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

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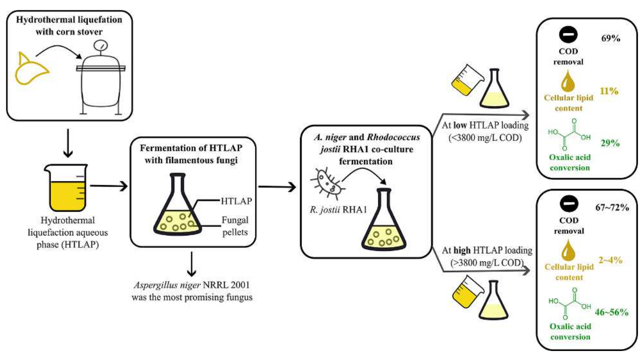
HIGHLIGHTS

- *A. niger* NRRL 2001 showed promising HTLAP fermentation abilities.
- *A. niger* removed 70 % COD in HTLAP with a COD of 7800 mg/L.
- 46–56% of total organic carbon in HTLAP was converted to oxalic acid by *A. niger.*
- Co-culturing *A. niger* with *R. jostii* RHA1 was beneficial at low HTLAP loading.

GRAPHICAL ABSTRACT



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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.

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1. Introduction

Crop residue represents one of the most abundant feedstocks of renewable energy (Toor et al., 2011). 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 an reactant and catalyst (Toor et al., 2011). 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 (Gu et al., 2019; Watson et al., 2020).

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 (Gu et al., 2019). Approximately 45% of organic carbon and 80% of other nutrients (e.g., nitrogen and phosphorous) of the feedstock end up in the HTLAP (Gu et al., 2019; Pham et al., 2013). 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. (Song et al., 2022; Tito et al., 2023; Watson et al., 2020), of which catalytic gasification and anaerobic digestion (AD) have been the most studied methods (Watson et al., 2020). 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 H_2 (used for HTL biocrude upgrading) (Jiang et al., 2024; Tito et al., 2023). 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 (Jiang et al., 2024; Pipitone et al., 2020). 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 (Du et al., 2012; Watson et al., 2020).

Aerobic fermentation is a widely used method for industrial wastewater valorization (Reddy et al., 2019; Wang et al., 2022a). 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 (Kosa and Ragauskas, 2012). He et al. examined *R. opacus* PD630, *R. jostii* RHA1, and *R. jostii* RHA1 VanA for biodegradation of HTLAP from algae and pine wood (He et al., 2017) 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 (Cordova et al., 2020). 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 (Liu et al., 2024). 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).

Little work has been done so far 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.

2. Materials and methods

2.1. Materials

2.1.1. Generation of 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 min, 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.

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 OD_{600} (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).

2.1.3. Supplementary nutrient stocks

Stock solutions of supplementary nutrients used in the study were prepared as described previously (Liu et al., 2024). 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 NH_4Cl, 0.1 g/L KH_2PO_4, 50 mg/L $MgSO_4·7H_2O$, 25 mg/L $CaCl_2·2H_2O$, and 5 mg/L $FeSO_4·7H_2O$, respectively.

2.2. Aerobic fermentation of hydrothermal liquefaction aqueous phase

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 (Liu et al., 2024). 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 (Liu et al., 2024). All experiments in this study were conducted in duplicate unless specified, otherwise.

2.2.2. Fungal cultivation followed by R. jostii inoculation

The pH, nutrient supplementation, and HTLAP dilution factor determined in Section 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 ($OD_{600} = 1.0$) prepared in Section 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.

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 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 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 2.2.1. During the fermentation, a 0.5-mL sample was taken from each flask every 2 days for COD measurement.

2.3. Analytical methods

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 (Liu et al., 2024). 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 μm 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 (Liu et al., 2024). 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).

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 (Lapeyrie et al., 1987). The concentration of oxalic acid in the wash solution was determined using HPLC, as described in Section 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 uL tridecannoic 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 × 0.25 mm, RESTEK, Bellefonte, PA), as described previously (Liu et al., 2023). 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. (1), in accordance with the NREL protocol (Van Wychen et al., 2016)

$$\%Total\ lipid = \left(\sum_{i=4}^{24} M_i \times \frac{M_{13-added}}{M_{13}}\right) \Big/ M_{Drycell} \times 100 \quad (1)$$

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).

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

$M_{Drycell}$: the sample dry cell mass

3. Results and discussion

3.1. Characteristics of hydrothermal liquefaction aqueous phase

Table 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. 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 (Liu et al., 2020). 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 (Panisko et al., 2015). 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 HTLAP 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 (Liu et al., 2020). As shown in Table 1, 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 (Watson et al., 2020).

Table 1 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 (Basar et al., 2023), distiller's dried grains with solubles (Biller et al., 2016), algae (Maddi et al., 2016), and lignocellulosic biomass (Davidson et al., 2019). Panisko et al. reported an acetic acid concentration of 7–10 g/L in HTLAP of corn stover (Panisko et al., 2015), 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 Na_2CO_3.

As shown in Table 1, 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 (Panisko et al., 2015; Pipitone et al., 2020). 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 (**see supplementary material**). The GC–MS chromatograph also confirmed most compounds in Table 1, except for acetic acid, formic acid, and propionic acid which can't 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 (**see supplementary material**) in HTLAP but were not quantified in this study due to their lower concentrations.

3.2. Optimization of fungal fermentation conditions in hydrothermal liquefaction aqueous phase

Fig. 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 (Fig. 1a). *T. versicolor*, and *P. chrysosporium* were more inhibited by HTLAP and did not have significant cell activity until 16× and 20× dilution of HTLAP, respectively (Fig. 1a). The original pH (pH 4) of HTLAP greatly inhibited fungal cell activity, as evidenced by the noticeably lower bars depicted in Fig. 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 (Fig. 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 (Liang et al., 2013).

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 (Fig. 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 (Cordell et al., 2009). In addition, phosphorus supplementation may encourage

Table 1
Inorganic element concentrations, water quality parameters, and organic compound concentrations of HTLAP[a].

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.

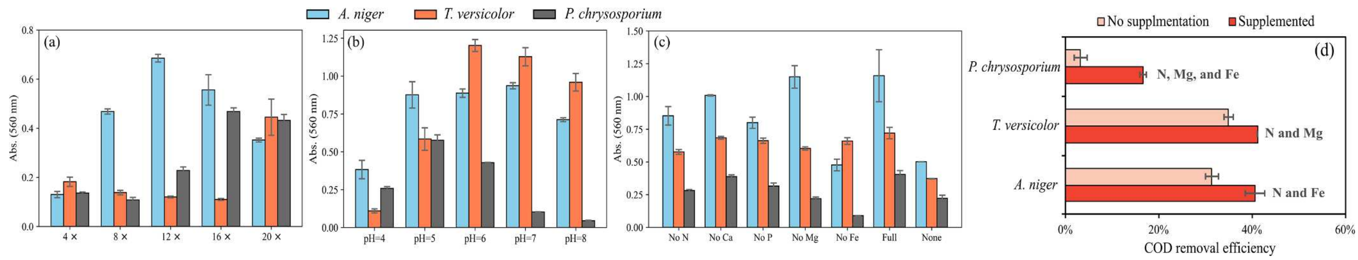

Fig. 1. Cell activity of the tested fungi under screened HTLAP dilution fold **(a)**, pH **(b)**, and nutrient supplementation **(c)**, and COD removal efficiencies by three fungi on day 12 of fermentation in HTLAP with and without nutrient supplementation **(d)**. In **(c)**, "Full" denotes the supplementation 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.

bacterial growth, making the fungal fermentation system more susceptible to contamination (Liu et al., 2024). Therefore, the decision was made not to supplement P for HTLAP fermentation, despite its positive impact on fungal cell activity.

Fig. 1d shows the COD removal efficiencies by 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 Fig. 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.

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 Fig. 2. Even with nutrient supplementation, it was found that organic acids, such as acetic acid (Fig. 2a), glycolic acid (Fig. 2b), malic acid (Fig. 2c), and succinic acid (Fig. 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 accounted for 8% and 2% of TOC in HTLAP, respectively. None of the three fungal stains were shown to consume glycolic acid or succinic acid (Fig. 2b and d). In contrast to organic acids, all fungal strains effectively degraded catechol (Fig. 2e) and hydroquinone (Fig. 2f). *T. versicolor* and *A. niger* completely removed catechol and hydroquinone by day 4 of incubation, regardless

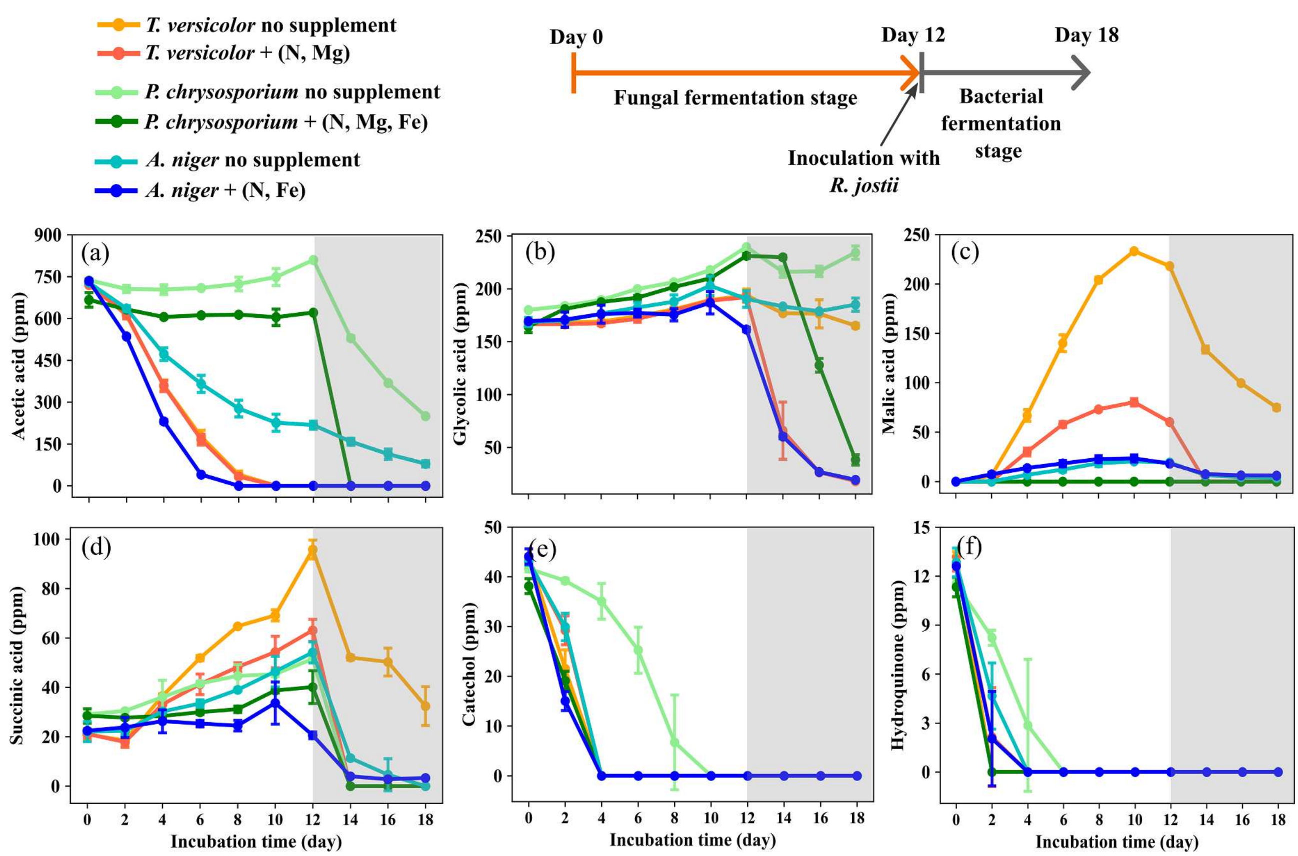

Fig. 2. Concentrations of acetic acid **(a)**, glycolic acid **(b)**, malic acid **(c)**, succinic acid **(d)**, catechol **(e)**, and hydroquinone **(f)** during fungal fermentation stage (day 0–12) and bacterial fermentation stage (day 12–18; shaded in gray) in HTLAP (n = 2) with or without nutrient supplementation described in **Table 3**. HTLAP was the only COD source in the media. Each data point represents the average and standard deviation of duplicate measurements.

of nutrient supplementation, whereas *P. chrysosporium* required longer time (6–10 days) for the degradation.

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 (Fig. 2). The compound degradation during the bacterial fermentation stage (days 12–18) is illustrated in Fig. 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 (Fig. 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 (Liu et al., 2024).

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 Fig. 1a, and recognizing the synergistic effect observed between *A. niger* and *R. jostii* (Fig. 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 **supplementary material**. The co-culture system achieved a higher COD removal of 63% compared to 46% achieved with single culture of *A. niger* (**see supplementary material**), which is consistent with the findings in Section 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 Fig. 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 (Fig. 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 2 were completely removed by the co-culture as evidenced by the HPLC chromatograms before and after fermentation (**Fig. 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 (**see supplementary material**).

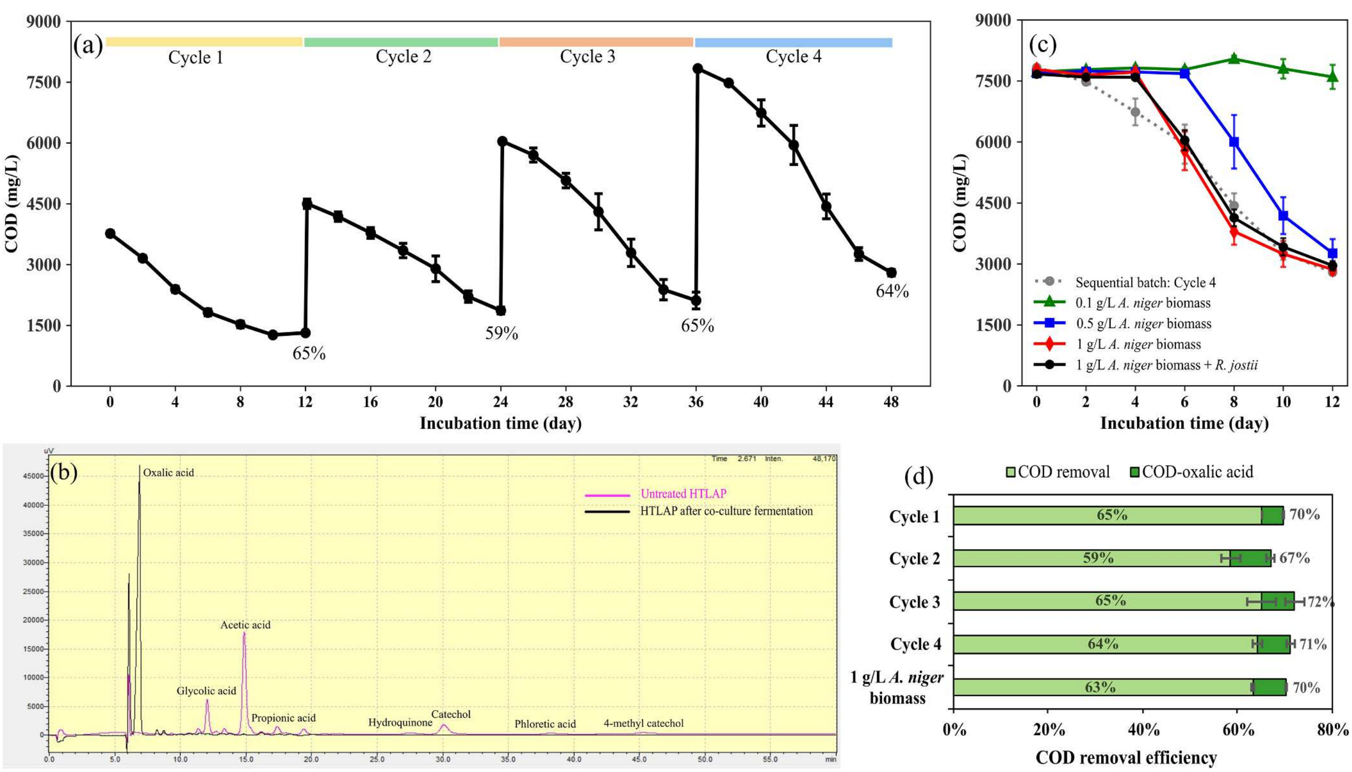

Fig. 3. COD profile during sequential batch fermentation with *A. niger* and *R. jostii* co-culture when subjecting to increasing initial COD loading (cycle 1: 3765 mg/L; cycle 2: 4500 mg/L; cycle 3: 6040 mg/L; and cycle 4: 7835 mg/L) from HTLAP **(a)**, HPLC chromatogram of HTLAP before (pink line) 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 by the 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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. Fig. 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 Fig. 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 (Fig. 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* (Fig. 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 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 (Fig. 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, the produced oxalic acid remained in the fermentation broth and thus contributed to the final COD measurement. Fig. 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 (Gu et al., 2019; Liu et al., 2024; Watson et al., 2020), the achieved COD removal in this study suggests promising potential for utilizing *A. niger* in HTLAP fermentation.

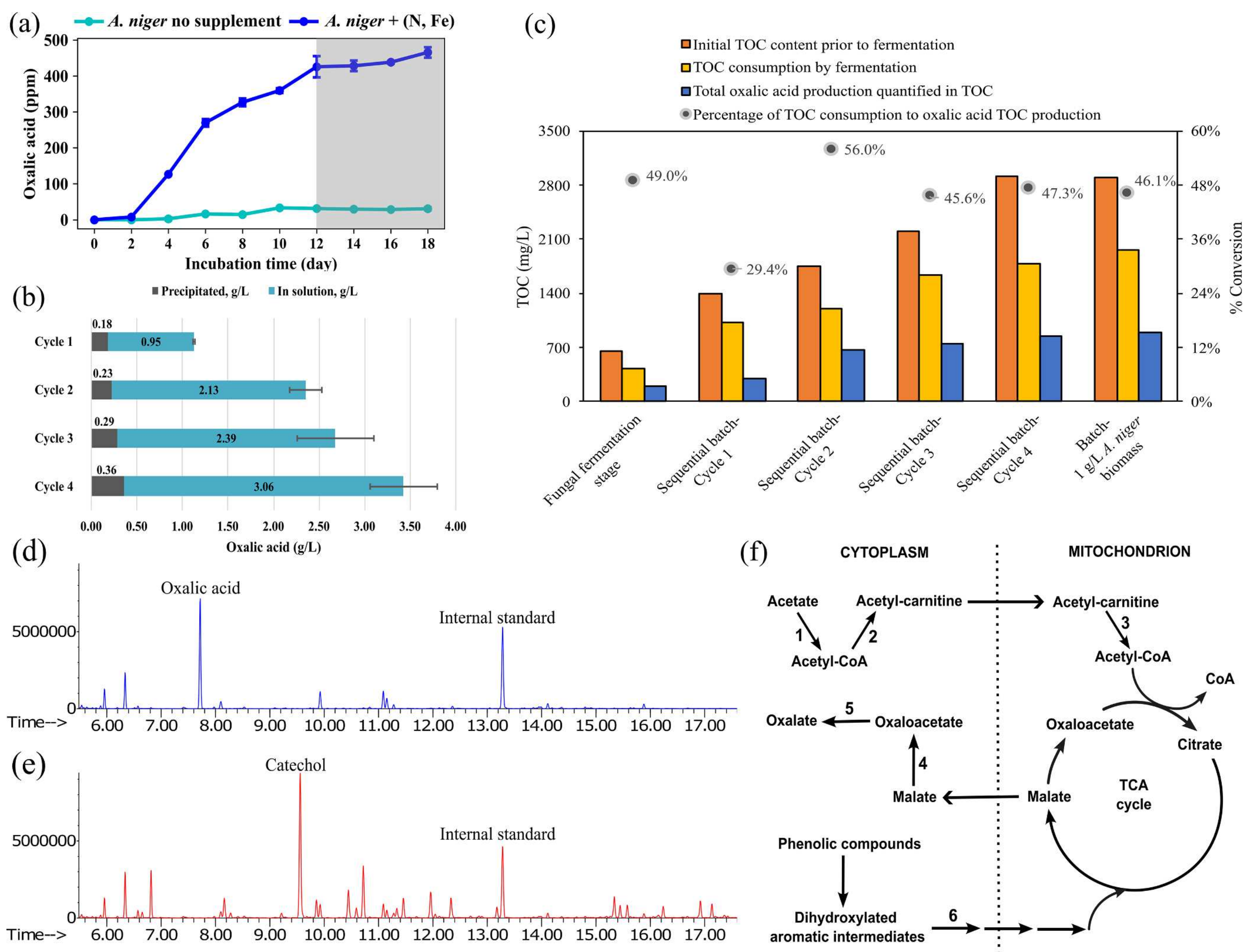

Fig. 4. Variation of oxalic acid concentration during HTLAP fermentation with and without N and Fe supplementations **(a)**, oxalic acid concentrations at the end of different cycles of sequential batch fermentation **(b)**, and percentage of HTLAP-originated TOC conversion to oxalic acid and the corresponding 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. Value-added product profiles during hydrothermal liquefaction aqueous phase fermentation

3.5.1. Oxalic acid production

A. niger was found to produce oxalic acid during HTLAP fermentation (Fig. 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 (Amenaghawon et al., 2024). 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 (Amenaghawon et al., 2024). Currently, the primary method for producing oxalic acid involves chemical synthesis using fossil resources (Schuler et al., 2021). 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 (Amenaghawon et al., 2024). This study suggested that HTLAP is a promising feedstock for oxalic acid production without competing with human food supply.

As shown in Fig. 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* (Gharieb, 2000). 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 (Gharieb, 2000; Wang et al., 2022b). Therefore, this fungus may produce more oxalic acid in response to low phosphorus level, possibly as a strategy to access insoluble phosphorus (Gharieb, 2000). 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 (Semana and Powlowski, 2019). 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 Fig. 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 (Fig. 2).

Fig. 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 2.3.2) and the calcium concentration in HTLAP. Fig. 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 (Strasser et al., 1994). 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 (Rosseto et al., 2024).

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 (Fig. 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 (Fig. 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 Fig. 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 (Ramón De Lucas et al., 2001). 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 (Lubbers et al., 2019). These intermediates then undergo ring cleavage via intradiol ring cleavage dioxygenases and are converted to TCA cycle intermediates (Sgro et al., 2023). Malate, generated from the TCA cycle is transported to cytoplasm, where it is converted to oxaloacetate and finally to oxalate (Poulsen et al., 2012).

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 lipid content, and cell mass concentration at the end of fermentation with *A. niger* cultures are illustrated in Fig. 5. As shown in Fig. 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 (Fig. 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 (Holder et al., 2011; Li et al., 2021). The cellular lipid content was on average 11% at the end of cycle 1 fermentation (Fig. 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 (Fig. 5b).

3.6. Effect of hydrothermal liquefaction aqueous phase loading on a. Niger and R. Jostii co-culture fermentation

The findings in this study suggest 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

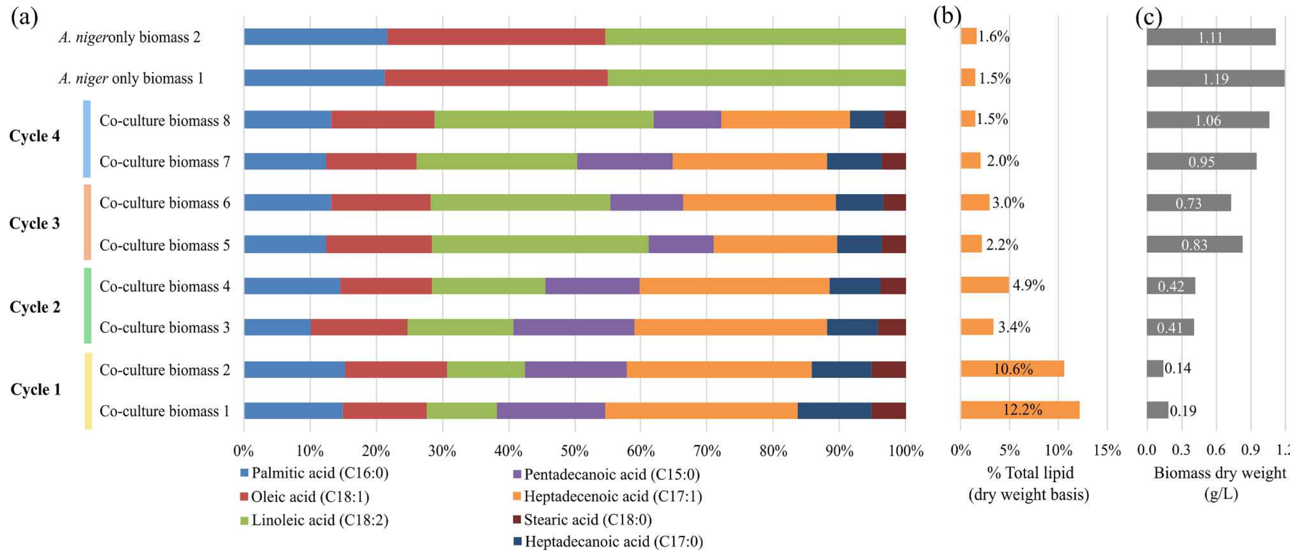

Fig. 5. Fatty acid profile **(a)**, cellular lipid content **(b)**, and dry weight concentration (g cell mass/L HTLAP) **(c)** of microbial cell mass at the end of batch fermentation with single *A. niger* culture, and different cycles of sequential batch fermentation with *A. niger* and *R. jostii* co-culture.

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 (Fig. 5b) suggests 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 (**see supplementary material**). 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 (Fig. 3c).

R. jostii demonstrated the ability to consume organic acids remaining after *A. niger* fermentation (Fig. 2), and their co-culture increased COD removal from 46% (single *A. niger* culture) to 63% (**see supplementary material**). However, at higher HTLAP loading, it was observed that *A. niger* alone achieved similar COD removals without co-culture with *R. jostii* (Fig. 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 (Andersen et al., 2009). 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 (Andersen et al., 2009).

3.7. Outlook on A. niger and R. jostii co-culture for hydrothermal liquefaction aqueous phase fermentation

This study suggests that *A. niger* and *R. jostii* co-culture fermentation could be 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 (Sánchez et al., 2021). 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 (Sánchez et al., 2008). 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 (SundarRajan et al., 2021). 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 (Basar et al., 2024). However, aerobic fermentation has drawbacks, such as high energy consumption and greenhouse gas emission. 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.

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.

CRediT authorship contribution statement

Meicen Liu: Writing – original draft, Visualization, Methodology, Investigation, Data curation. **Jiefu Wang:** Resources, Methodology, Investigation. **Isamu Umeda:** Resources, Methodology. **Zhiwu Wang:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition. **Sandeep Kumar:** Writing – review & editing, Resources, Methodology, Funding acquisition. **Yi Zheng:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131240.

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