the ratios of consumed TOC to produced oxalic acid were 46-56%.

Oxalic acid fermentation using *A. niger* has been studied previously, but it encountered challenges such as the co-production of other organic acids and the need for feedstock that competes with food supplies. For example, fermentation of sucrose using *A. niger* was shown to produce a mixture of oxalic acid and gluconic acid. Despite converting up to 80% of sucrose to organic acids, the selectivity for oxalic acid was only 39% due to the high concentration of co-produced gluconic acid [33]. In contrast, fermentation of lactose with *A. niger* yields oxalic acid as the sole product which is desirable from the standpoint of conversion and separation economy. However, the utilization of lactose-rich feedstock, such as whey permeate, for oxalic acid fermentation is limited due to its potential for producing lactose powder, whey protein, and animal feed [34].

This study reports that fermentation using HTLAP as a non-food competing feedstock with *A. niger* yielded oxalic acid as the only product while effectively removing the vast majority

of organic compounds in HTLAP (Figure 4.3b and 4d). This is supported by the HPLC and GC-MS chromatograms before and after fermentation with *A. niger*, showing oxalic acid as the predominate peak in HTLAP post-fermentation broth (Figure 4.3b and 4d). HTLAP fermentation with *A. niger* couples HTLAP treatment with oxalic acid production, contributing to improved process economy.

It is reasonable to observe the oxalic acid production in HTLAP fermentation with *A. niger*, since the corresponding metabolic pathways have been established in the literature. The major metabolic pathways and enzymes responsible for oxalic acid production by *A. niger* are depicted in Figure 4.4f based on established pathways found in *Aspergillus* species. As a dominant organic compound in HTLAP, acetate undergoes conversion to acetyl-CoA in the cytoplasm via cytosolic acetyl-CoA synthetase. It is then transported to the mitochondria as acetyl-carnitine and subsequently converted back to acetyl-CoA for entry into the TCA cycle in the mitochondria [35]. To utilize various phenolic compounds, they are transformed into one of the seven dehydroxylated aromatic intermediates, including protocatechuic acid, catechol, hydroxyquinol, gentisic acid, gallic acid, hydroquinone, and pyrogallol [36]. These intermediates then undergo ring cleavage via intradiol ring cleavage dioxygenases and are converted to TCA cycle intermediates [37]. Malate, generated from the TCA cycle is transported to cytoplasm, where it is converted to oxaloacetate and finally to oxalate [38].

4.3.5.2 Cellular fatty acid profile

Lipid-rich microbial cells hold the potential to be converted into biofuel, thereby contributing to the process economy. The fatty acid profile, cellular fatty acid content (total fatty acid mg/dry cell mass mg $\times 100\%$), and cell mass concentration at the end of fermentation with *A. niger* cultures are illustrated in Figure 4.5. As shown in Figure 4.5a, pentadecanoic (C15:0),

palmitic (C16:0), heptadecanoic (C17:0), heptadecenoic (C17:1), steric (C18:0), oleic (C18:1), and linoleic (C18:2) acids were found in microbial cells harvested at the end of cycles 1 to 4 sequential batch cultivation. Since the cells from HTLAP fermentation with *A. niger* pure culture consisted only of palmitic, oleic, and linoleic acids (Figure 4.5a), the additional fatty acids identified in the cells from sequential batch fermentation could originate from *R. jostii*. This hypothesis is supported by the knowledge that pentadecanoic, heptadecanoic, heptadecenoic, and steric acids have been previously found in the cells of oleaginous *Rhodococcus* strains [39,40]. The cellular lipid content was on average 11% at the end of cycle 1 fermentation (Figure 4.5b). However, this percentage decreased to 4.2%, 2.6%, and 1.8% at the end of cycles 2, 3, and 4 of sequential batch fermentation, gradually approaching the lipid content of 1.6% in single *A. niger* culture cell mass (Figure 4.5b).

4.3.6 Effect of hydrothermal liquefaction aqueous phase loading on *A. niger* and *R. jostii* co-culture fermentation

Our finding suggested that employing a co-culture of *A. niger* and *R. jostii* is a promising strategy for HTLAP fermentation, capable of achieving 67-72% COD removal while producing lipids and oxalic acid as value-added products. At low HTLAP loading, with COD less than 3800 mg/L, the products of the fermentation were oxalic acid at 0.5-1 g/L and cell mass containing 11% lipid. At high HTLAP loading, with COD ranging from 4500 to 7800 mg/L, the fermentation predominantly produced oxalic acid at 2.4-3.4 g/L, representing 46-56% of the TOC consumed by the co-culture.

The gradually decreasing cellular lipid content in the co-culture with increasing HTLAP loading from cycle 1 to 4 (Figure 4.5b) suggested that the co-culture cell mass was gradually taken over by *A. niger* at higher HTLAP loadings. Compared to *A. niger*, *R. jostii* was more

susceptible to inhibition by HTLAP, as it was unable to grow in 15× diluted HTLAP, likely due to the high phenolic compound concentration (Appendix Figure B.3). Therefore, at higher HTLAP loading, *R. jostii* may struggle to stay active and therefore was overtaken by *A. niger*. This is further verified by the finding that, at high HTLAP loading, the COD removal by *A. niger* and the co-culture of *A. niger* and *R. jostii* was nearly identical (Figure 4.3c).

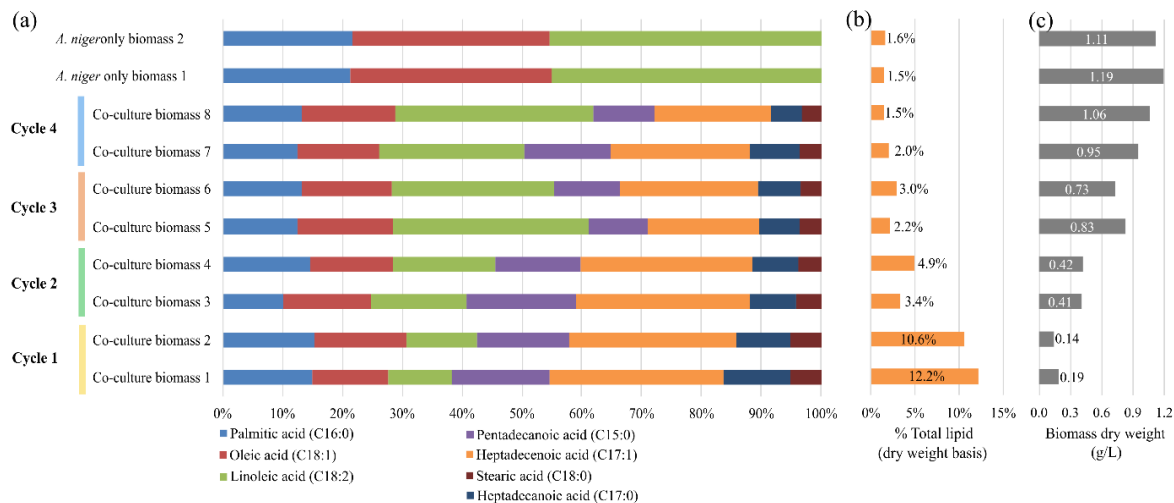


Figure 4.5 Fatty acid profile (a), cellular lipid content (b), and dry weight concentration (g cell mass/L HTLAP) (c) of microbial cell mass at the end of batch fermentation with single *A. niger* culture, and different cycles of sequential batch fermentation with *A. niger* and *R. jostii* co-culture.

R. jostii demonstrated the ability to consume organic acids remaining after *A. niger* fermentation (Figure 4.2), and their co-culture increased COD removal from 46% (single *A. niger* culture) to 63% (Appendix Figure B.2). However, at higher HTLAP loading, it was observed that *A. niger* alone achieved similar COD removals without co-culture with *R. jostii* (Figure 4.3c). This phenomenon may be attributed to pH shift during fermentation at higher HTLAP loading, facilitated by increased oxalic acid concentration produced by *A. niger*.

Specifically, it was found that the spent fermentation broth after *A. niger* cultivation at lower HTLAP loading (<3800 mg/L COD) had a pH of 6.5-7.5, while fermentation at higher HTLAP loading of 4500, 6040, and 7800 mg/L COD resulted in a lower ending pH of 4.5-5. pH is a critical factor influencing *A. niger* metabolism. The optimal pH for *A. niger* to produce oxalic acid, gluconic acid, and citric acid have been reported to be 5-8, 5.5, and below 2, respectively [41]. Furthermore, Previous studies showed that over 2000 genes of *A. niger* were significantly affected by cultivation pH levels of 2.5, 4.5, and 6.0, highlighting the substantial impact of pH on fungal metabolism [41].

4.3.7 Outlook on using *A. niger* and *R. jostii* co-culture in hydrothermal liquefaction aqueous phase fermentation

This study suggests that *A. niger* and *R. jostii* co-culture fermentation is a promising approach for treating and valorizing HTLAP, achieving over 70% COD removal in 12 days while enabling effective oxalic acid production without co-production of other organic acids. Given the encouraging results from this batch fermentation study, future efforts should focus on developing batch reactors for industrial-scale HTLAP treatment. Batch reactors are advantageous in terms of process control and can generally achieve better COD removal [42]. Considering the high complexity and recalcitrance of HTLAP, batch setups are more suitable for its fermentation. Specifically, employing a agitated airlift reactor has been shown to benefit *Aspergillus sp.* fermentation while successfully maintaining the desired fungal pellet morphology [43]. This type of reactor could potentially be employed for HTLAP fermentation.

Apart from aerobic fermentation, AD, microalgal cultivation, and aerobic treatment at wastewater treatment plants have also been examined for the biological treatment and valorization of HTLAP. Compared to AD and microalgal cultivation, aerobic fermentation is less

prone to inhibition by HTLAP and has a shorter incubation time [44]. Compared to aerobic treatment at wastewater treatment plants, aerobic fermentation converts the abundant organic compounds in HTLAP into value-added products without generating excess sludge [45]. However, aerobic fermentation has drawbacks such as high energy consumption and greenhouse gas emissions. Therefore, techno-economic analysis and life cycle assessment will be necessary to evaluate the economic feasibility and environmental performance of the aerobic fermentation process for HTLAP treatment and valorization.

4.4 Conclusions

Aspergillus niger NRRL 2001 demonstrated promising HTLAP fermentation capabilities. In a 3.75× diluted HTLAP with a COD of 7800 mg/L, it achieved over 70% COD removal and produced 3 g/L of oxalic acid within 12 days of fermentation. Approximately 46-56% of consumed total organic carbon in HTLAP was converted to oxalic acid by the fungus. Co-culturing with *R. jostii* RHA1 showed benefits at lower HTLAP loadings (<3800 mg/L COD), yielding 69% COD removal and 11% lipid of dry cell mass. These findings highlighted *A. niger*'s potential for HTLAP valorization, promoting the sustainability of HTL for biofuel production from corn stover.

References

- [1] S.S. Toor, L. Rosendahl, A. Rudolf, Hydrothermal liquefaction of biomass: A review of subcritical water technologies, *Energy* 36 (2011) 2328–2342.
<https://doi.org/10.1016/j.energy.2011.03.013>.
- [2] Y. Gu, X. Zhang, B. Deal, L. Han, Biological systems for treatment and valorization of wastewater generated from hydrothermal liquefaction of biomass and systems thinking: A review, *Bioresour Technol* 278 (2019) 329–345. <https://doi.org/10.1016/j.biortech.2019.01.127>.

- [3] J. Watson, T. Wang, B. Si, W.-T. Chen, A. Aierzhati, Y. Zhang, Valorization of hydrothermal liquefaction aqueous phase: pathways towards commercial viability, *Progress in Energy and Combustion Science* 77 (2020) 100819. <https://doi.org/10.1016/j.pecs.2019.100819>.
- [4] M. Pham, L. Schideman, B.K. Sharma, Y. Zhang, W.-T. Chen, Effects of hydrothermal liquefaction on the fate of bioactive contaminants in manure and algal feedstocks, *Bioresource Technology* 149 (2013) 126–135. <https://doi.org/10.1016/j.biortech.2013.08.131>.
- [5] H. Song, T. Yang, B. Li, Y. Tong, R. Li, Hydrothermal liquefaction of sewage sludge into biocrude: Effect of aqueous phase recycling on energy recovery and pollution mitigation, *Water Research* 226 (2022) 119278. <https://doi.org/10.1016/j.watres.2022.119278>.
- [6] E. Tito, G. Zoppi, G. Pipitone, E. Miliotti, A.D. Fraia, A.M. Rizzo, R. Pirone, D. Chiaramonti, S. Bensaid, Conceptual design and techno-economic assessment of coupled hydrothermal liquefaction and aqueous phase reforming of lignocellulosic residues, *Journal of Environmental Chemical Engineering* 11 (2023) 109076. <https://doi.org/10.1016/j.jece.2022.109076>.
- [7] Y. Jiang, L. Ou, L. Snowden-Swan, H. Cai, S. Li, K. Ramasamy, A. Schmidt, H. Wang, D.M. Santosa, M.V. Olarte, M. Guo, M.R. Thorson, Aqueous-phase product treatment and monetization options of wet waste hydrothermal liquefaction: Comprehensive techno-economic and life-cycle GHG emission assessment unveiling research opportunities, *Bioresource Technology* 397 (2024) 130504. <https://doi.org/10.1016/j.biortech.2024.130504>.
- [8] G. Pipitone, G. Zoppi, S. Bocchini, A.M. Rizzo, D. Chiaramonti, R. Pirone, S. Bensaid, Aqueous phase reforming of the residual waters derived from lignin-rich hydrothermal liquefaction: investigation of representative organic compounds and actual biorefinery streams, *Catalysis Today* 345 (2020) 237–250. <https://doi.org/10.1016/j.cattod.2019.09.040>.

- [9] Z. Du, B. Hu, A. Shi, X. Ma, Y. Cheng, P. Chen, Y. Liu, X. Lin, R. Ruan, Cultivation of a microalga *Chlorella vulgaris* using recycled aqueous phase nutrients from hydrothermal carbonization process, *Bioresource Technology* 126 (2012) 354–357. <https://doi.org/10.1016/j.biortech.2012.09.062>.
- [10] M.V. Reddy, Y. Mawatari, R. Onodera, Y. Nakamura, Y. Yajima, Y.-C. Chang, Bacterial conversion of waste into polyhydroxybutyrate (PHB): A new approach of bio-circular economy for treating waste and energy generation, *Bioresource Technology Reports* 7 (2019) 100246. <https://doi.org/10.1016/j.biteb.2019.100246>.
- [11] S.-K. Wang, K.-X. Yang, Y.-R. Zhu, X.-Y. Zhu, D.-F. Nie, N. Jiao, I. Angelidaki, One-step co-cultivation and flocculation of microalgae with filamentous fungi to valorize starch wastewater into high-value biomass, *Bioresource Technology* 361 (2022) 127625. <https://doi.org/10.1016/j.biortech.2022.127625>.
- [12] M. Kosa, A.J. Ragauskas, Bioconversion of lignin model compounds with oleaginous *Rhodococci*, *Appl Microbiol Biotechnol* 93 (2012) 891–900. <https://doi.org/10.1007/s00253-011-3743-z>.
- [13] Y. He, X. Li, X. Xue, M.S. Swita, A.J. Schmidt, B. Yang, Biological conversion of the aqueous wastes from hydrothermal liquefaction of algae and pine wood by *Rhodococci*, *Bioresource Technology* 224 (2017) 457–464. <https://doi.org/10.1016/j.biortech.2016.10.059>.
- [14] L.T. Cordova, B.C. Lad, S.A. Ali, A.J. Schmidt, J.M. Billing, K. Pomraning, B. Hofstad, M.S. Swita, J.R. Collett, H.S. Alper, Valorizing a hydrothermal liquefaction aqueous phase through co-production of chemicals and lipids using the oleaginous yeast *Yarrowia lipolytica*, *Bioresource Technology* 313 (2020) 123639. <https://doi.org/10.1016/j.biortech.2020.123639>.

- [15] M. Liu, C. Mahata, Z. Wang, S. Kumar, Y. Zheng, Comparative exploration of biological treatment of hydrothermal liquefaction wastewater from sewage sludge: Effects of culture, fermentation conditions, and ammonia stripping, *Journal of Environmental Management* 349 (2024) 119527. <https://doi.org/10.1016/j.jenvman.2023.119527>.
- [16] S. Sankaran, S.K. Khanal, N. Jasti, B. Jin, A.L. Pometto, J.H. Van Leeuwen, Use of Filamentous Fungi for Wastewater Treatment and Production of High Value Fungal Byproducts: A Review, *Critical Reviews in Environmental Science and Technology* 40 (2010) 400–449. <https://doi.org/10.1080/10643380802278943>.
- [17] F. Lapeyrie, G.A. Chilvers, C.A. Bhém, OXALIC ACID SYNTHESIS BY THE MYCORRHIZAL FUNGUS *PAXILLUS INVOLUTUS* (BATSCH. EX FR.) FR., *New Phytologist* 106 (1987) 139–146. <https://doi.org/10.1111/j.1469-8137.1987.tb04797.x>.
- [18] M. Liu, S. Li, T. Weiss, Y. Li, D. Wang, Y. Zheng, Solid-state fermentation of grain sorghum to produce Chinese liquor: Effect of grain properties and fermenting culture, *Journal of Cereal Science* 114 (2023) 103776. <https://doi.org/10.1016/j.jcs.2023.103776>.
- [19] S. Van Wychen, K. Ramirez, L.M.L. Laurens, Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by in situ Transesterification: Laboratory Analytical Procedure (LAP), National Renewable Energy Lab. (NREL), Golden, CO (United States), 2016. <https://doi.org/10.2172/1118085>.
- [20] H. Liu, Y. Chen, H. Yang, F. G. Gentili, U. Söderlind, X. Wang, W. Zhang, H. Chen, Conversion of high-ash microalgae through hydrothermal liquefaction, *Sustainable Energy & Fuels* 4 (2020) 2782–2791. <https://doi.org/10.1039/C9SE01114E>.
- [21] E. Panisko, T. Wietsma, T. Lemmon, K. Albrecht, D. Howe, Characterization of the aqueous fractions from hydrotreatment and hydrothermal liquefaction of lignocellulosic

feedstocks, *Biomass and Bioenergy* 74 (2015) 162–171.

<https://doi.org/10.1016/j.biombioe.2015.01.011>.

[22] I.A. Basar, H. Liu, C. Eskicioglu, Incorporating hydrothermal liquefaction into wastewater treatment – Part III: Aqueous phase characterization and evaluation of on-site treatment, *Chemical Engineering Journal* 467 (2023) 143422.

<https://doi.org/10.1016/j.cej.2023.143422>.

[23] P. Biller, R.B. Madsen, M. Klemmer, J. Becker, B.B. Iversen, M. Glasius, Effect of hydrothermal liquefaction aqueous phase recycling on bio-crude yields and composition, *Bioresource Technology* 220 (2016) 190–199. <https://doi.org/10.1016/j.biortech.2016.08.053>.

[24] B. Maddi, E. Panisko, T. Wietsma, T. Lemmon, M. Swita, K. Albrecht, D. Howe, Quantitative characterization of the aqueous fraction from hydrothermal liquefaction of algae, *Biomass and Bioenergy* 93 (2016) 122–130. <https://doi.org/10.1016/j.biombioe.2016.07.010>.

[25] S.D. Davidson, J.A. Lopez-Ruiz, Y. Zhu, A.R. Cooper, K.O. Albrecht, R.A. Dagle, Strategies To Valorize the Hydrothermal Liquefaction-Derived Aqueous Phase into Fuels and Chemicals, *ACS Sustainable Chem. Eng.* 7 (2019) 19889–19901.

<https://doi.org/10.1021/acssuschemeng.9b05308>.

[26] Y. Liang, X. Zhao, Z. Chi, M. Rover, P. Johnston, R. Brown, L. Jarboe, Z. Wen, Utilization of acetic acid-rich pyrolytic bio-oil by microalga *Chlamydomonas reinhardtii*: Reducing bio-oil toxicity and enhancing algal toxicity tolerance, *Bioresource Technology* 133 (2013) 500–506. <https://doi.org/10.1016/j.biortech.2013.01.134>.

[27] D. Cordell, J.-O. Drangert, S. White, The story of phosphorus: Global food security and food for thought, *Global Environmental Change* 19 (2009) 292–305.

<https://doi.org/10.1016/j.gloenvcha.2008.10.009>.

- [28] A.N. Amenaghawon, J.E. Ayere, U.O. Amune, I.C. Otuya, E.C. Abuga, C.L. Anyalewechi, O.V. Okoro, J.A. Okolie, P.K. Oyefolu, S.O. Eshiemogie, B.E. Osahon, M. Omede, S.A. Eshiemogie, S. Igemhokhai, M.O. Okedi, H.S. Kusuma, O.E. Muojama, A. Shavandi, H. Darmokoesoemo, A comprehensive review of recent advances in the applications and biosynthesis of oxalic acid from bio-derived substrates, *Environmental Research* 251 (2024) 118703. <https://doi.org/10.1016/j.envres.2024.118703>.
- [29] E. Schuler, M. Demetriou, N.R. Shiju, G.M. Gruter, Towards Sustainable Oxalic Acid from CO₂ and Biomass, *ChemSusChem* 14 (2021) 3636–3664. <https://doi.org/10.1002/cssc.202101272>.
- [30] M.M. Gharieb, Nutritional effects on oxalic acid production and solubilization of gypsum by *Aspergillus niger*, *Mycological Research* 104 (2000) 550–556. <https://doi.org/10.1017/S0953756299001707>.
- [31] L. Wang, H. Guan, J. Hu, Y. Feng, X. Li, K.K. Yusef, H. Gao, D. Tian, *Aspergillus niger* Enhances Organic and Inorganic Phosphorus Release from Wheat Straw by Secretion of Degrading Enzymes and Oxalic Acid, *J. Agric. Food Chem.* 70 (2022) 10738–10746. <https://doi.org/10.1021/acs.jafc.2c03063>.
- [32] P. Semana, J. Powlowski, Four Aromatic Intradiol Ring Cleavage Dioxygenases from *Aspergillus niger*, *Appl Environ Microbiol* 85 (2019) e01786-19. <https://doi.org/10.1128/AEM.01786-19>.
- [33] H. Strasser, W. Burgstaller, F. Schinner, High-yield production of oxalic acid for metal leaching processes by *Aspergillus niger*, *FEMS Microbiol Lett* 119 (1994) 365–370. <https://doi.org/10.1111/j.1574-6968.1994.tb06914.x>.

- [34] M. Rosseto, C.V.T. Rigueto, K.S. Gomes, D.D.C. Krein, R.A. Loss, A. Dettmer, N.S.P. dos S. Richards, Whey filtration: a review of products, application, and pretreatment with transglutaminase enzyme, *Journal of the Science of Food and Agriculture* 104 (2024) 3185–3196. <https://doi.org/10.1002/jsfa.13248>.
- [35] J. Ramón De Lucas, O. Martínez, P. Pérez, M. Isabel López, S. Valenciano, F. Laborda, The *Aspergillus nidulans* carnitine carrier encoded by the *acuH* gene is exclusively located in the mitochondria, *FEMS Microbiol Lett* 201 (2001) 193–198. <https://doi.org/10.1111/j.1574-6968.2001.tb10756.x>.
- [36] R.J.M. Lubbers, A. Dilokpimol, J. Visser, M.R. Mäkelä, K.S. Hildén, R.P. de Vries, A comparison between the homocyclic aromatic metabolic pathways from plant-derived compounds by bacteria and fungi, *Biotechnology Advances* 37 (2019) 107396. <https://doi.org/10.1016/j.biotechadv.2019.05.002>.
- [37] M. Sgro, N. Chow, F. Olyaei, M. Arentshorst, N. Geoffrion, A.F.J. Ram, J. Powlowski, A. Tsang, Functional analysis of the protocatechuate branch of the β -ketoadipate pathway in *Aspergillus niger*, *Journal of Biological Chemistry* 299 (2023). <https://doi.org/10.1016/j.jbc.2023.105003>.
- [38] L. Poulsen, M.R. Andersen, A.E. Lantz, J. Thykaer, Identification of a transcription factor controlling pH-dependent organic acid response in *Aspergillus niger*, *PLoS One* 7 (2012) e50596. <https://doi.org/10.1371/journal.pone.0050596>.
- [39] J.W. Holder, J.C. Ulrich, A.C. DeBono, P.A. Godfrey, C.A. Desjardins, J. Zucker, Q. Zeng, A.L.B. Leach, I. Ghiviriga, C. Dancel, T. Abeel, D. Gevers, C.D. Kodira, B. Desany, J.P. Affourtit, B.W. Birren, A.J. Sinskey, Comparative and Functional Genomics of *Rhodococcus*

opacus PD630 for Biofuels Development, PLoS Genet 7 (2011) e1002219.

<https://doi.org/10.1371/journal.pgen.1002219>.

[40] X. Li, Z. Xu, J.R. Cort, W.-J. Qian, B. Yang, Lipid production from non-sugar compounds in pretreated lignocellulose hydrolysates by *Rhodococcus jostii* RHA1, Biomass and Bioenergy 145 (2021) 105970. <https://doi.org/10.1016/j.biombioe.2021.105970>.

[41] M.R. Andersen, L. Lehmann, J. Nielsen, Systemic analysis of the response of *Aspergillus niger* to ambient pH, Genome Biol 10 (2009) R47. <https://doi.org/10.1186/gb-2009-10-5-r47>.

[42] J.B. Sánchez, M. Vuono, D. Dionisi, Model-based comparison of sequencing batch reactors and continuous-flow activated sludge processes for biological wastewater treatment, Computers & Chemical Engineering 144 (2021) 107127. <https://doi.org/10.1016/j.compchemeng.2020.107127>.

[43] O. Sánchez, F. Guio, D. Garcia, E. Silva, L. Caicedo, Fructooligosaccharides production by *Aspergillus* sp. N74 in a mechanically agitated airlift reactor, Food and Bioprocess Processing 86 (2008) 109–115. <https://doi.org/10.1016/j.fbp.2008.02.003>.

[44] P. SundarRajan, K.P. Gopinath, J. Arun, K. GracePavithra, A. Adithya Joseph, S. Manasa, Insights into valuing the aqueous phase derived from hydrothermal liquefaction, Renewable and Sustainable Energy Reviews 144 (2021) 111019. <https://doi.org/10.1016/j.rser.2021.111019>.

[45] I.A. Basar, A. Stokes, C. Eskicioglu, Evaluation of on-site biological treatment options for hydrothermal liquefaction aqueous phase derived from sludge in municipal wastewater treatment plants, Water Research 252 (2024) 121206. <https://doi.org/10.1016/j.watres.2024.121206>.

Chapter 5 - Impact of Hydrothermal Liquefaction Conditions on the Chemical Composition and Biodegradability of Aqueous Phase

Paper title:

Impact of Hydrothermal Liquefaction Conditions on the Chemical Composition and Biodegradability of Aqueous Phase

Submitted to:

Water Research, 31 August 2024

Authors' names:

Meicen Liu¹, Isamu Umeda², Zhiwu Wang³, Sandeep Kumar², Yi Zheng¹

Authors' affiliations:

1. Department of Grain Science and Industry, Kansas State University, 1980 Kimball Avenue, Manhattan, KS 66506, USA
2. Department of Civil and Environmental Engineering, Old Dominion University, Norfolk, VA 23529, USA
3. Department of Biological Systems Engineering, Virginia Tech, 1230 Washington St. SW, Blacksburg, VA, 24061, USA

Abstract

Aerobic fermentation is a simple and environmentally friendly method to treat and recover nutrients from hydrothermal liquefaction aqueous phase (HTLAP). This study evaluated the chemical composition and biodegradability of HTLAP derived from corn stover under different hydrothermal liquefaction (HTL) conditions. The compositions of HTLAP followed specific patterns corresponding to HTL reaction severity, with high severity leading to increased concentrations of certain phenolic compounds. The elevated phenolics contributed to stronger inhibition of HTLAP on *Aspergillus niger* and *Rhodococcus jostii*. The half-maximal inhibitory concentration (IC_{50}) of HTLAP for both microbes can be well predicted by linear regression models with HTL reaction temperature and time as the input variables, while corn stover solid loading showed no significant effect on IC_{50} and was not included in the models. Furthermore, aerobic fermentation with *A. niger* followed by *R. jostii* was demonstrated to be a versatile treatment process for HTLAP derived from a broad range of HTL conditions, achieving 45-70% of COD removal. This research provides a quantitative understanding of the relationships between HTL reaction conditions, HTLAP composition, and biodegradability, laying the foundation for integrating HTLAP aerobic fermentation into the HTL system, potentially enhancing its overall cost-efficiency and sustainability.

5.1 Introduction

Biofuels derived from lignocellulosic biomass are considered carbon neutral, even carbon negative alternatives to fossil fuels because lignocellulose is generated through photosynthesis, thus being a type of renewable feedstock. Although utilizing lignocellulose to produce biofuels is an attractive option for accomplishing a circular economy, the biochemical conversion process is hard to achieve economic competitiveness taking into account of the costs of logistics, chemical or enzymatic pretreatments, and detoxification [1].

Hydrothermal liquefaction (HTL) is a wet thermochemical conversion process that has been developed as one of the most promising technologies for sustainable biofuel production from lignocellulosic materials [2]. In HTL, feedstocks, such as lignocellulosic biomass, municipal solid waste, microalgal biomass, or sewage sludge are added to water to achieve a weight percentage of 5-20% and react at 250-370°C and 4-22 MPa [3,4]. At the elevated temperature and pressure, water serves as both the reaction medium and reactant in a complex series of reactions that hydrolyze, thermal-recompose, and recombine the feedstock into four product phases in a short period of time. These product phases include biocrude, gas, solid residue, and aqueous phase (i.e., hydrothermal liquefaction aqueous phase, HTLAP). The HTL process effectively degrades antibiotics, pharmaceuticals, and genetic materials of pathogens, while avoiding the energy-intensive drying step which is required for pyrolysis and gasification. Consequently, HTL has recently received increasing attention to be a favorable method for the disposal and valorization of a variety of organic waste streams.

HTLAP is a by-product generated at high volume due to the water-rich nature of HTL process. It needs to be properly treated to prevent the direct discharge into the environment [5]. As a spent reaction medium, HTLAP extracts roughly 45% of organic carbon and 80% of other

nutrients from the feedstock, leading to its high contaminant levels and significant energy loss of unutilized [6,7]. Consequently, treatment and valorization of HTLAP presents a major challenge towards an economically feasible HTL technology. HTLAP contains hundreds of organic compounds resulting from a complex series of reactions during HTL. Variations in HTL conditions (e.g., reaction temperature, time, and feedstock loading) lead to significant differences in HTLAP compositions. So far, the understanding of the relationship between HTL conditions and HTLAP compositions is very limited. Basar et al. examined the total carbohydrate, total phenol, and total short-chain fatty acids of HTLAP from nine HTL conditions within 290-360°C in 0-30 min [8]. It was found that total carbohydrates, total phenols, and glycerol were inversely proportional to the reaction temperature and time [8]. However, no clear linear trend was identified for the composition of individual organic acids, phenolic compounds, and N-heterocyclic compounds [8]. Existing studies on HTLAP generated at different conditions have focused on the study of wastewater parameters, such as pH, total nitrogen, total phosphorous, and COD [8,9]. There is a significant lack of knowledge on the detailed chemical compositions of HTLAP at different reaction conditions, which is important for the development of targeted HTLAP treatment and valorization technologies.

Biological valorization through anaerobic digestion (AD), microalgal cultivation, and aerobic fermentation offers a set of common options for transforming HTLAP due to their simplicity and environmental friendliness. However, AD and microalgal cultivation suffer from long incubation time and heavy dilution requirement due to the presence of inhibitors in HTLAP [5,6]. In comparison, aerobic culture is less affected by the inhibitory compounds [8,10–12]. Recently, an aerobic co-culture of *Aspergillus niger* and *Rhodococcus jostii* have been developed to treat and valorize HTLAP. At the initial chemical oxygen demand (COD) ranging from 4500

to 7800 mg/L (i.e., 3.75- to 6-fold diluted HTLAP), this co-culture fermentation achieved approximately 60% COD removal while converting around 50% of the consumed organic carbon into oxalic acid as a value-added product [13]. Evaluating the performance of these two microorganisms in the fermentation of HTLAP generated under different conditions offers an excellent opportunity to establish a correlation between HTL conditions and the HTLAP composition/biodegradability. However, such an evaluation hasn't been done.

This study used corn stover (CS) as a representative lignocellulosic feedstock for HTL and examined the chemical compositions of 27 different HTLAP from HTL of CS at three reaction temperatures (250, 300, and 350°C), times (5, 30, and 60 min), and CS solid loadings (5, 10, and 15%, w/v). To evaluate the HTLAP biodegradability, a subset of HTLAP samples were chosen based on the design of experiment (DoE) methodology. The use of DoE statistical tools allows for the construction of a response surface model to define the relationship between the response (biodegradability) and input variables (HTL temperature, time, and CS loading) while requiring fewer sample runs than changing one factor at a time. Additionally, DoE randomizes the experimental error to each experimental point and equally distributes experimental points in the investigated space, thus increasing the precision and reliability of the response surface model [14].

The goals of this study are to reveal the chemical compositions of HTLAP generated under various HTL conditions and to associate both the HTL conditions and HTLAP compositions with the biodegradability of HTLAP during *A. niger* and *R. jostii* fermentations. This work aims to: 1) elucidate the correlation between HTL conditions and the resulting HTLAP chemical compositions; 2) build response surface models for the half-maximal inhibitory concentration (IC₅₀) of HTLAP against *A. niger* and *R. jostii* using HTL conditions

(temperature, time, and CS loading) as input variables; and 3) evaluate the fermentation outcomes of HTLAP from different HTL conditions and identify chemical compounds that significantly impact HTLAP fermentation with *A. niger* and *R. jostii*. Applying the DoE methodology allows us to deliver quantitative insights into the relationship between HTL parameters and HTLAP biodegradability through the construction of a multiple linear regression model. Such knowledge represents a significant step toward incorporating aerobic fermentation treatment of HTLAP into the HTL process to improve the overall cost-efficiency and environmental sustainability.

5.2 Materials and Methods

5.2.1 Hydrothermal liquefaction aqueous phase preparation

The HTL was conducted in a 280-mL high pressure reactor (GC-3, High Pressure Equipment Co., USA) with an induction heating unit (HI-HEATER 4020, DHF, Japan). The HTL conditions included temperature (250, 300, and 350°C), time (5, 30, and 60 min), and CS loading (5, 10, and 15%, w/v), and each treatment was run in triplicates unless specified, otherwise. CS with a particle size between 2 and 12.7 mm was added to the reactor at a solid loading of 5, 10, or 15% (w/v). The heating rate of the reactor was approximately 50°C/min from 100°C to the targeted temperature and the heating from room temperature to 100°C took less than 1 min. Once reached the desired temperature, the reaction was maintained at the temperature for 5, 30, or 60 min, after which the reaction was immediately quenched by cooling the reactor with tap water in 5 min. The solid and liquid products were separated by filtration through a 11- μ m filter paper. To the filtrate, 225 mL of dichloromethane was added to extract light bio-oil, and the remaining aqueous phase was the HTLAP used in this study. The severity of the HTL reaction was calculated as Eq. 5.1 [8]. The severity within the range of room

temperature to base temperature (100°C) is negligible when the heating rate is high, therefore dropped from Eq. 5.1.

$$Severity = \log \left[\int_0^t e^{\frac{T(t)-T_b}{\omega}} dt \right] \approx \log \left[\left(\frac{\omega}{c} + t_R \right) e^{\frac{T_{max}-T_b}{\omega}} \right] \quad (5.1)$$

t : time (min)

$T(t)$: temperature (°C) at a given time

T_b : base temperature of 100°C

ω : fitted parameter of 14.75°C

c : rate of heating (50°C/min)

T_{max} : desired temperature (250, 300, and 350°C)

t_R : reaction time (min) after reached desired temperature (5, 30, and 60 min)

The pH of the HTLAP was adjusted to pH 5 for *A. niger* cultivation, and pH 7 for *R. jostii* cultivation with potassium hydroxide. The HTLAP was then sterilized by filtration through a 0.22-µm sterile syringe filter before used.

5.2.2 Microbial inoculum preparation

A. niger NRRL 2001 was obtained from the Agricultural Research Service Culture Collection (Northern Regional Research Laboratory, U.S. Department of Agriculture). The bacterium *R. jostii* strain RHA1 was a gift from Dr. Lindsay D. Eltis (Departments of Microbiology and Biochemistry, University of British Columbia, Vancouver, Canada). *A. niger* and *R. jostii* were cultivated on potato dextrose agar and tryptic soy agar, respectively, at 25°C. All microbial cultivation in this study was conducted under sterile conditions. To prepare *R. jostii* inoculum, cells from a 1-day-old agar plate were harvested, washed three times with ultrapure water, and made into a cell suspension with an optical density at $\lambda=600$ nm (OD₆₀₀) of 1.0. For *A. niger* inoculum preparation, agar pellets and broth pellets were used for the IC₅₀ assay

and HTLAP fermentation assay, respectively. Agar pellets of *A. niger* were prepared by growing a 2-day-old lawn of fungal hyphae on potato dextrose agar. A 1-mm diameter disk was cut from the fungal hyphae lawn. To prepare *A. niger* broth pellets, the fungus was cultured in potato dextrose broth to form pellets around 1 mm in diameter. These pellets were harvested, thoroughly washed 5 times, and used as the broth pellet inoculum.

5.2.3 Determination of IC₅₀

The toxicity of HTLAP for *A. niger* and *R. jostii* was assessed by IC₅₀ value, which served as a quantitative measure of HTLAP biodegradability. The IC₅₀ assay for *R. jostii* was conducted in 24-well microplates in duplicates. In each microplate, a series of two-fold dilutions (1×, 2×, 4×, ...1024×) of HTLAP with tryptic soy broth were prepared. To initiate the IC₅₀ assay, *R. jostii* inoculum was added at 2.5% (v/v) into each well. The microplates were incubated for 1 day at 25°C without agitation. After incubation, the microplates were placed in an ice bath, and samples from each well were subjected to an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The absorbance at 560 nm, determined in the MTT assay, is directly correlated with active cell biomass [11]. For the MTT assay, a 200-μL homogeneous sample from each well was mixed with 800 μL tryptic soy broth. Then, 100 μL MTT solution (5 mg/mL MTT in phosphate-buffered saline) was added and incubated in the dark at 25°C for 1 h for color development in active cells. After incubation, the mixture was centrifuged, and the color of the cell pellet was dissolved in 1 mL DMSO and measured for absorbance at 560 nm (Abs₅₆₀).

IC₅₀ assay for *A. niger* was conducted on serially diluted agar plates for better consistency compared to the cultivation in the liquid medium [15]. To prepare the agar plates, a series of two-fold dilutions (1×, 2×, 4×, ...256×) of HTLAP with potato dextrose agar were

made, ensuring a constant agar concentration of 1.5% (w/v) at each dilution level. Filtration-sterilized HTLAP was added to autoclaved potato dextrose agar rather than autoclaving it with the medium to prevent potential side reactions. Freshly prepared agar for each HTLAP dilution was poured into a 100-mm diameter Petri dish with four compartments, with 8 mL of agar per compartment. After solidification, a 1-mm diameter *A. niger* agar pellet, prepared as described in section 5.2.2, was placed at the center of each agar compartment. The inoculated agar plates were then incubated at 25°C for 2 days before the measurement of the fungal colony diameter.

To determine IC₅₀, a four-parameter inhibitor response model, as shown in Eq. 5.2, was fitted to the Abs560 and colony diameter data at various dilution levels for *R. jostii* and *A. niger*, respectively. The R² values of all regressions were all above 0.9.

$$y = l + \frac{u - l}{1 + \exp[b(\log(x) - \log(e))]} = l + \frac{u - l}{1 + (x/e)^b} \quad (5.2)$$

y: response variable (Abs560 for *R. jostii*, and colony diameter for *A. niger*)

x: dose variable (the HTLAP concentration in g/L COD)

l: lower limit of response

u: upper limit of response

b: slope at the inflection point

e: IC₅₀ of the HTLAP

5.2.4 Fermentation assay

The HTLAP fermentation assay was conducted to evaluate the degradation of various compounds by *A. niger* and *R. jostii* at different time points, offering detailed insights into the biodegradation progress of HTLAP by these microbes. HTLAP was diluted with sterile ultrapure water to achieve a COD of 4500 mg/L. The HTLAP medium was supplemented with 0.4 g/L NH₄Cl and 5 mg/L FeSO₄·7H₂O to address nutrient deficiencies, as described previously [13]. *A.*

niger fermentation was initiated by adding 1-mm diameter broth pellets, prepared as described in section 5.2.2, at a rate of 4 pellets per mL into the diluted HTLAP. The fermentation with *A. niger* proceeded for 15 days, after which the fungal pellets were removed, and *R. jostii* inoculum, prepared as described in section 5.2.2, was added at 5% (v/v) to start the fermentation of the fungal-treated HTLAP. The fermentation assay was performed in sterile 100-mL flasks, each containing 30 mL of HTLAP medium. Incubation was conducted at 25°C with agitation at 150 rpm and humidification. Samples were taken every 5 days to determine COD and analyze the degradation of organic acids and phenolic compounds.

5.2.5 Analytical methods

The chemical composition of HTLAP was analyzed on a high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) [11]. The HPLC (Shimadzu, Kyoto, Japan) was equipped with an Aminex HPX-87H column (7.8×300 mm, Bio-Rad Laboratories, CA, USA) and a refractive index detector (RID). A five-point standard curve was generated for each compound and used for their quantification. The GC-MS (Agilent GC 6890 coupled with a MS 5973, Agilent Technologies, CA, USA) was equipped with a 30-meter long Zebron ZB-1701 column (0.25 mm inner diameter and 0.25 μ m film thickness) (Phenomenex, CA, USA). The samples were derivatized with MSTFA + 1% TMCS (TS-48915, Thermo Fisher Scientific, MA, USA) with trans-cinnamic acid-d6 as an internal standard.

Total organic carbon (TOC), COD, and total nitrogen (TN) were measured by using test kits (Hach Company, IA, USA). Inorganic elements, including K, Ca, Mn, Mg, and Fe were measured on an inductively coupled plasma optical emission spectroscopy (ICP-OES) (Agilent 5800, Agilent Technologies, CA, USA). The pH was measured using a pH meter (Accumet AE150, Fisher Scientific, NH, USA).

5.2.6 Experimental design and statistical analysis

A subset of HTLAP samples were chosen following DoE methodology and were examined with the IC₅₀ and fermentation assays described in sections 5.2.3 and 5.2.4, respectively. The DoE methodology allows for the examination of HTLAP samples across all tested ranges of temperature, time, and CS loading without the need to test every possible combination individually, as required in a one-factor-at-a-time approach. It also incorporates replicated samples for error estimation, thus reducing the total number of samples needed for examination while allowing for a comprehensive evaluation of HTL parameters on HTLAP biodegradability. Specifically, a uniform precision central composite design (CCD) was utilized, consisting of 20 HTLAP samples, including six replicated samples at the center point, to assess the interactions among the input variables: temperature (x_1), time (x_2), and loading (x_3), and the response variables: IC₅₀ for *A. niger* and *R. jostii*. This design was also applied to the subsequent fermentation assay to track the degradation of various compounds in the HTLAP samples, providing additional insights into the biodegradability of HTLAP under different HTL conditions. The input variables were coded with Eq. 5.3 to have values between -1 and 1, allowing for comparison of their coefficients, which correspond to the impact of each input variable on the response. The experimental arrangement was carried out by using JMP Pro 16 software (SAS Institute, Cary, NC, USA).

$$x_i = \frac{2X_i - (U + L)}{U - L} \quad (5.3)$$

x_i : coded value of input variable (between -1 and 1)

X_i : actual value of the input variable.

U : upper limit of X_i

L : lower limit of X_i

The uniform precision CCD is suitable for fitting quadratic response surface with the generalized form of second-order multiple regression equation (Eq. 5.4).

$$Y = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^n \beta_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^n \beta_{ij} x_i x_j \quad (5.4)$$

Y : response variable (IC₅₀ in g/L COD)

β_0 : coefficient for intercept

β_i : coefficient for linear term

β_{ii} : coefficient for quadratic term

β_{ij} : coefficient for interaction term

x_i and x_j : input variables in coded form

n : number of input variables

Analysis of variance (ANOVA) was employed to assess the interaction between the input variables and the response. The quality of fit for Eq. 5.3 was evaluated using the correlation coefficient (R^2), and the model significance was checked using the lack of fit value at 95% confidence level. Contour plots of the response surface were constructed using the fitted models. All statistical and correlation analyses in this study were performed using JMP Pro 16 software (SAS Institute, Cary, NC, USA).

5.3 Results and Discussion

5.3.1 Effect of hydrothermal liquefaction conditions on the aqueous phase chemical compositions

Figure 5.1 shows the concentration variation of different groups of compounds in HTLAP generated under 27 HTL reaction conditions. Distinct patterns of variation were observed for short-chain organic acids, dihydroxybenzene isomers, phenolic acids, phenolic

aldehydes, and furanic compounds with the increase of HTL reaction temperature and time (at a constant temperature). Temperature and time were lumped into a single severity factor for evaluating the pulping process and HTL in the past (Eq. 5.1) [8,16]. This work also used such a way to calculate the HTL reaction severity. Due to the presence of reaction temperature ($T_{\max}$) in the exponent of the integrant, these temperature levels tested in this study (250, 300, and 350°C) contribute more to the severity than time does, as a consequence of which the horizontal axes in Figure 5.1 from left to right represent increasing HTL reaction severity from 5.1 to 9.1.

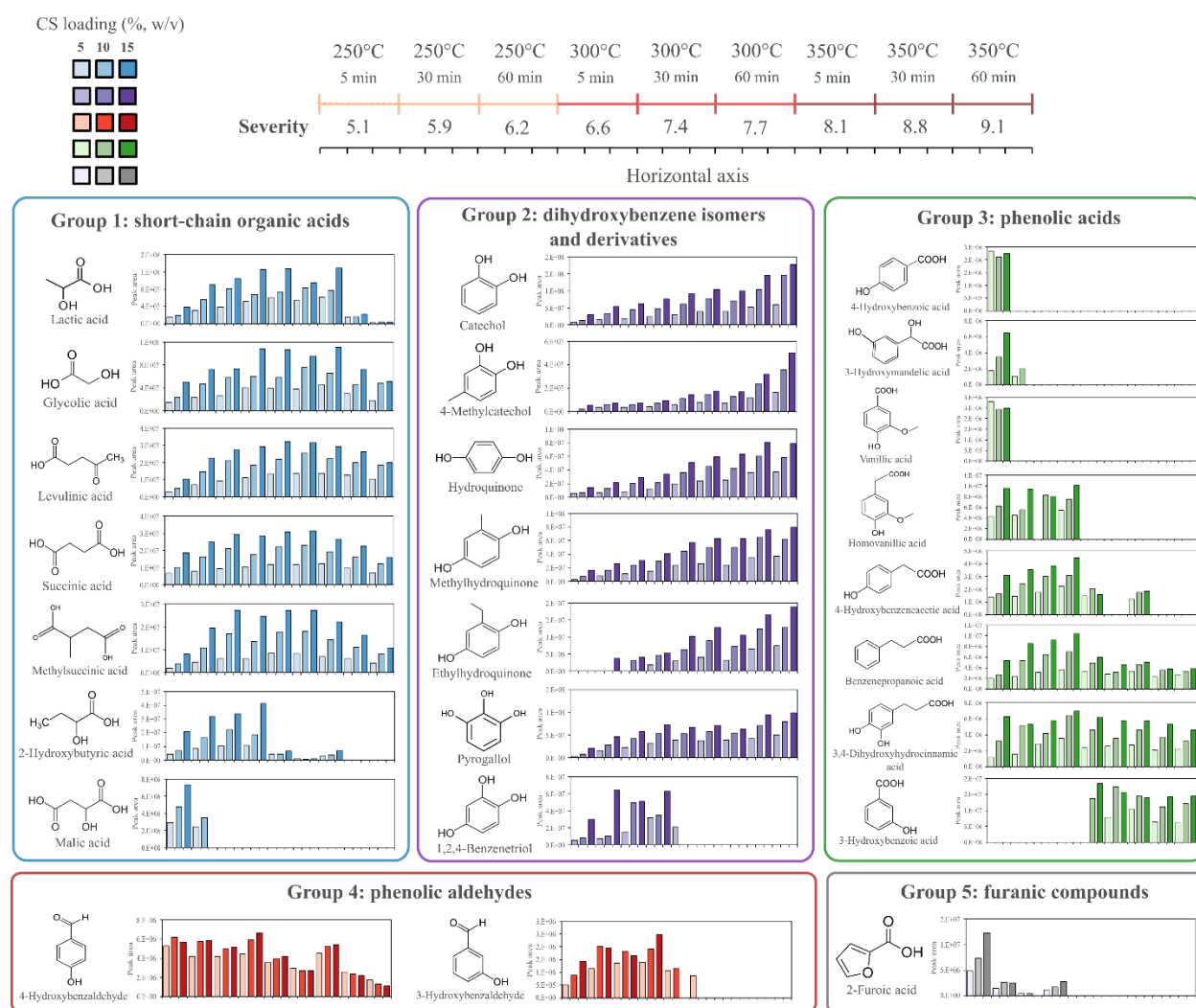


Figure 5.1 Concentration variation of short-chain organic acids (group 1, blue), dihydroxybenzene isomers and derivatives (group 2, purple), phenolic acids (group 3, green),

phenolic aldehydes (group 4, red), and furanic compounds (group 5, gray) when subjected to increased HTL reaction severity. CS loadings during HTL (5, 10, and 15%, w/v) are illustrated with the increasing saturation of the corresponding colors.

As reaction severity increased from 5.1 to 9.1, the concentrations of organic acids including lactic, glycolic, levulinic, succinic, methyl succinic, and 2-hydroxybutyric acids roughly followed an arch-shaped pattern (i.e., increased and then decreased) (Figure 5.1). Organic acids in HTLAP mainly result from the degradation of cellulose, hemicellulose, and carbohydrates [17]. In cellulose HTL, increasing HTL reaction temperature from 200 to 275°C was shown to increase formic, lactic, and glycolic acid concentrations [18]. In this study, a similar trend at temperature from 250 to 300°C for most organic acids was observed, suggesting that increasing HTL reaction severity from 5.1 to 7.4 broke down lignocellulosic biomass more completely into more organic acids that were extracted into HTLAP. Further increase of temperature to 350°C with the increase of reaction severity from 7.4 to 9.1 resulted in the decrease of organic acid concentrations (Figure 5.1). This is likely due to the degradation of these acids to acetic, formic, and propionic acids, and potentially to gaseous products, such as CO₂ that was found as a predominant component in HTL gas phase [5,19].

The concentrations of all dihydroxybenzene isomers and derivatives except for 1,2,4-benzenetriol were increased with the increase of HTL reaction severity from 5.1 to 9.1 (Figure 5.1). Phenolics in HTLAP mainly originated from lignin of CS which underwent hydrolysis during HTL and was degraded into monophenolics [17,20]. As shown in Figure 5.1, the higher HTL reaction severity facilitated the degradation reaction, leading to an increase in the concentration of phenolic compounds, such as catechol, 4-methylcatechol, hydroquinone, methylhydroquinone, and pyrogallol, which is in accordance with the results of lignin HTL [21].

1,2,4-benzenetriol likely came from cellulose of CS because it is known that HTL converts cellulose into 5-hydroxymethylfurfural which can be further converted into 1,2,4-benzenetriol [22]. However, this compound was only detected at low reaction severities of 5.1~6.2. The reason for the absence of this compound at high reaction severities is unknown.

Phenolic acids and phenolic aldehydes were detected in HTLAP, and their concentrations followed different patterns with the variation of the HTL severity (Figure 5.1). Specifically, 4-hydroxybenzoic acid, 3-hydroxymandelic acid, vanillic acid, homovanillic acid, and 4-hydroxybenzeneacetic acid were only present in HTLAP at low reaction severities less than 5.1, 5.9, 5.1, 6.6, and 8.1, respectively. This is likely due to the demethylation and decarboxylation of these compounds at high reaction severities, resulting in the increased concentrations of dihydroxybenzene isomers, such as catechol and hydroquinone. Such reactions have been proposed to describe the decomposition of vanillin and isovanillin under hydrothermal conditions [23]. Other phenolic acids, including benzenepropanoic acid, 3,4-dihydrocinnamic acid, and 3-hydroxybenzoic acid were present at the reaction severity above 8.1, suggesting their higher stability than the aforementioned phenolic acids. The phenolic aldehyde 4-hydroxybenzenealdehyde was present in HTLAP in the entire tested reaction severity ranging from 5.1 to 9.1, although its concentration decreased at the severity of 8.8 and 9.1. On the other hand, 3-hydroxybenzaldehyde was only present in HTLAP when the reaction severity was between 5.1 and 7.7. Due to the complexity of HTL reaction and the limited understanding of its reaction mechanisms, the reason for the observed concentration variation of the phenolic aldehydes remains unclear.

Furanic compounds are formed due to the dehydration of carbohydrates during hydrothermal conversion [22]. As shown in Figure 5.1, one furanic compound, 2-furoic acid was

detected in HTLAP in this study and it was only present at low reaction severities below 6.6. Furfural was not detected in HTLAP in this study. This is in agreement with the literature where the degradation of furanic compounds, such as 5-hydroxymethyl furfural and furfural have been reported at high hydrothermal reaction severities [24]. Unlike phenolic compounds, furanic compounds were not a major compound in HTLAP of lignocellulosic biomass, which could be due to transient presence of these compounds at high HTL temperature and/or pressure. It is noteworthy that both phenolic compounds and furanic compounds are known to inhibit microbial growth and activities [25,26]. Less furanic compounds than phenolic compounds could suggest that the microbial inhibitory effect of HTLAP could be mainly attributed to the presence of phenolic compounds rather than furanic compounds.

5.3.2 Characterization of hydrothermal liquefaction aqueous phase used for biodegradability assay

Figure 5.2a shows the subset of HTLAP samples selected for biodegradability assays based on their HTL conditions as described in section 5.2.6, highlighting the combinations of temperature, time, and CS loading for the individual HTLAP samples. The design incorporated combinations of reaction temperature and time that covered all severities in Figure 5.1 while enabling the examination of CS loading effect on the biodegradability of HTLAP. The remaining analyses, which focus on the evaluation of biodegradability through both IC_{50} and compound degradation during fermentation, were conducted using this twenty-sample design.

Prior to the biodegradability analysis, it is important to examine the characteristics of the selected HTLAP samples, including COD, TOC, TN, and elemental composition. Figure 5.2b-h illustrate the characteristics of HTLAP generated under the conditions selected as shown in Figure 5.2a. The COD and TOC of the HTLAP ranged from 13 to 38 g/L, and from 3.5 to 16

g/L, respectively (Figure 5.2a and b). Both parameters demonstrated a strong linear correlation with CS loading (correlation coefficients > 0.9 , $p < 0.0001$), which indicated that the higher CS loading in HTL led to more organic compounds being extracted into HTLAP. The TN of HTLAP was low, falling between 0.06 and 0.17 g/L (Figure 5.2d). The measured COD, TOC, and TN for HTLAP aligned with literature on HTLAP of lignocellulosic biomass with a high carbohydrate to protein ratio [5]. Calcium concentration in HTLAP ranged from 77 to 264 ppm and had a strong linear correlation coefficient of 0.99 ($p < 0.0001$) with CS loading, suggesting the soluble nature of the calcium fraction regardless of reaction severity (Figure 5.2e). The variation in magnesium concentration closely mirrored that of calcium at reaction severity below 8.1, indicating that this element also remained soluble in HTLAP at these severities (Figure 5.2f). However, increasing reaction severity to 8.8 and 9.1 significantly reduced the magnesium concentration in HTLAP, likely due to its precipitation into the solid residue under those conditions (Figure 5.2f) [27]. The iron concentration in HTLAP ranged from 0 to 11 ppm, while it became undetectable with the reaction severity above 7.7 (Figure 5.2g). The manganese concentration varied from 1.0 to 7.8 ppm and was linearly associated with CS loading (correlation coefficient=0.83, $p=0.0001$) (Figure 5.2h). The absence of iron in multiple HTLAP samples and the low TN in all samples necessitate the supplementation of FeSO_4 and NH_4Cl to HTLAP for aerobic fermentation [13].

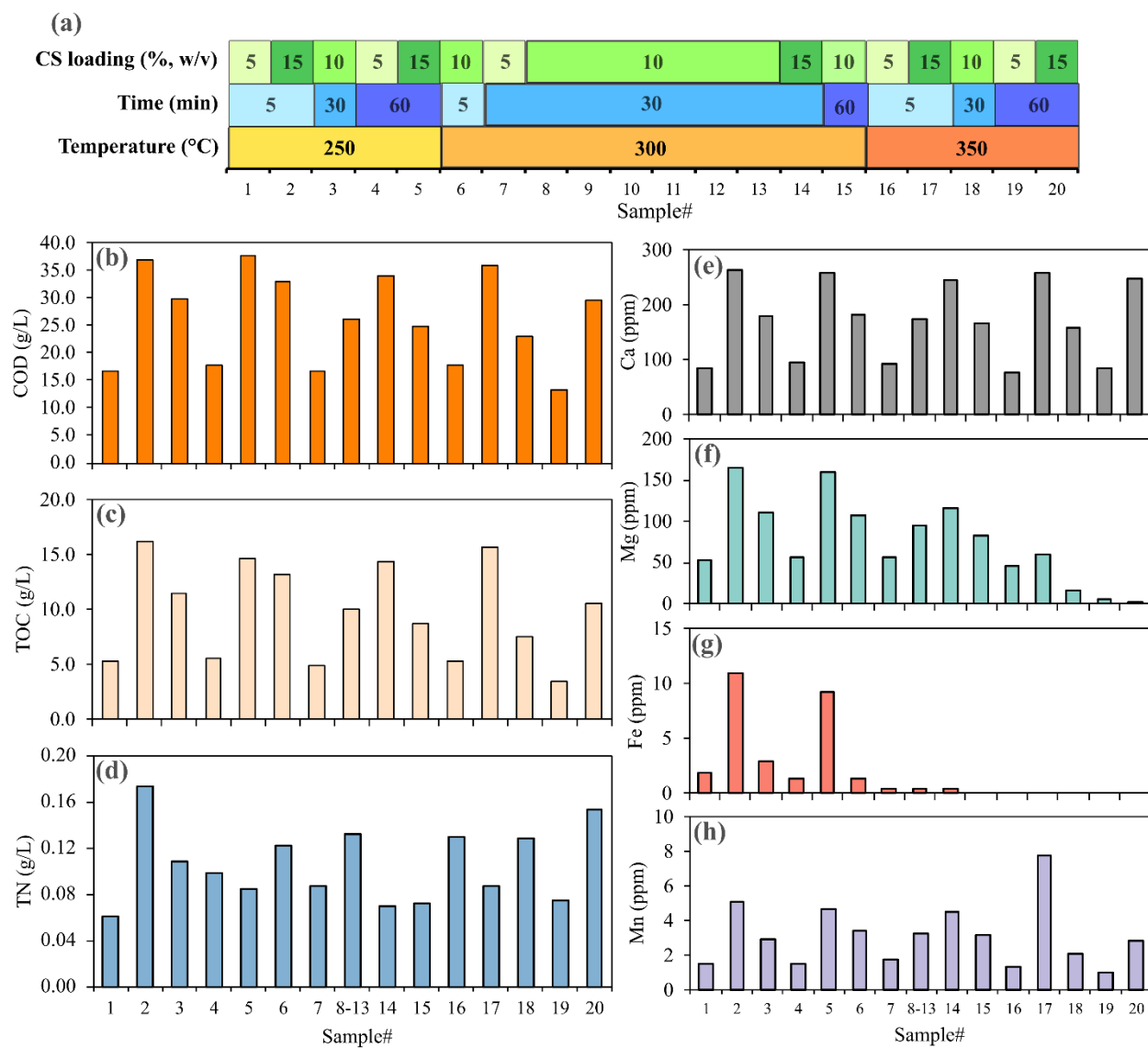


Figure 5.2 Schematic diagram of the twenty HTLAP samples used for subsequent biodegradability analyses, illustrating the HTL conditions used to generate the HTLAP samples (a). Water quality parameters including COD (b), TOC (c), and TN (d), and concentration of calcium (e), magnesium (f), iron (g), and manganese (h) elements of the selected HTLAP samples. Since samples 8 to 13 were subsets taken from pooled HTLAP produced under the same HTL condition, their water quality parameters and elemental compositions were measured together rather than individually.

5.3.3 Impact of hydrothermal liquefaction conditions on IC₅₀ of the aqueous phase

Table 5.1 shows the IC₅₀ values of the selected HTLAP samples (shown in Figure 5.2a) for *A. niger* and *R. jostii*. IC₅₀ is a quantitative measure representing the amount of a particular inhibitory substance required to interfere with a biological process or biological component by 50% *in vitro* [28]. In this study, IC₅₀ represents the inhibitory effect of HTLAP on *A. niger* and *R. jostii* and serves as a quantitative measure of HTLAP biodegradability, expressed as the g/L of HTLAP-derived COD required to inhibit microbial growth by 50%. Overall, the IC₅₀ of HTLAP decreased as the HTL reaction severity increased (Table 5.1), inferring that the HTLAP became more inhibitory to the two microbes. In addition, the IC₅₀ for *R. jostii* decreased more rapidly than that for *A. niger*, suggesting that *A. niger* is more tolerant of HTLAP.

Further insight on the IC₅₀ data shown in Table 5.1 was developed by fitting them to a second-order response surface model (Eq. 5.4). The statistical significance of the model coefficients was evaluated using ANOVA based on their corresponding F and p-values at 5% confidence level. To simplify the model and ensure an insignificant lack of fit, any non-significant terms were removed. The ANOVA results and estimated value of coefficients are shown in Table 5.2. The simplified IC₅₀ models for both *R. jostii* and *A. niger* were significant with p-values of less than 0.0001 and had insignificant lack-of-fit p-values, indicating that the models fit the data well. CS loading (x_3) was found to be insignificant based on the ANOVA and was therefore completely removed from the model. As the IC₅₀ is expressed in g/L of HTLAP-derived COD, which already accounts for the linear effect of CS loading on the concentration of organic compounds in HTLAP. In this respect, such results confirm the accuracy of the response surface model and the IC₅₀ assays, indicating that decreasing CS loading in HTL is nearly equivalent to the dilution of the resulted HTLAP.

The coefficient estimates in the response surface model can be used for direct comparison of the effects of temperature (x_1) and time (x_2) on the IC_{50} of HTLAP. The reason is that the input variables x_1 and x_2 were rescaled to values between -1 and 1 for model fitting, as described in Eq. 5.3. As shown in Table 5.2, the IC_{50} for both *R. jostii* and *A. niger* was predominantly affected by temperature, as indicated by the x_1 coefficients being 2-3 times the absolute value of the x_2 coefficients. The negative values of both x_1 and x_2 coefficients suggest that both variables negatively impact the IC_{50} of HTLAP for *R. jostii* and *A. niger*.

The resulting models that express the empirical relationships between HTL conditions and the IC_{50} of the resulting HTLAP are presented in Eqs. 5.5 and 5.6, for *R. jostii* and *A. niger*, respectively.

$$IC_{50}(R.jostii) = 15.03 - 0.0390 \times Temperature - 0.0207 \times Time \quad (5.5)$$

$$IC_{50}(A.niger) = 6.77 - 0.00699 \times Temperature + 0.0883 \times Time - 3.49 \times 10^{-4} \times Temperature \times Time \quad (5.6)$$

Figure 5.3a and b illustrate the response surfaces of these equations. The models consist of only first-order terms, with the model for *A. niger* including an additional interaction term. The models in Eqs. 5.5 and 5.6 have R^2 of 0.78 and 0.77, respectively, indicating that 78% and 77% of the variation in the response can be explained by these models. This satisfactory fit is also evident in Figure 5.3a and b, where the empirical data points closely align with the response surfaces. Additionally, the response surfaces clearly demonstrate the negative impacts of temperature and time on IC_{50} and highlight the higher IC_{50} of HTLAP for *A. niger* (Figure 5.3a) compared to *R. jostii* (Figure 5.3b).

As shown in Figure 5.3c, the concentrations of certain compounds in HTLAP were linearly correlated with the IC_{50} for *A. niger* and *R. jostii*. Compounds were ranked by decreasing

peak area in the GC-MS chromatograph, from catechol to vanillic acid. Strong negative correlations, with coefficients between -0.68 and -0.84 ($p < 0.0001$), were found between the concentrations of catechol/pyrogallol/methylhydroquinone/hydroquinone/4-methylcatechol and the IC_{50} values for *A. niger* and *R. jostii*, suggesting that increasing concentrations of these five phenolic compounds in HTLAP likely contributed to the lower IC_{50} observed for both *A. niger* and *R. jostii*. Phenolic compounds are known to inhibit microbial growth and considered a major group of compounds contributing to the microbial toxicity of HTLAP [6,29]. This study provides strong evidence that increasing concentrations of certain phenolic compounds, including catechol, pyrogallol, methylhydroquinone, hydroquinone, and 4-methylcatechol, are responsible for the increased microbial inhibition of HTLAP.

Table 5.1 Central composite design with the selected HTLAP samples and their IC_{50} results

Sample#	Temperature (°C)	Time (min)	CS loading (% w/v)	Reaction severity	IC_{50} (g/L COD)*	
					<i>A. niger</i>	<i>R. jostii</i>
1	250	5	5	5.1	5.49	5.31
2	250	5	15	5.1	3.95	5.19
3	250	30	10	5.9	5.60	5.23
4	250	60	5	6.2	5.61	3.87
5	250	60	15	6.2	4.29	4.82
6	300	5	10	6.6	4.94	4.03
7	300	30	5	7.4	4.60	4.95
8	300	30	10	7.4	4.23	2.05
9	300	30	10	7.4	4.11	1.84
10	300	30	10	7.4	4.43	2.25
11	300	30	10	7.4	4.21	2.03
12	300	30	10	7.4	4.29	1.90
13	300	30	10	7.4	4.13	1.89
14	300	30	15	7.4	3.85	2.04
15	300	60	10	7.7	3.48	1.63
16	350	5	5	8.1	4.35	2.05
17	350	5	15	8.1	3.95	0.88
18	350	30	10	8.8	2.75	0.77
19	350	60	5	9.1	2.48	0.74
20	350	60	15	9.1	2.33	0.50

* The IC_{50} was determined by fitting data from duplicated runs to Eq. 5.2.

Table 5.2 ANOVA results and estimated coefficients for IC₅₀ models

Response	Source	Coefficient estimate	df	F-value	p-value (Prob. > F)
IC ₅₀ (<i>R. jostii</i>)	Model		2	29.93	<0.0001
	β_0	2.673	1		
	x_1	-1.948	1	55.15	<0.0001
	x_2	-0.568	1	4.71	0.0445
	Lack of fit		6	0.58	0.7384
IC ₅₀ (<i>A. niger</i>)	Model		3	18.19	<0.0001
	β_0	4.133	1		
	x_1	-0.917	1	37.73	<0.0001
	x_2	-0.453	1	9.24	0.0078
	$x_1 x_2$	-0.480	1	8.30	0.0109
	Lack of fit		5	0.93	0.5006

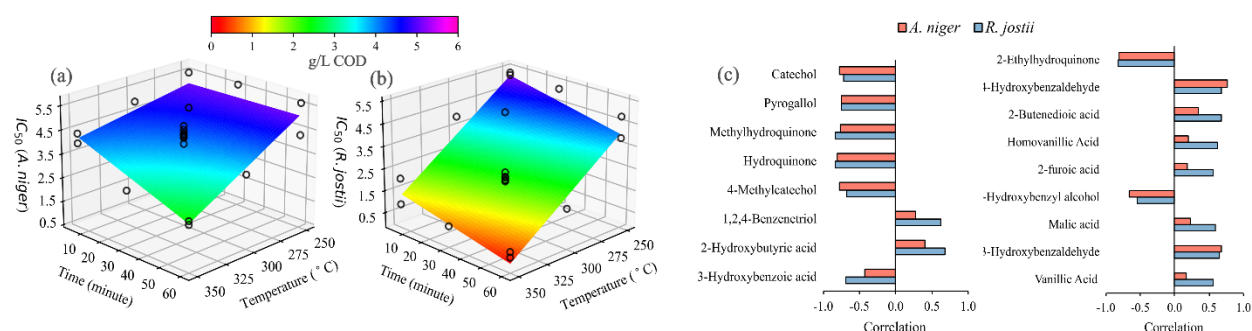


Figure 5.3 Response surface plots showing the effects of HTL reaction temperature and time on the IC₅₀ (in g/L COD) of the resulting HTLAP for *A. niger* (a) and *R. jostii* (b). The actual IC₅₀ data points are plotted as black-edged circles along with the response surfaces. The statistically significant ($p < 0.05$) Pearson correlation coefficients between the concentrations of different compounds in HTLAP and the IC₅₀ of HTLAP for *A. niger* and *R. jostii* are shown in (c).

5.3.4 Biodegradation of hydrothermal liquefaction aqueous phase via aerobic fermentation

In addition to IC₅₀, examining the removal of individual compounds during aerobic fermentation of HTLAP is necessary to gain further insight into its biodegradability. Following

the IC₅₀ examination, the selected HTLAP samples shown in Figure 5.2a were subjected to the fermentation assay described in section 5.2.4. Specifically, a sequential fermentation assay was conducted, which included fermentation with *A. niger* from day 0 to 15, followed by *R. jostii* from day 15 to 25. The sequential fermentation approach was chosen to accommodate the lower IC₅₀ of HTLAP for *R. jostii* than *A. niger*. Previous studies showed that initial fermentation with *A. niger* removed most phenolic compounds in HTLAP, thereby facilitating the subsequent growth of *R. jostii* [13]. To ensure consistent comparisons on the degradation across the twenty HTLAP samples, they were diluted to a uniform initial COD of 4500 mg/L, as described in section 5.2.4, before undergoing aerobic fermentation.

As shown in Figure 5.4a, seven major compounds in HTLAP were monitored during fermentation and their combined concentration accounted for 31-64% of the TOC in HTLAP. Lower percentages of quantified TOC, ranging from 31% to 44%, were observed for HTLAP samples obtained at low reaction severities (5.1~6.6) (Figure 5.4a). This result may be due to the high concentrations of unreacted high-molecular-weight compounds present in HTLAP generated at low reaction severities. A previous study indicated that the average molecular weight of dissolved organic matter in HTLAP corresponded to formulas of C₁₈H₁₈N₁O₇ and C₂₁H₂₃N₄O₇, suggesting a significant presence of high-molecular-weight compounds in HTLAP [30]. Since these high-molecular-weight compounds were not quantifiable with the analytical methods used in this study, their concentrations were not included in Figure 5.4a.

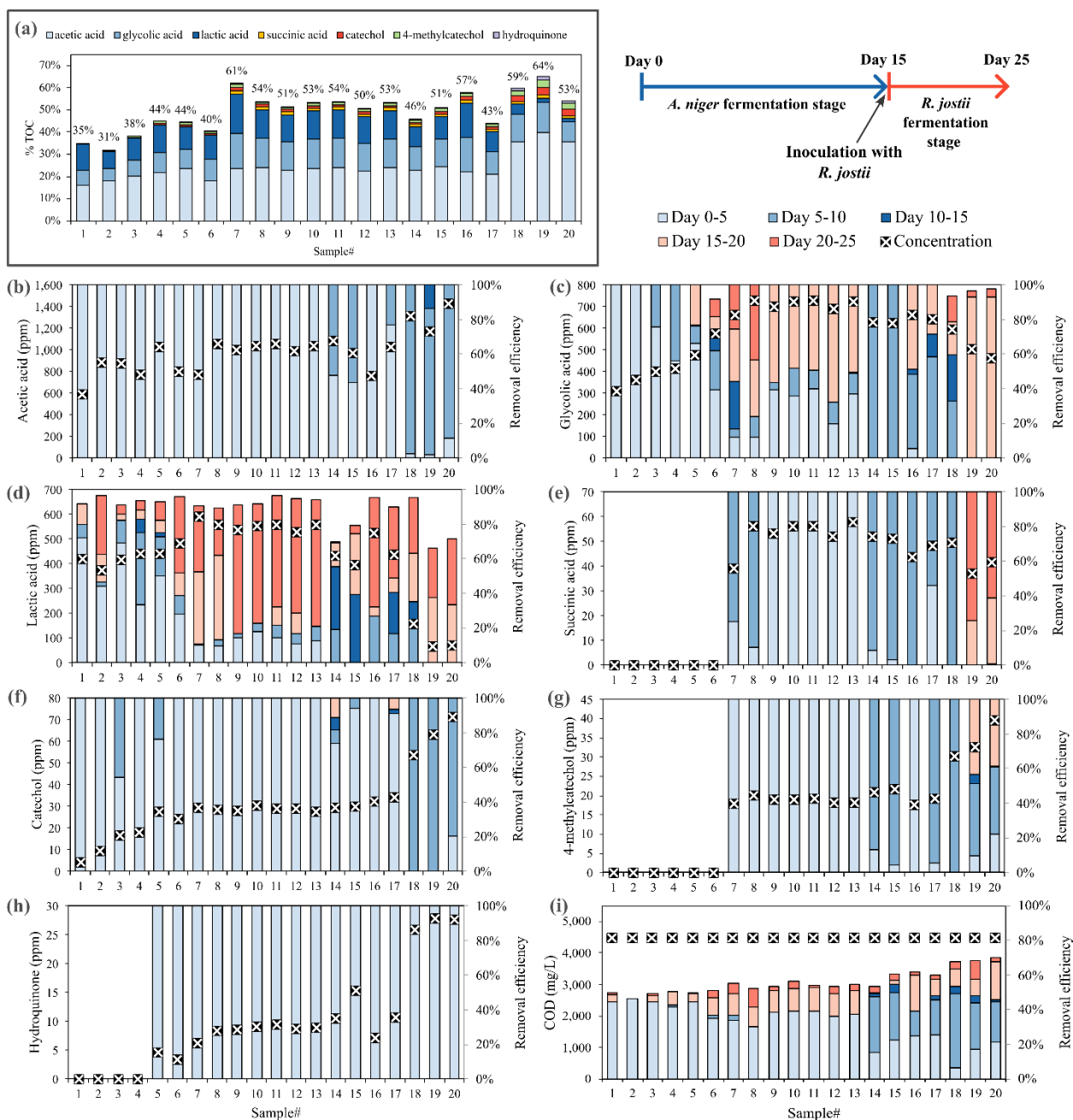


Figure 5.4 Concentrations of monitored compounds in HTLAP expressed as percentages of TOC (a), and their degradation during fermentation with *A. niger* (day 0-15) and *R. jostii* (day 15-25) in each of the twenty samples selected via CCD (b-h). The overall COD removal during the fermentation is shown in (i). Starting concentrations of COD and individual compounds for

each sample are marked with a cross, while removal efficiencies are illustrated with bars colored according to the fermentation time points.

Acetic acid is the most abundant compound in HTLAP, accounting for 16-40% of TOC depending on HTL reaction conditions, and its concentration increased as reaction severity rose (Figure 5.4a). As shown in Figure 5.4b, acetic acid was degraded rapidly during fermentation and completely removed in 5 days for 14 out of 20 samples. This result indicates that acetic acid is a readily consumable carbon source in HTLAP for *A. niger* fermentation, consistent with the literature [13]. Glycolic acid and lactic acid were also major organic acids present in HTLAP, representing 6-16% and 2-18% of TOC, respectively (Figure 5.4a). However, the degradation of these two acids by *A. niger* was incomplete, with less than 60% removal for most samples during the 15-day fungal fermentation stage (Figure 5.4c and d). The remaining organic acids were largely consumed by *R. jostii* during the subsequent bacterial fermentation stage. Specifically, following *R. jostii* fermentation, the removal of glycolic acid in all samples exceeded 90% (Figure 5.4c), and lactic acid removal in 16 out of 20 samples also exceeded 90% (Figure 5.4d). Succinic acid was present at low concentration in HTLAP, representing less than 2% of TOC (Figure 5.4a), which likely contributed to its completed removal in 18 out of 20 samples during the *A. niger* fermentation (Figure 5.4e). Overall, these results suggested that glycolic acid and lactic acid are less preferred carbon sources for *A. niger* compared to acetic acid, with glycolic acid consumed slightly faster than lactic acid by this fungus. Since *A. niger* alone cannot remove all the glycolic acid and lactic acid in HTLAP, *R. jostii* needs to be inoculated at a later stage of fermentation. *R. jostii* can consume most glycolic acid and lactic acid left behind by *A. niger*. Similar to *A. niger*, *R. jostii* also prioritized the consumption of glycolic acid over lactic acid.

Phenolic compounds, such as catechol, 4-methylcatechol, and hydroquinone only represent less than 3.5% TOC in HTLAP (Figure 5.4a). However, they are primarily responsible for the microbial toxicity of HTLAP (Figure 5.3c). The degradation of catechol, 4-methylcatechol, and hydroquinone during HTLAP fermentation is shown in Figure 5.4f, g, and h, respectively. Both catechol and hydroquinone were readily degraded by *A. niger*, with complete degradation occurring within the first 5 days of fermentation for most of HTLAP samples. In comparison, 4-methylcatechol was degraded more slowly, with only minor degradation observed in many samples during the first 5 days. This slower degradation was likely due to the need for fungi and bacteria to convert 4-methylcatechol into intermediates, such as catechol, protocatechuic acid, and/or hydroquinone prior to the ring-cleavage reactions, which slowed down its degradation during HTLAP fermentation [31]. Figure 5.4b-h also show that when the concentrations of catechol, hydroquinone, and 4-methylcatechol were high, as in samples 18, 19, and 20, the degradation of these phenolic compounds and organic acids was much slower, further confirming the negative impact of these phenolic compounds on microbial activity as shown in Figure 5.3c.

The overall biodegradation performance was quantified as COD removal (Figure 5.4i). *A. niger* fermentation removed 29-53% of COD, and the sequential fermentation with *R. jostii* further increased the COD removal to 45-70%. A trend was observed where HTLAP from lower reaction severities exhibited lower COD removal efficiencies at the end of the 25-day fermentation. This was likely because HTLAP from lower reaction severities contains larger fractions of high molecular weight compounds, which were known to resist microbial degradation [30].

Based on the results shown in Figure 5.4, the biodegradation profiles of HTLAP during *A. niger* and *R. jostii* fermentation can be categorized into three distinct types, as illustrated in Figure 5.5. In detail, Figure 5.5 highlights the chemical compositions of the corresponding HTLAP samples and tracks the degradation of various compounds throughout the 25-day fermentation period, offering a quantitative overview of compound degradation during aerobic fermentation. The first type of profile corresponds to samples 1 through 6, derived from HTLAP with severities of 5.1 to 6.6. These HTLAP samples were characterized by low concentrations of phenolic compounds and showed rapid degradation of all quantified compounds, primarily within the first 5 days of incubation. The second type of degradation profile, covering samples 7 to 18 (severities 7.4 to 8.8), involved HTLAP with higher concentrations of phenolics and organic acids. In this group, the *A. niger* fermentation period (day 0-15) resulted in incomplete removal of glycolic acid and lactic acid, leaving a substantial portion for *R. jostii* fermentation from day 15 to 25. The third type of profile includes samples 19 and 20, representing HTLAP derived from a reaction severity of 9.1. These samples contained the highest phenolic compound concentrations observed in this study, leading to significantly slower biodegradation compared to the other two types.

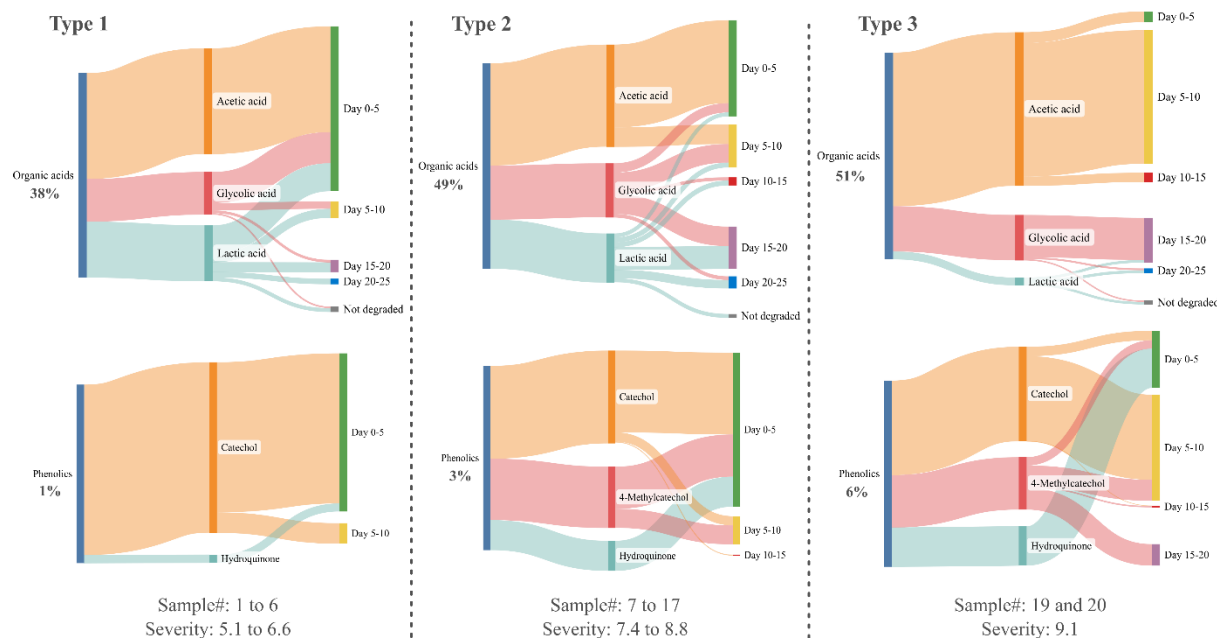


Figure 5.5 Schematics of three types of compound degradation profiles during aerobic fermentation with *A. niger* (day 0-15) followed by *R. jostii* (day 15-25). Each type of degradation profile is separated by dashed lines, with the corresponding sample number and severity range labeled at the bottom. The width of each node on the diagram quantitatively represents the average percentage of an individual compound in terms of TOC, with nodes representing zero percentage removed from the diagram. Compounds categorized as organic acids and phenolics are illustrated separately, with the corresponding total percentage of TOC labeled on the graphs.

5.3.5 Perspectives on integrating aerobic fermentation into hydrothermal liquefaction technology

To integrate aerobic fermentation treatment of HTLAP into HTL technology, techno-economic analysis and life cycle assessment are necessary to systematically evaluate the viability of the fermentation process. While aerobic fermentation has shown advantages, such as rapid HTLAP degradation and high tolerance to microbial inhibition, it also requires energy input for aeration and generates carbon dioxide, which are drawbacks compared to other biological

HTLAP treatment methods, e.g., AD and microalgal cultivation. This study lays the groundwork for conducting a techno-economic analysis and life cycle assessment of aerobic fermentation by establishing the relationship between HTL conditions and HTLAP biodegradability in aerobic fermentation. The IC_{50} and biodegradability data for HTLAP derived from various HTL conditions can help set process thresholds, such as minimum HTLAP dilution and minimum treatment time, for the fermentation of HTLAP from different HTL conditions. Further work will be done to establish the relationship between HTL reaction conditions and the yield and quality of biocrude oil produced. These studies will reveal systematic and quantitative insights into the feasibility of incorporating aerobic fermentation treatment of HTLAP into HTL technology.

5.4 Conclusions

The chemical compositions of HTLAP derived under various HTL conditions demonstrated distinct patterns as reaction severity increased. HTLAP produced at higher reaction severities was more inhibitory to *A. niger* and *R. jostii*, with the increased microbial inhibition being attributed to the elevated concentrations of specific phenolic compounds. Additionally, the IC_{50} of HTLAP for both *A. niger* and *R. jostii* could be effectively predicted using multiple linear regression models with HTL reaction temperature and time as the input variables. This study also showed that aerobic fermentation with *A. niger* and *R. jostii* is a versatile process capable of treating HTLAP derived from the HTL of CS across a wide range of conditions, i.e., temperature (250, 300, and 350°C), time (5, 30, and 60 min), and CS loading (5, 10, and 15%, w/v), while achieving 45-70% COD removal depending on the specific HTL conditions. Overall, the findings of this work provide a quantitative understanding of the relationships between the HTL condition and the chemical composition and biodegradability of the resulting HTLAP, laying the groundwork for the integration of HTLAP aerobic fermentation into HTL technology.

Acknowledgement

This work was supported by the National Science Foundation [Grant number: 2001593]. The authors thank Dr. Lindsay Eltis from the Departments of Microbiology and Biochemistry at The University of British Columbia, Vancouver, Canada, for providing the *R. jostii* RHA1 strain. We also appreciate the support from the U.S. Department of Agriculture Agricultural Research Service Culture Collection (Northern Regional Research Laboratory) for supplying the *A. niger* strain used in this study. Additionally, we are grateful to Prof. Ganga Hettiarachchi and Ph.D. student Chandima Wekumbura Wekumbure Gedara from the Department of Agronomy at Kansas State University for their assistance with ICP-OES analysis.

References

- [1] X.-Q. Zhao, C.-G. Liu, F.-W. Bai, Making the biochemical conversion of lignocellulose more robust, *Trends in Biotechnology* 42 (2024) 418–430.
<https://doi.org/10.1016/j.tibtech.2023.09.014>.
- [2] A. Swetha, S. ShriVigneshwar, K.P. Gopinath, R. Sivaramakrishnan, R. Shanmuganathan, J. Arun, Review on hydrothermal liquefaction aqueous phase as a valuable resource for biofuels, bio-hydrogen and valuable bio-chemicals recovery, *Chemosphere* 283 (2021) 131248. <https://doi.org/10.1016/j.chemosphere.2021.131248>.
- [3] A.R.K. Gollakota, N. Kishore, S. Gu, A review on hydrothermal liquefaction of biomass, *Renewable and Sustainable Energy Reviews* 81 (2018) 1378–1392.
<https://doi.org/10.1016/j.rser.2017.05.178>.
- [4] L. Cao, C. Zhang, H. Chen, D.C.W. Tsang, G. Luo, S. Zhang, J. Chen, Hydrothermal liquefaction of agricultural and forestry wastes: state-of-the-art review and future prospects, *Bioresource Technology* 245 (2017) 1184–1193. <https://doi.org/10.1016/j.biortech.2017.08.196>.

- [5] J. Watson, T. Wang, B. Si, W.-T. Chen, A. Aierzhati, Y. Zhang, Valorization of hydrothermal liquefaction aqueous phase: pathways towards commercial viability, *Progress in Energy and Combustion Science* 77 (2020) 100819. <https://doi.org/10.1016/j.pecs.2019.100819>.
- [6] Y. Gu, X. Zhang, B. Deal, L. Han, Biological systems for treatment and valorization of wastewater generated from hydrothermal liquefaction of biomass and systems thinking: A review, *Bioresource Technology* 278 (2019) 329–345. <https://doi.org/10.1016/j.biortech.2019.01.127>.
- [7] M. Pham, L. Schideman, B.K. Sharma, Y. Zhang, W.-T. Chen, Effects of hydrothermal liquefaction on the fate of bioactive contaminants in manure and algal feedstocks, *Bioresource Technology* 149 (2013) 126–135. <https://doi.org/10.1016/j.biortech.2013.08.131>.
- [8] I.A. Basar, H. Liu, C. Eskicioglu, Incorporating hydrothermal liquefaction into wastewater treatment – Part III: Aqueous phase characterization and evaluation of on-site treatment, *Chemical Engineering Journal* 467 (2023) 143422. <https://doi.org/10.1016/j.cej.2023.143422>.
- [9] C. Gai, Y. Zhang, W.-T. Chen, Y. Zhou, L. Schideman, P. Zhang, G. Tommaso, C.-T. Kuo, Y. Dong, Characterization of aqueous phase from the hydrothermal liquefaction of *Chlorella pyrenoidosa*, *Bioresource Technology* 184 (2015) 328–335. <https://doi.org/10.1016/j.biortech.2014.10.118>.
- [10] I.A. Basar, A. Stokes, C. Eskicioglu, Evaluation of on-site biological treatment options for hydrothermal liquefaction aqueous phase derived from sludge in municipal wastewater treatment plants, *Water Research* 252 (2024) 121206. <https://doi.org/10.1016/j.watres.2024.121206>.

- [11] M. Liu, C. Mahata, Z. Wang, S. Kumar, Y. Zheng, Comparative exploration of biological treatment of hydrothermal liquefaction wastewater from sewage sludge: Effects of culture, fermentation conditions, and ammonia stripping, *Journal of Environmental Management* 349 (2024) 119527. <https://doi.org/10.1016/j.jenvman.2023.119527>.
- [12] Y. He, X. Li, X. Xue, M.S. Swita, A.J. Schmidt, B. Yang, Biological conversion of the aqueous wastes from hydrothermal liquefaction of algae and pine wood by *Rhodococci*, *Bioresource Technology* 224 (2017) 457–464. <https://doi.org/10.1016/j.biortech.2016.10.059>.
- [13] M. Liu, J. Wang, I. Umeda, Z. Wang, S. Kumar, Y. Zheng, Harnessing filamentous fungi and fungal-bacterial co-culture for biological treatment and valorization of hydrothermal liquefaction aqueous phase from corn stover, *Bioresource Technology* 409 (2024) 131240. <https://doi.org/10.1016/j.biortech.2024.131240>.
- [14] D. Baş, İ.H. Boyacı, Modeling and optimization II: Comparison of estimation capabilities of response surface methodology with artificial neural networks in a biochemical reaction, *Journal of Food Engineering* 78 (2007) 846–854. <https://doi.org/10.1016/j.jfoodeng.2005.11.025>.
- [15] J.-R. Kuate, J.M. Bessière, P.H.A. Zollo, S.P. Kuate, Chemical composition and antidermatophytic properties of volatile fractions of hexanic extract from leaves of *Cupressus lusitanica* Mill. from Cameroon, *Journal of Ethnopharmacology* 103 (2006) 160–165. <https://doi.org/10.1016/j.jep.2005.07.022>.
- [16] R.P. Overend, E. Chornet, J.A. Gascoigne, B.S. Hartley, P.M.A. Broda, P.J. Senior, Fractionation of lignocellulosics by steam-aqueous pretreatments, *Philosophical Transactions of the Royal Society of London. Series A, Mathematical and Physical Sciences* 321 (1997) 523–536. <https://doi.org/10.1098/rsta.1987.0029>.

- [17] W.-T. Chen, Y. Zhang, J. Zhang, G. Yu, L.C. Schideman, P. Zhang, M. Minarick, Hydrothermal liquefaction of mixed-culture algal biomass from wastewater treatment system into bio-crude oil, *Bioresource Technology* 152 (2014) 130–139.
<https://doi.org/10.1016/j.biortech.2013.10.111>.
- [18] G. Nazeri, S.B. Liaw, Y. Yu, H. Wu, Formation of organic acids during cellobiose decomposition in hot-compressed water, *Fuel* 218 (2018) 174–178.
<https://doi.org/10.1016/j.fuel.2018.01.016>.
- [19] A.T. Quitain, M. Faisal, K. Kang, H. Daimon, K. Fujie, Low-molecular-weight carboxylic acids produced from hydrothermal treatment of organic wastes, *Journal of Hazardous Materials* 93 (2002) 209–220. [https://doi.org/10.1016/S0304-3894\(02\)00024-9](https://doi.org/10.1016/S0304-3894(02)00024-9).
- [20] C. Peng, G. Zhang, J. Han, X. Li, Hydrothermal conversion of lignin and black liquor for phenolics with the aids of alkali and hydrogen donor, *Carbon Resources Conversion* 2 (2019) 141–150. <https://doi.org/10.1016/j.crcon.2019.06.004>.
- [21] K.R. Arturi, M. Strandgaard, R.P. Nielsen, E.G. Søgaaard, M. Maschietti, Hydrothermal liquefaction of lignin in near-critical water in a new batch reactor: Influence of phenol and temperature, *The Journal of Supercritical Fluids* 123 (2017) 28–39.
<https://doi.org/10.1016/j.supflu.2016.12.015>.
- [22] A.J. Kumalaputri, C. Randolph, E. Otten, H.J. Heeres, P.J. Deuss, Lewis Acid Catalyzed Conversion of 5-Hydroxymethylfurfural to 1,2,4-Benzenetriol, an Overlooked Biobased Compound, *ACS Sustain Chem Eng* 6 (2018) 3419–3425.
<https://doi.org/10.1021/acssuschemeng.7b03648>.

- [23] D. Alam, M.Y. Lui, A.K.L. Yuen, Z. Li, X. Liang, T. Maschmeyer, B.S. Haynes, A. Montoya, Substituted Aromatic Aldehyde Decomposition under Hydrothermal Conditions, *Energy Fuels* 36 (2022) 5375–5383. <https://doi.org/10.1021/acs.energyfuels.2c00361>.
- [24] M. Kröger, F. Hartmann, M. Klemm, Hydrothermal Treatment of Carboxy-methyl Cellulose Salt: Formation and Decomposition of Furans, Pentenes and Benzenes, *Chem. Eng. Technol.* 36 (2013) 287–294. <https://doi.org/10.1002/ceat.201200468>.
- [25] W.-M. Chai, X. Liu, Y.-H. Hu, H.-L. Feng, Y.-L. Jia, Y.-J. Guo, H.-T. Zhou, Q.-X. Chen, Antityrosinase and antimicrobial activities of furfuryl alcohol, furfural and furoic acid, *International Journal of Biological Macromolecules* 57 (2013) 151–155. <https://doi.org/10.1016/j.ijbiomac.2013.02.019>.
- [26] F. Monlau, C. Sambusiti, A. Barakat, M. Quéméneur, E. Trably, J.-P. Steyer, H. Carrère, Do furanic and phenolic compounds of lignocellulosic and algae biomass hydrolyzate inhibit anaerobic mixed cultures? A comprehensive review, *Biotechnology Advances* 32 (2014) 934–951. <https://doi.org/10.1016/j.biotechadv.2014.04.007>.
- [27] J. Lu, J. Watson, Z. Liu, Y. Wu, Elemental migration and transformation during hydrothermal liquefaction of biomass, *Journal of Hazardous Materials* 423 (2022) 126961. <https://doi.org/10.1016/j.jhazmat.2021.126961>.
- [28] M. Rahimifard, H. Haghi-Aminjan, Levels of effect in toxicology assessment, in: P. Wexler (Ed.), *Encyclopedia of Toxicology (Fourth Edition)*, Academic Press, Oxford, 2024: pp. 813–815. <https://doi.org/10.1016/B978-0-12-824315-2.00103-2>.
- [29] J. Wang, L. Zhang, Y. He, R. Ji, Biodegradation of phenolic pollutants and bioaugmentation strategies: A review of current knowledge and future perspectives, *Journal of Hazardous Materials* 469 (2024) 133906. <https://doi.org/10.1016/j.jhazmat.2024.133906>.

- [30] S. Hao, S. Ren, N. Zhou, H. Chen, M. Usman, C. He, Q. Shi, G. Luo, S. Zhang, Molecular composition of hydrothermal liquefaction wastewater from sewage sludge and its transformation during anaerobic digestion, *Journal of Hazardous Materials* 383 (2020) 121163. <https://doi.org/10.1016/j.jhazmat.2019.121163>.
- [31] R.J.M. Lubbers, A. Dilokpimol, J. Visser, M.R. Mäkelä, K.S. Hildén, R.P. de Vries, A comparison between the homocyclic aromatic metabolic pathways from plant-derived compounds by bacteria and fungi, *Biotechnology Advances* 37 (2019) 107396. <https://doi.org/10.1016/j.biotechadv.2019.05.002>.

Chapter 6 - Conclusions and Future Work

HTL is a promising technology for converting wet biomass feedstock into clean biofuels. Development of a reliable method for the treatment and valorization of HTL aqueous phase (HTLAP) is crucial for making HTL economically viable. In response to the growing need for sustainable and renewable energy sources, this thesis research explored the potential of aerobic fermentation as a viable method for treating HTLAP.

Methods such as thermochemical conversion, algae cultivation, and anaerobic digestion (AD) have been extensively studied for HTLAP treatment. In comparison, aerobic fermentation is less explored. Aerobic microorganisms exhibit greater tolerance to the inhibitors present in HTLAP than algae and the microbial consortia used in AD, and the fermentation process can be operated under milder temperature than the thermochemical conversion. Given these advantages, it is worth investigating the potential of aerobic fermentation for HTLAP treatment.

Important findings achieved in this dissertation project include:

1. Adjusting pH and supplementing nutrients greatly improved COD removal and yields of value-added products in HTLAP fermentation.
2. Sludge HTLAP showed resistance to biodegradation, likely due to the presence of N-heterocyclic compounds, which were removed by less than 40% across all tested microbes.
3. The co-culture of *A. niger* and *R. jostii* proved effective in treating and valorizing corn stover HTLAP, with initial COD values ranging from 3.8 to 7.8 g/L. This co-culture achieved approximately 70% COD removal while producing oxalic acid and cellular lipids as value-added products during HTLAP fermentation.

4. The concentration of organic compounds in HTLAP exhibited distinct patterns as the severity of HTL reactions increased.
5. A quantitative relationship was established between HTL operating parameters (temperature, retention time, and feedstock loading) and the biodegradability of the resulting HTLAP, indicating that higher reaction temperatures and longer reaction times negatively affected biodegradability during aerobic fermentation with *A. niger* and *R. jostii*. The reduced biodegradability of HTLAP produced under high temperature and prolonged time was linked to the increased concentration of certain phenolic compounds resulting from these conditions.
6. The co-culture of *A. niger* and *R. jostii* demonstrated versatility in fermenting HTLAP derived from various HTL operating conditions, achieving COD removal efficiencies between 45% and 70%, depending on the specific parameters used.

In conclusion, this dissertation makes a significant contribution to the advancement of HTLAP treatment technologies by developing and optimizing an aerobic fermentation method for HTLAP treatment and valorization. The robustness of this method in handling HTLAP derived from a wide range of conditions has been demonstrated, enhancing our understanding on HTLAP variation and the application of aerobic fermentation for effective HTLAP management. By advancing these areas, we can enhance the economic and environmental viability of HTL technology, making a significant contribution to the sustainable management of biomass resources.

Based on the findings of this dissertation, the following future work is recommended:

- Develop batch reactors for industrial-scale HTLAP fermentation, such as agitated airlift reactors.
- Conduct a techno-economic analysis and life cycle assessment of HTLAP aerobic fermentation to evaluate its feasibility and environmental impact.
- Identify the optimal HTL conditions to maximize both biocrude oil yield and HTLAP biodegradability.
- Characterize and quantify the high molecular weight compounds responsible for residual COD after HTLAP aerobic fermentation and explore methods for their removal.
- Identify microbial strains or consortia capable of effectively degrading N-heterocyclic compounds in HTLAP derived from protein-rich feedstocks.
- Investigate the fermentation of HTLAP derived from other feedstocks, such as microalgae and municipal solid waste, as originally proposed. Due to time constraints, this work has not yet been completed.
- Explore genetic engineering of microbial strains to enhance COD removal and increase the yield of value-added products during HTLAP fermentation.

Appendix A - Supplementary Figures and Tables from Chapter 3

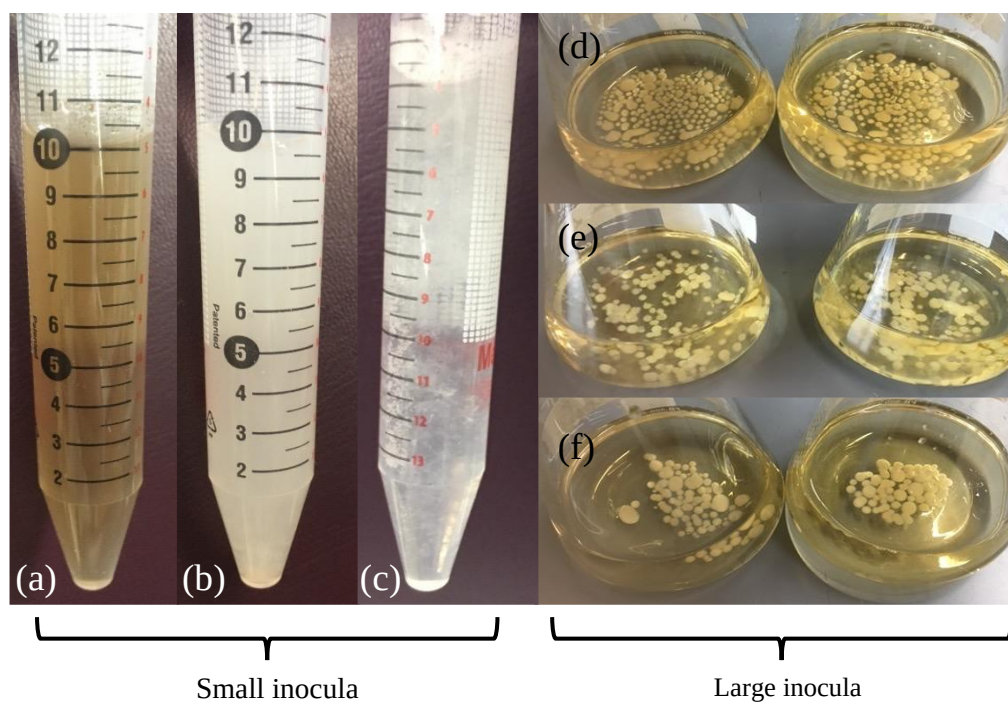
ITS region sequence of strain RHM:

```
NNNNNNNNNNNACCTGATTTGAGATCTAATCTTAAAATGTAGACATTCT
GATTAGAAGCTTCCTTTAACCCAACCCGGCTCTAGTCCGAAGACTAGAAT
TCCTCAGCGAATAGTCTATTACGCCAAGTCAATCCGAAGTTCGATTGCGG
ATGCTAATGCATTACGAACGAGCTAGACCGTAAAGGCCAGCAGCGCTCAG
AAACCAAACACCTCTTCAATCATTAAGAAAGAGGAGGGTTGAAGTATTCA
TGACACTCAAACAGGCATGCTCCACGGAATACCATGGAGCGCAAGGTGCG
TTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGC
ATTTGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGTTGAA
AGTTTTATTTTGTATAAAAATTTAATACATTCATAGACTTTGTGTTTATA
AGTGAATAGGAGTTCGCTCTCTTGCGAGAGTTACTATCCCAAACAAGTGC
ACAGGGTTAGAAAGTGAGAGTTCGGACTCCAAGTTAAGTTGGACGTCCTA
TATTCATAATGATCCTTCCGCAGGTTACCTACGGAACCTTGTTANCC
TTTTTTTNTTCCANATCTNCTANGGGTNGAGGGNGGGGACGCATATTTGG
NNNAAAACTACCGNGGTTACAAAAAGGGTGCTACGACNGCGGAAGACCCC
GNGTTGCCCCGAAGAGANNCCGCGCNCAGCCNANACGTCCNNNCAGCNTA
ACCCCCCCCCATGGAAGGGCGGTTTCGGGTCCGTAAAAAGCTTTTACGT
CTGGGCGGGATTGTTACCCGAGCTCCCAGCCNAATCCCNCCNNNTGGAATANN
```

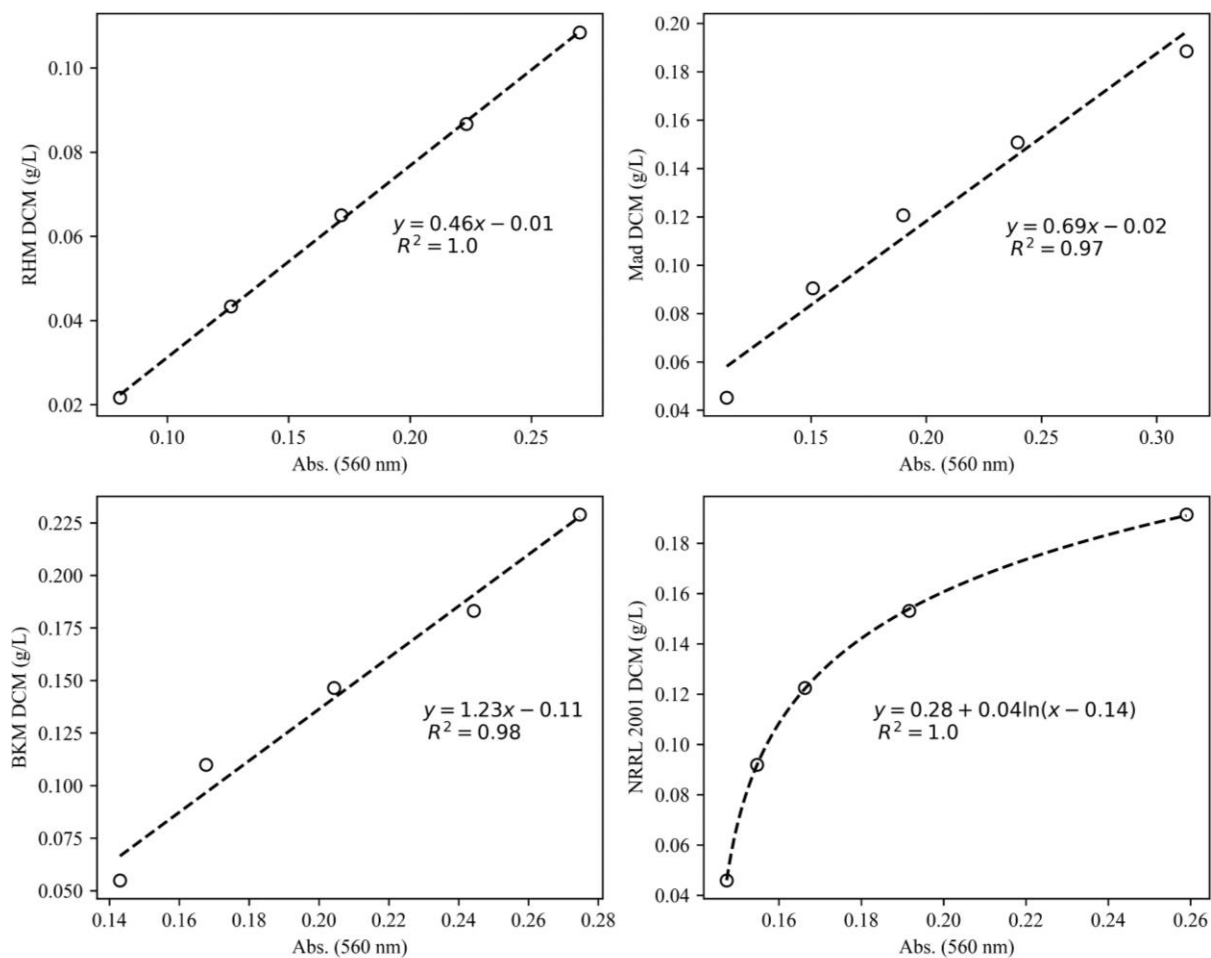
	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☒	Rhodotorula mucilaginosa CBS 316 ITS region: from TYPE material	Rhodotorula mucilaginosa	1057	1057	67%	0.0	99.83%	620	NR_073296.1
☐	Rhodotorula alborubescens JCM 5352 ITS region: from TYPE material	Rhodotorula alborubescens	1038	1038	66%	0.0	99.65%	570	NR_153197.1
☐	Rhodotorula taiwanensis CBS 11729 ITS region: from TYPE material	Rhodotorula taiwanensis	909	909	68%	0.0	94.90%	611	NR_157462.1
☐	Rhodotorula sphaerocarpa CBS 5939 ITS region: from TYPE material	Rhodotorula sphaerocarpa	876	876	67%	0.0	94.29%	616	NR_073269.1
☐	Rhodotorula paludigena CBS 6566 ITS region: from TYPE material	Rhodotorula paludigena	756	756	67%	0.0	90.69%	609	NR_073265.1

Appendix Figure A.1 ITS region sequence of the lab isolated strain RHM and its BLAST search

result



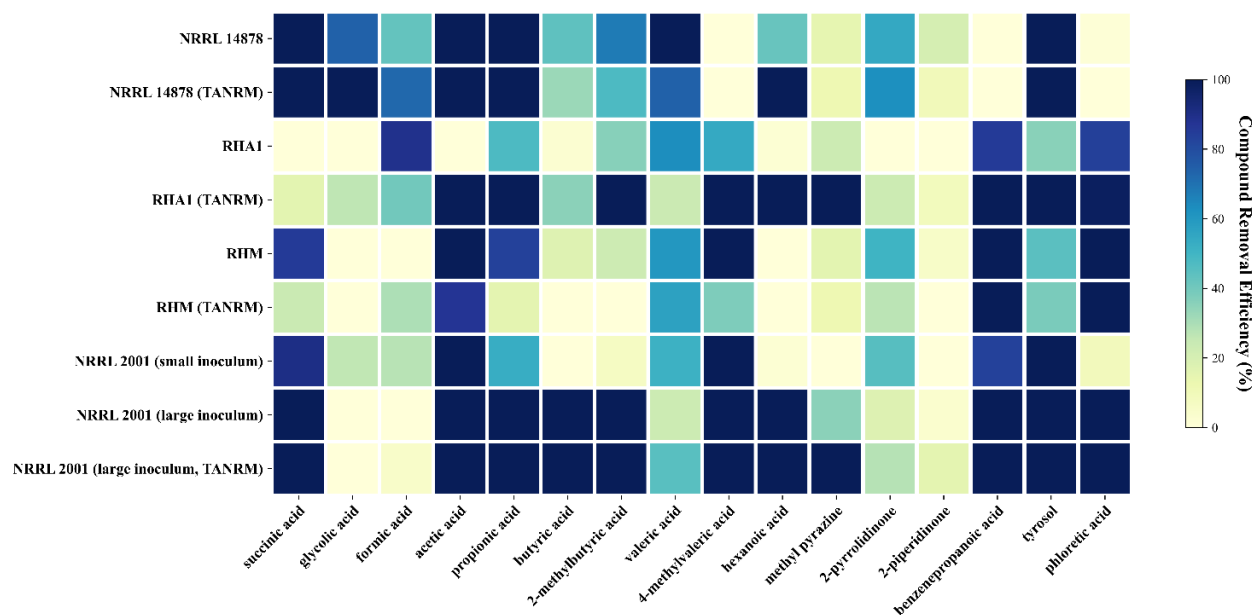
Appendix Figure A.2 Small (a-c) and large (d-f) inocula of filamentous fungal strains: spore stock of NRRL 2001 (a), spore stock of BKM (b), small hyphal clumps of Mad (c), and fungal pellets of NRRL 2001 (d), BKM (e), and Mad (f).



Appendix Figure A.3 Direct relationship between fungal dry cell mass and the absorbance at 560 nm determined by MTT assay.



Appendix Figure A.4 Reactor setup for adsorption assay. Nitrogen gas was continuously blown into the tubes



Appendix Figure A.5 Comparison of compound biodegradation in sludge HTLWW with and without TAN removal (TANRM).

Appendix Table A.1 Biological treatment of wastewater from HTL of nitrogen-rich feedstocks in other studies and this study

HTL feedstock	Pretreatment method ^a	Cultivation species	Treatment time (day)	COD (or TOC)/TN concentration (g/L)	COD (or TOC) removal efficiency (%)	References
Microalga cultivation						
Microalga, <i>Spirulina</i>	None	<i>Chlorella protothecoides</i>	14	4.2 COD	17	Pham et al. (2013b)
Mixed algal-bacterial biomass		Algae and bacteria consortium	7.7	1.2 /0.23	63	Zhou et al. (2013)
Swine manure		<i>Chlorella vulgaris</i>	7.5	2.0/0.1	20	Yang et al. (2018a)
Anaerobic digestion						
Swine manure	None		50	10/0.5	43	Yang et al. (2018a)
	Ozone				66	
	Granular activated carbon adsorption				97	
Microalga, <i>Spirulina</i>	None	Mixed culture of AD	90	5.8/1.5	36	Zheng et al. (2017)
Sewage sludge	None		19/3	8	Wang et al. (2021)	
	Biochar adsorption		17/2.6	7.5		
	Struvite precipitation	15/1.9	35			
	Biochar adsorption, struvite precipitation	13/1.7	48			
Microalga, <i>Chlorella</i> 1067	None		50	4/1.1	49	Li et al. (2019)
Microalga, <i>Chlorella</i> 1067	Zeolite adsorption		48	4/0.45	60	
Pure culture aerobic fermentation						
Marine <i>Tetraselmis</i> alga		<i>Rhodococcus opacus</i> PD630	11	55/8.3	85	He et al. (2017)
		<i>Rhodococcus jostii</i> RHA1			93	
		<i>Rhodococcus jostii</i> RHA1 VanA ⁻			93	
Microalga, <i>Nannochloropsis</i>	None	<i>Pseudomonas putida</i> KT2440	2	2.0 (TOC)/1.3	40 (TOC)	Nelson et al. (2013)
		<i>Escherichia coli</i> K12 MG1655			43 (TOC)	
		<i>Pseudomonas putida</i>			36	
Sewage sludge	Ammonia nitrogen removal		15	7.2/0.9	18.5	This study
		<i>Rhodococcus jostii</i> RHA1		5.8/0.5	44	
		<i>Phanerochaete chrysosporium</i>	30		31	
	None	<i>Aspergillus niger</i>	12	3.6/0.47	47	
		<i>Trametes versicolor</i>			45.5	
		<i>Rhodotorula sp.</i>	30		36	

^a Pretreatment of HTLWW prior to biological treatment.

HTL: hydrothermal liquefaction; COD: chemical oxygen demand; TOC: total organic carbon; and TN: total nitrogen.

Appendix Table A.2 Standard curve equations and detection limits of organic compounds quantified in the study

Compound	Equation	Detection limit	unit	Category
oxalic acid	(area-357.41)/222	4.83	ppm	organic acid
malic acid	(area+77.78)/207.56	1.12	ppm	organic acid
succinic acid	(area+42.66)/189.36	0.68	ppm	organic acid
glycolic acid	(area+79.669)/180.18	1.33	ppm	organic acid
formic acid	(area+99.12)/113.79	2.61	ppm	organic acid
acetic acid	(area-142.04)/118.34	3.60	ppm	organic acid
propionic acid	(area+1423.6)/162.2	26.3	ppm	organic acid
butyric acid	(area+10924)/324.13	101	ppm	organic acid
2-methylbutric acid	(area+725.53)/182.18	11.9	ppm	organic acid
valeric acid (height)	(height+11.149)/4.9296	6.79	ppm	organic acid
4-methylvaleric acid	(area+2001.8)/193.8	31.0	ppm	organic acid
hexanoic acid	(area+649.34)/124.04	15.7	ppm	organic acid
pyrazine	(area+60203)/24811	7.28	ppm	n-heterocyclic
methyl pyrazine	(area+351260)/52906	19.9	ppm	n-heterocyclic
2-pyrrolidinone	(area+385871)/19136	60.5	ppm	n-heterocyclic
2-piperidinone	(area+220908)/28597	23.2	ppm	n-heterocyclic
benzenepropanoic acid	(area+4774345.59)/1663565.65	11.5	ppm	phenolic
tyrosol	(area+930246.42)/2206774.14	1.69	ppm	phenolic
phloretic acid	(area+634439.84)/2441233.81	1.04	ppm	phenolic

The R² values of all standard curves were above 0.99.

Appendix Table A.3 Unquantified compounds in sludge HTLWW and their degradation by different microbes

Compound	Degradation of unquantified compounds						
	NRRL 14878	ATCC 31800	RHA1	BKM	Mad	NRRL 2001	RHM
Butanoic acid, 2-methyl	+	+	+	-	+	+	-
Pentanoic acid	+	+	+	+	+	+	+
Pentanoic acid, 4-methyl	-	-	+	+	+	+	+
3-aminoisobutyric acid	-	-	-	-	-	-	-
2-hydroxyisocaproic acid	-	-	-	-	-	-	-
Phenylpropanamide	-	-	-	+	+	+	+
Phenol	+	+	+	+	+	+	+
p-cresol	-	+	+	+	+	+	+
Benzyl alcohol	-	+	+	-	+	+	+
Benzeneacetic acid	+	-	+	+	+	+	-
Benzoic acid	-	-	-	-	-	-	-
Benzoic acid, 4-ethoxy-, ethyl ester	-	-	-	-	-	-	-
Pyrazine, 2-ethyl-6-methyl	-	-	-	-	-	-	-
Pyrrolidine, 1-acetyl	-	-	-	-	-	-	-

(+): compound was degraded by the microorganism (peak area was reduced by more than 50%)

(-): compound was not significantly degraded by the microorganism

Appendix Table A.4 Significantly different COD removal rate as determined by Tukey-Kramer test at $\alpha=0.05$

Microorganism	Nutrient supplementation	Letter from Tukey-Kramer test
NRRL 14878	yes	A
ATCC 31800	yes	A
BKM	yes	A
NRRL 2001	yes	A
RHM	yes	A
RHA1	yes	B
Mad	yes	B
NRRL 2001	no	B
RHM	no	B
NRRL 14878	no	C
ATCC 31800	no	CD
RHA1	no	CD
Mad	no	DE
BKM	no	E

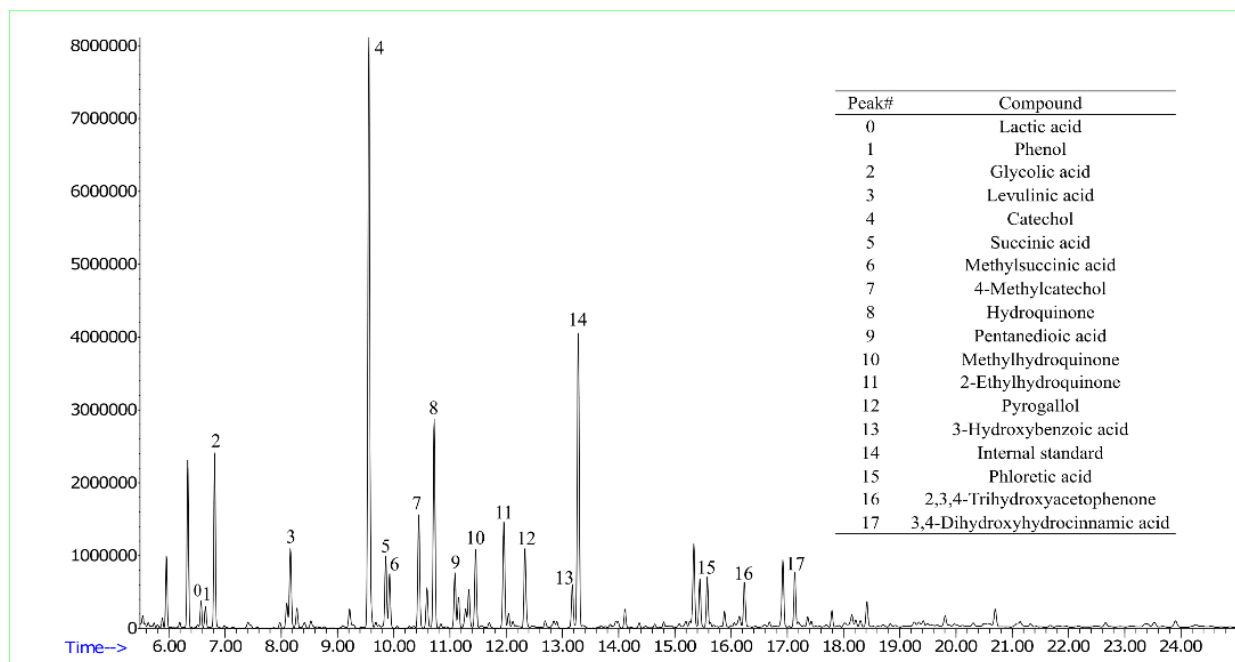
Microorganism and nutrient supplementation combinations not connected by the same letter have significantly different COD removal rates. Letters were assigned alphabetically based on COD removal rate, and “A” and “E” denote those with the highest and the lowest COD removal rates, respectively.

Appendix Table A.5 Significantly different COD removal rate after treatment with small and large fungal inoculums as determined by Tukey-Kramer test at $\alpha=0.05$

Microorganism	Inoculum	Nutrient supplementation	Letter from Tukey-Kramer test
NRRL 2001	large inoculum	yes	A
NRRL 2001	large inoculum	no	A
Mad	large inoculum	no	A
NRRL 2001	small inoculum	yes	B
BKM	small inoculum	yes	B
Mad	small inoculum	yes	C
Mad	large inoculum	yes	CD
BKM	large inoculum	yes	D

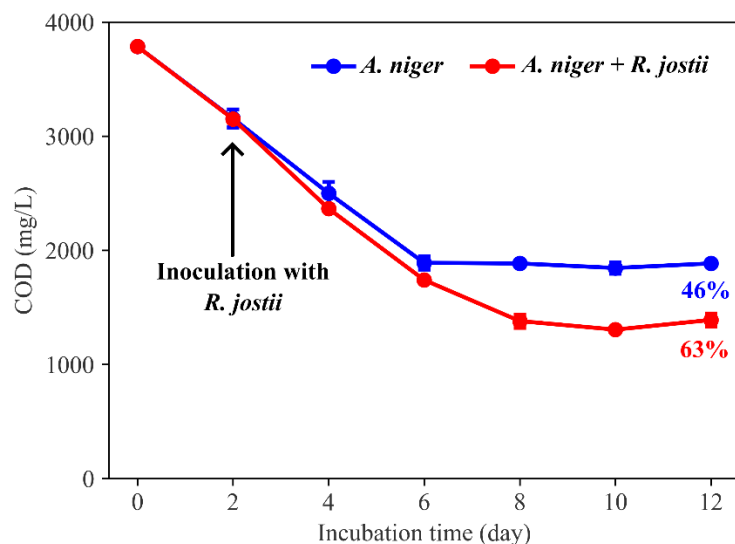
Microorganism and nutrient supplementation combinations not connected by the same letter have significantly different COD removal rates. Letters were assigned alphabetically based on COD removal rate, and “A” and “D” denote those with the highest and the lowest COD removal rates, respectively.

Appendix B - Supplementary Figures and Tables from Chapter 4

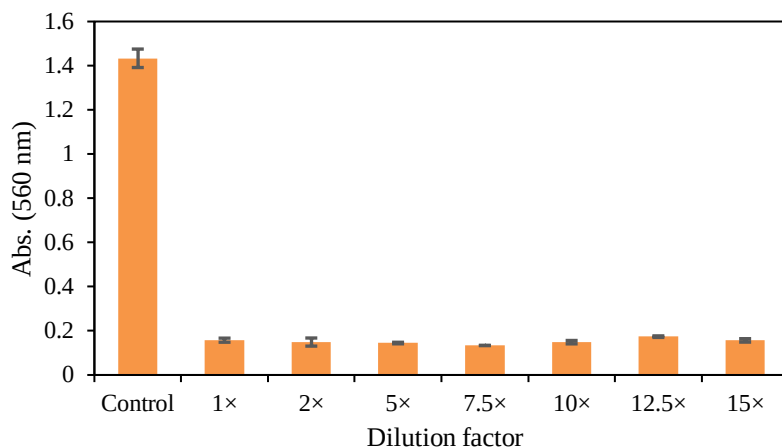


Appendix Figure B.1 GC-MS chromatogram of the corn stover HTLAP used in this study.

Major compounds identified with a match score over 90 were labeled.



Appendix Figure B.2 COD variation during batch fermentation with *A. niger* pure culture and co-culture of *A. niger* with *R. jostii* (n=2). The COD removal at the end of 12-day fermentation were labeled on the graph. To ensure favorable conditions for *R. jostii* growth and activity, we inoculated the bacterium on day 2 of the incubation period, thereby avoiding exposure to potentially inhibitory levels of phenolic compounds.



Appendix Figure B.3 Cell activity of *R. jostii* under screened HTLAP dilution folds (n=2). The control represents the cell activity of *R. jostii* grown in 15× diluted HTLAP that has been detoxified by removing various phenolic compounds, including 4-methylcatechol, catechol, and hydroquinone, by 34%, 89%, and 79%, respectively. Detoxification was performed via overliming by adjusting the pH of HTLAP to 10 with $\text{Ca}(\text{OH})_2$ followed by incubation at 60°C for 5 h with hourly manual shaking. After incubation, any precipitates were removed by centrifugation at 5000 rpm. The pH of overlimed HTLAP was then adjusted to match that of untreated HTLAP using 6 M HCl and sterilized by filtration through a 0.22- μm syringe filter before being used for microbial cultivation.

Appendix Table B.1 Unquantified compounds in HTLAP and their degradation by single *A. niger* culture and co-culture of *A. niger* and *R. jostii*

Compound	Degradation of unquantified compounds	
	<i>A. niger</i>	<i>A. niger</i> + <i>R. jostii</i>
Phenol	+++	++++
Levulinic acid	-	++++
4-Hydroxybutanoic acid	-	++++
Methylsuccinic acid	-	-
4-Methylcatechol	++++	++++
Pentanedioic acid	-	-
Methylhydroquinone	++++	++++
2-Ethylhydroquinone	++++	++++
Pyrogallol	++++	++++
3-Hydroxybenzoic acid	+	++++
Phloretic acid	-	++++
2,3,4-Trihydroxyacetophenone	++++	++++
3,4-Dihydroxyhydrocinnamic acid	++++	++++

(-): compound was not significantly degraded by the microorganism

(+): compound was slightly degraded by the microorganism (peak area reduction: 25-50%)

(++): compound was degraded by the microorganism (peak area reduction: 50-75%)

(+++): compound was extensively degraded by the microorganism (peak area reduction: 75-99%)

(++++): compound was completely degraded by the microorganism (peak area reduction: >99%)