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Research Article

# Changes in pH Values Allow for a Visible Detection of the End Point in Submerged Liquid Biodetoxification during Biorefinery Processing

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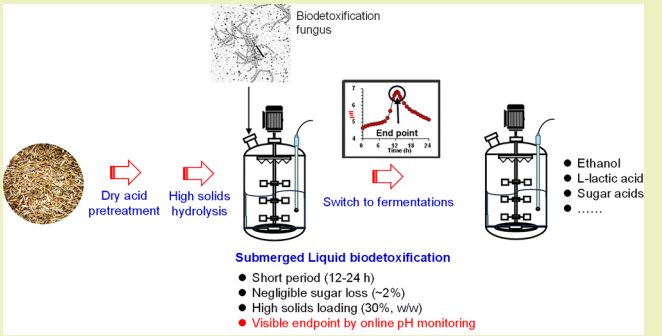
**ABSTRACT:** Detoxification of inhibitory compounds in pretreated lignocellulose feedstock is a crucial step prior to consequent fermentations in the biorefinery chain. Biological detoxification offers a fast and highly selective method for biodegradation of inhibitory compounds into $CO_2$ and water. This study focused on the enhanced online monitoring of the end point of the submerged liquid biodetoxification by *Paecilomyces variotii* FN89 in high solids loading wheat straw hydrolysate. Submerged liquid biodetoxification reduces reactor volume and improves mass transfer with greater ease of operation but faces the challenge of timely switching of the biodetoxification stage into the fermentation stage of biodetoxification microbes in the lignocellulose hydrolysate with highly concentrated sugars and inhibitors. In other words, the challenge is to quickly identify the end point of biodetoxification to ensure a timely completion of inhibitors while maintaining the fermentable sugars untouched. The inadequate biodetoxification results in poor fermentability in the subsequent bioconversion step, while excessive biodetoxification leads to the unwanted consumption of fermentable sugars. The results indicate that all furfural and 5-hydroxymethylfurfural (HMF) and over 80% of acetic acid in the wheat straw hydrolysate were effectively removed within a short period (12−24 h) under moderate operating parameters and negligible loss of fermentable sugars (below 2%). We observed that the change of pH value had a certain regularity and exactly corresponded to the biodetoxification process due to the consumption of inhibitory acids (acetic acid) and the generation of acidic byproducts (fatty acids). Upon reaching the maximum pH value through the visible online observation, the biodetoxification stage could be promptly transitioned to the fermentation stage by switching-off the aeration, inoculating the fermentation microbe seed and changing the fermentation parameters. The method was applied to the productions of chiral L-lactic acid, ethanol, and sugar acids from wheat straw after dry acid pretreatment, enzymatic saccharification, simultaneous saccharification, and co-fermentation. This pH peak value can be used as a timely, easily visible, and detectable indicator of submerged liquid biodetoxification completion with rapid and complete removal of inhibitors and negligible loss of fermentable sugars.




**KEYWORDS:** *lignocellulose, submerged liquid biodetoxification, end point, pH variation, bioproducts*

## 1. INTRODUCTION

Pretreatment is the starting and determinative step of a biorefinery chain to overcome the biorecalcitrance of lignocellulose biomass and enables the release of fermentable sugars.[1,2] Toxic compounds are generated during pretreatment, including furan aldehydes from excessive pentose and hexose degradation, weak organic acids from acetyl group oxidation, or phenolic aldehydes from lignin degradation. These inhibitory compounds strongly inhibit the viability and metabolism of fermentation microbes. Therefore, a detoxification (or conditioning) step must be followed to eliminate the inhibitors from the pretreated lignocellulose feedstock. The general detoxification methods such as water-washing, activated carbon adsorption, and overliming inevitably result in much loss of fermentable sugars or generation of wastewater.[7] For practical biorefinery processes under high lignocellulose solids loading conditions, biological detoxification (biodetoxification) using specific microorganisms is considered as the only proper approach among various available options by its effectiveness on complete degradation of inhibitors into $CO_2$ and water with the good preservation of fermentable sugars.[3−6]

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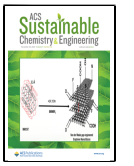




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Early stage biodetoxification was performed in the way of solid-state fermentation by inoculating spores or filamentous mycelia of *Amorphotheca resinae* (*A. resinae*) ZN1 or *Paecilomyces variotii* (*P. variotii*) FN89 onto the dry pretreated lignocellulose solids.[7,8] Solid-state biodetoxification is effective at bench-scale but not proper at large-scale operation due to (i) the high capital cost of aerobic solid-state fermentation in large scale;[9] (ii) high electricity consumption for mixing, aeration, pH, and heat removal;[9,10] (iii) long operation time;[7,8] (iv) low efficient transport of solid biomass; (v) vulnerability to contamination; and (vi) an inability to allow end point determination. One approach to overcome these inherent drawbacks of solid-state biodetoxification is to perform biodetoxification by submerged liquid fermentation. This approach involves enzymatically hydrolyzing the solid pretreated lignocellulose into the liquid hydrolysate slurry and then inoculating the biodetoxification microbes into the hydrolysate under appropriate aeration and mixing conditions to perform the submerged liquid biodetoxification. Several drawbacks in solid-state biodetoxification could be overcome directly by submerged liquid biodetoxification: (i) bioreactor number is cut into one for both saccharification and biodetoxification; (ii) cell growth is improved and inhibitor degradation is accelerated by the better heat and mass transfer;[11] (iii) transportation of the biodetoxified hydrolysate slurry in closed vessels, pipelines, and pumps prevents contamination.

A practical submerged liquid biodetoxification operation faces a significant challenge of identifying a visible end point indicator to ensure rapid completion of inhibitors while maintaining the fermentable sugars untouched. Inadequate biodetoxification results in poor fermentability in the subsequent bioconversion step, while excessive biodetoxification leads to the unwanted consumption of fermentable sugars. The biodetoxification stage should be promptly transitioned to the fermentation stage when the inhibitors are completely eliminated with a negligible loss of fermentable sugars.

*Paecilomyces variotii* is a common cosmopolitan fungus found in soils, plants, animals, and foodstuffs and able to grow in common agroindustrial derivatives at low oxygen level.[12] *P. variotii* strains had been isolated for phenol degradation,[13] or castor bean residue detoxification,[14] but no reports on degradation on complicated lignocellulose pretreatment derived inhibitors such as furfural, HMF, acetic acid, and phenolic aldehydes. This study used a newly isolated *P. variotii* FN89 from the pretreated corn stover solids in our previous study[15] and established a practical submerged liquid biodetoxification method using *P. variotii* FN89. *P. variotii* FN89 showed faster cell growth, improved detoxification performance, and higher tolerance to low pH than the previously isolated biodetoxification strain *Amorphotheca resinae* ZN1.[7,8] A unique phenomenon was observed that the change of pH value exactly corresponded to the submerged liquid biodetoxification process due to the consumption of inhibitory acids (such as acetic acid) and the generation of acidic byproducts (such as furoic acid, benzoic acid, and fatty acids). Upon reaching the maximum pH value through the visible online observation, the biodetoxification stage could be promptly transitioned to the fermentation stage with successful applications to the productions of chiral L-lactic acid, ethanol, and sugar acids from wheat straw. This pH peak value can be used as a timely, easily visible, and detectable indicator of submerged liquid biodetoxification completion with rapid and complete removal of inhibitors and negligible loss of fermentable sugars.

## 2. MATERIALS AND METHODS

**2.1. Raw Feedstock.** Raw wheat straw was harvested from Nanyang, Henan province, China, in May 2021. It was coarsely chopped through the mesh with a 10 mm diameter. The main compositions of wheat straw were determined according to the National Renewable Energy Laboratory (NREL) protocols,[16] which contained 35.6 ± 0.4% cellulose, 24.3 ± 0.2% xylan, 18.7 ± 0.1% lignin, and 9.7 ± 0.3% ash on dry weight.

**2.2. Enzymes and Reagents.** Cellulase Cellic CTec 2.0 was purchased from Novozymes (Beijing, China) with the measured protein content of 86.3 ± 4.4 mg/mL.[17] Glucoamylase GA-L NEW was purchased from Genencor (Jilin, China) with the activity of 103900 U/mL according to the manufacturer's instruction. Sorbitol was purchased from Macklin Reagent (Shanghai, China). Yeast extract was purchased from Oxoid (Hamspshire, U.K.). Sulfuric acid, glucose, and other reagents were purchased from Sinopharm Chemical Reagent (Shanghai, China).

**2.3. Microorganisms and Medium.** Filamentous fungus *Paecilomyces variotii* FN89 (Chinese General Microorganism Collection Central, CGMCC, with the registry number 17665) was used as biodetoxification strain.[8] *P. variotii* FN89 was cultured on a potato dextrose agar (PDA) plate. The spores were washed and collected using 0.05% (w/w) Tween-80. The seed medium for *P. variotii* FN89 contained 2 g/L $KH_2PO_4$, 0.5 g/L $CaCl_2$, 1 g/L yeast extract (YE), 1 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4{\cdot}7H_2O$, and 20 g/L glucose.

Engineered lactic acid bacterium *Pediococcus acidilactici* (*Pe. acidilactici*) ZY271 (CGMCC 13611) was used for chiral L-lactic acid fermentation from lignocellulose derived sugars (glucose, xylose, arabinose, mannose, and galactose).[18,19] *Pe. acidilactici* ZY271 was originally derived from *Pe. acidilactici* DQ2.[20] The *ldhD* gene was knocked-out to a chiral L-lactic acid producing *Pe. acidilactici* TY112.[21] Then the pentose phosphate pathway (PPP) was integrated into the genome by inserting the four genes encoding xylose isomerase (*xylA*), xylulokinase (*xylB*), transketolase (*tkt*), transaldolase (*tal*), and the phosphoketolase pathway (PK) was blocked by disrupting the phosphoketolase (*pkt*) gene for reducing acetic acid generation.[19] The seed was cultured in simplified Man−Rogosa−Sharp (MRS) medium containing 10 g/L peptone, 10 g/L YE, 5 g/L $CH_3COONa$, 2 g/L ammonium citrate dibasic, 2 g/L $K_2HPO_4$, 0.58 g/L $MgSO_4{\cdot}7H_2O$, 0.25 g/L $MnSO_4{\cdot}H_2O$, and 20 g/L glucose. The nutrients for fermentation contained 20 g/L yeast extract, 2 g/L ammonium citrate dibasic, and 0.25 g/L $MnSO_4{\cdot}H_2O$.

Engineered yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) XH7 was used for ethanol fermentation.[22] *S. cerevisiae* XH7 was derived from the wild-type *S. cerevisiae* strain BSIF by the integration of *Ru-xylA* encoding xylose isomerase, the overexpression of *XKS1* encoding endogenous xylulokinase and four genes on the nonoxidative pentose phosphate pathway. The *GRE3* and *PHO13* genes encoding aldose reductase and alkaline phosphatase, respectively, were also inactivated.[22] *S. cerevisiae* XH7 was activated in yeast extract peptone dextrose (YPD) medium composed of 20 g/L peptone, 10 g/L YE, and 20 g/L glucose. The nutrients for fermentation contained 10 g/L YE, 2 g/L $K_2HPO_4$, 2 g/L $(NH_4)_2SO_4$, and 1 g/L $MgSO_4{\cdot}7H_2O$.

Adaptively evolved bacterium *Gluconobacter oxydans* (*G. oxydans*) RM7 (CGMCC 14801) was derived from *Gluconobacter oxydans* DSM 2003 purchased from German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany, and used for gluconic and xylonic acids production.[23] The medium for seed culture contained 20 g/L YE, 1.5 g/L $K_2HPO_4$, 1.5 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 80 g/L of sorbitol. The nutrients for fermentation contained 20 g/L YE, 1.5 g/L $K_2HPO_4$, 1.5 g/L $(NH_4)_2SO_4$, and 0.5 g/L of $MgSO_4{\cdot}7H_2O$.

**2.4. Dry Acid Pretreatment.** Wheat straw was pretreated by a modified acid pretreatment method, dry acid pretreatment, where 1200 g of dry wheat straw feedstock was fed into the 20 L reactor in


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dry particle form with ~70% (w/w) solids loading (solid/liquid ratio of 2:1) and discharged from the reactor in dry particle form containing ~50% (w/w) solids with the cofeeding of sulfuric acid solution.[24,25] The sulfuric acid dosage was 3.8% (w/w) of dry wheat straw.[26] The reactor equipped with helical impeller can efficiently mix the wheat straw, acid solution, and steam. The pretreatment was conducted at 175 °C and 50 rpm for 5 min. The wheat straw absorbed all the condensed water from vapor steam but was still in granular form owing to its favorable hygroscopicity. The main compositions of pretreated wheat straw contained 350.2 ± 8.2 mg/g of cellulose, 14.0 ± 5.7 mg/g of xylan, 36.7 ± 0.8 mg/(g of glucose), 136.8 ± 2.6 mg/(g of xylose), 22.9 ± 0.3 mg/(g of acetic acid), 5.4 ± 1.0 mg/(g of HMF), and 2.8 ± 0.5 mg/(g of furfural) on dry weight.

**2.5. Enzymatic Hydrolysis and Submerged Liquid Biodetoxification.** The sulfuric acid in the pretreated wheat straw solids was neutralized by adding 20% (w/w) $Ca(OH)_2$ slurry with good mixing based on the stoichiometric calculation. The enzymatic hydrolysis of pretreated wheat straw was conducted under 30% (w/w) solids loading in a 5 L bioreactor equipped with single helical stirring at 50 °C, 150 rpm for 12 h (ethanol and L-lactic acid fermentation cases) or 48 h (gluconic and xylonic acids fermentation cases), respectively.[27] In the cases of L-lactic acid and ethanol production, enzymatic hydrolysis was conducted in two steps. The first step is the direct hydrolysis of the pretreated wheat straw (called prehydrolysis), and the second step is in the simultaneous saccharification and co-fermentation (SSCF). In the case of sugar acids production, the enzymatic hydrolysis was conducted in one step (48 h) and the cellulase (4 mg of protein/(g of DM)) was all added in the step because of the strong inhibition on the intermediate glucono-γ-lactone on cellulase activity.[28] The cellulase dosage was 4 mg of total proteins per gram of dry wheat straw matter for each case, and no further cellulase enzyme addition during each case of fermentation. The obtained wheat straw hydrolysate was transferred into a 3 L bioreactor equipped with a Rushton impeller for submerged liquid biodetoxification and product fermentations (Baoxing Biotech Co., Shanghai, China).

The spore suspension of *P. variotii* FN89, in which the magnitude of spores reaches about $10^8$/mL, was inoculated into the seed medium at the ratio of 10% (v/v) and then cultured at 37 °C and 300 rpm for 20 h. The prepared seed culture with the dry cell weight (DCW) of ~8 g/L was inoculated into the hydrolysate to initiate the submerged liquid biodetoxification with the ratio of 1% (w/w), 5% (w/w), 10% (w/w), or 20% (w/w). The parameters of the submerged liquid biodetoxification process including initial pH value (3.5, 4.0, 4.6, 5.0, or 5.5), agitation rate (300, 500, or 750 rpm), and aeration rate (0.5, 1.0, or 1.5 vvm) were optimized. No nutrients were added in the submerged liquid biodetoxification process. The pH values and dissolved oxygen were detected online using pH electrode (D0912-0016, HAN-STAR Analytical Sensor Co., Ltd., Suzhou, China) and dissolved oxygen electrode (CH7402, Hamilton Bonaduz AG, Switzerland). The biodetoxified hydrolysate was then maintained at 50 °C and 100 rpm under anaerobic conditions for 12 h to inactivate the strain *P. variotii* FN89.[15] *P. variotii* FN89 grows in mycelium form in liquid medium or undetoxified hydrolysate under proper temperature (below 37 °C) and aerobic condition, but survives in spore form under a high stress environment such as anaerobic condition and/or higher temperature (50 °C).[15]

**2.6. Production of Diverse Lignocellulose-Based Products.** The biodetoxified hydrolysate was used for bioethanol and chiral L-lactic acid by simultaneous saccharification and co-fermentation (SSCF) under anaerobic conditions. For ethanol production, the primary seed of *S. cerevisiae* XH7 was prepared by inoculating the activated seed into 5% (w/w) solids loading of biodetoxified hydrolysate. The secondary seed was obtained by inoculating the primary seed into 10% (w/w) solid loading of biodetoxified hydrolysate. All of the seeds were cultured at 30 °C and 180 rpm for 24 h. The secondary seed was inoculated into the biodetoxified and inactivated hydrolysate at the ratio of 20% (w/w) for bioethanol production.[15] The ethanol fermentation was conducted at 30 °C and 300 rpm for 48 h. The pH value was maintained at 5.5 by automatically adding a 5 M NaOH solution.

For chiral L-lactic acid production, the seed of *P. acidilactici* ZY271 was prepared according to our previously reported protocols.[29] The seed was then inoculated into the biodetoxified hydrolysate at a ratio of 10% (w/w). The L-lactic acid fermentation was conducted at 42 °C and 300 rpm for 72 h. The pH value was maintained at 5.5 by automatically adding 20% (w/w) $Ca(OH)_2$ slurry.

In gluconic acid fermentation, an intermediate glucono-γ-lactone is oxidized from glucose by *Gluconobacter oxydans* strain before spontaneously being hydrolyzed into gluconic acid; however, it strongly inhibits cellulase enzyme activity, and thus SSF and SSCF are unable to operate for gluconic acid fermentation.[28] Separate hydrolysis and fermentation (SHF) were applied for sugar acids production. In this study, in order to maximize the release of fermentable sugars from the pretreated wheat straw, the period of hydrolysis was set at 48 h, which is the same as in our previous study.[23] The seed culture of *G. oxydans* RM7 was prepared according to our previous study.[23] The seed was inoculated into the biodetoxified hydrolysate at a ratio of 10% (w/w). The fermentation was conducted at 30 °C, 500 rpm, and 2.4 vvm. The pH value was maintained at 5.5 by automatically adding 5 M NaOH and 2 M $H_2SO_4$.

**2.7. Gas Chromatography–Mass Spectrometer (GC-MS).** The acid components during the submerged liquid biodetoxification were determined by GC-MS.[30] The nonderivatization method was used to avoid the interference of the derived fatty acid for measurement of the original free acid in culture broth. The supernatant of hydrolysate was mixed with 50% (w/w) sulfuric acid and anhydrous ether in a ratio of 10:1:4. The mixture was oscillated for at least 30 min and then chilled at 4 °C for 30 min. The upper layer of ether was pipetted for GC-MS analysis. The main acids were determined by Agilent 6890-5975I (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent DB-WAX capillary column (30 cm × 250 μm × 0.25 μm). The database is the NTST05 library. The oven temperature was initially maintained at 60 °C for 2 min, then increased at 10 °C/min to 170 °C, and finally increased at 60 °C/min to 280 °C (held for 5 min). The split ratio was 5:1, and helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector and electron impact (EI) ion source were set at 280 and 230 °C, respectively. The mass spectrometer was operated in the EI mode at 70 eV in the scan range of 50–500 $m/z$. The volume of each injection sample was 1.0 μL.

**2.8. Analytical Methods.** In addition to the samples produced during sugar acids fermentation, the components of glucose, xylose, acetic acid, furfural, 5-HMF, ethanol, and lactic acid in other samples were all determined by a HPLC system (LC-20AD, Shimadzu, Japan) equipped with refractive index RID-10A detector and Biorad Aminex HPX-87H column according to our previously reported protocols.[25] Glucose and xylose in samples from sugar acids fermentation were analyzed by HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with HPX-87P column (Biorad, Hercules, CA, USA) at 80 °C with the sterilized and degassed deionized water as mobile phase at a flow rate of 0.6 mL/min.[31] Gluconic acid and xylonic acid were determined by HPLC system (LC-20AD, Shimadzu, Japan) fitted with UV/vis detector SPD-20A and Biorad Aminex HPX-87H column according to our previously reported method.[28]

## 3. RESULTS AND DISCUSSION

**3.1. Submerged Liquid Biodetoxification Efficiency and End Point Identification.** The feasibility and efficacy of submerged liquid biodetoxification by *P. variotii* FN89 were evaluated in the high solid loading wheat straw hydrolysate (~30%, w/w). The inhibitor concentrations in the wheat straw hydrolysate were represented by three major inhibitors, furfural, 5-hydroxymethylfurfural (HMF), and acetic acid. The end point of biodetoxification was defined as the time


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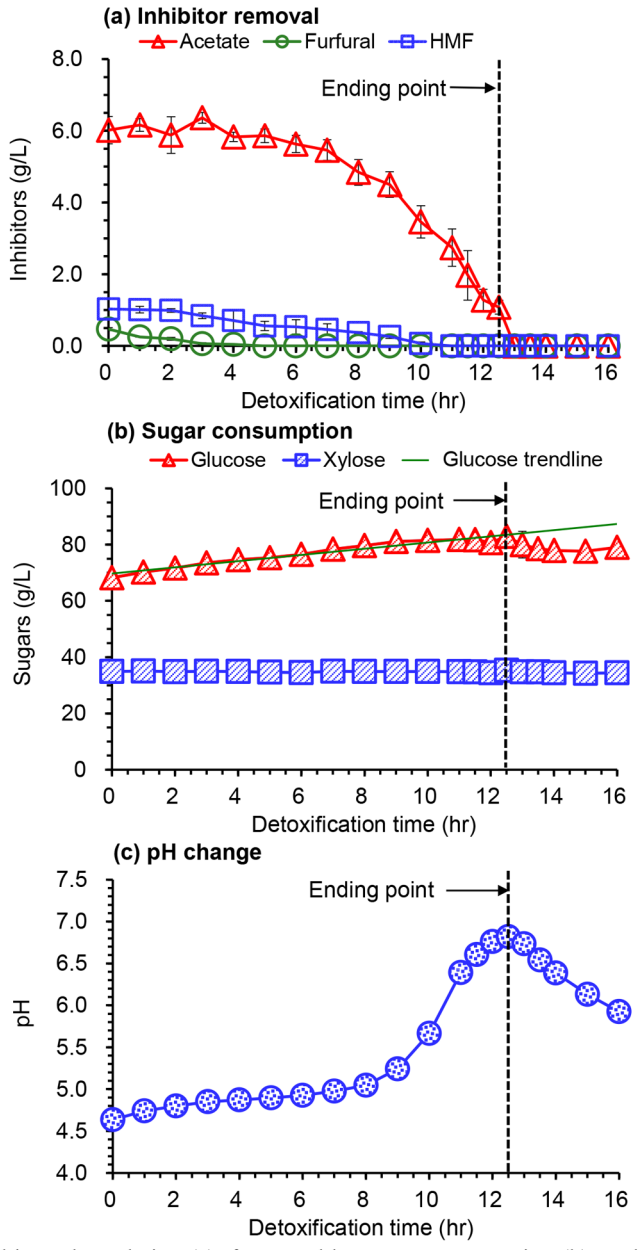


**Figure 1.** Inhibitors degradation (a), fermentable sugar consumption (b), and pH value change (c) during submerged liquid biodetoxification in high solid loading wheat straw hydrolysate. Hydrolysis: 30% (w/w) solids, 12 h, 50 °C, mild stirring (50 rpm), 4 mg protein/g. Biodetoxification: 20% (w/w) inoculum ratio, 37 °C, 1.5 vvm, 750 rpm. Each case was performed in triplicate.

point with complete removal of furfural and HMF, and ~80% removal of acetic acid. The loss of glucose and xylose was used as the second indicator of biodetoxification.

Figure 1 shows that all furfural and HMF (from 0.47 ± 0.04 and 1.03 ± 0.02 g/L to the undetectable levels) and 82.1% of acetate (from 6.02 ± 0.38 to 1.08 ± 0.30 g/L) were removed within 12.5 h of submerged liquid biodetoxification under vigorous agitation (750 rpm) and aeration (1.5 vvm) conditions (Figure 1a). According to the end point definition, the time point of 12.5 h was the end point of the biodetoxification case in Figure. 1. At the end point of biodetoxification (12.5 h), glucose concentration increased from the initial 68.21 ± 0.30 to 82.93 ± 1.32 g/L due to the continuous enzymatic hydrolysis during the biodetoxification period, while xylose was essentially unchanged (Figure 1b). If the submerged liquid biodetoxification continued after the end point of 12.5 h, the sugar loss obviously increased as indicated by the margin between the real glucose concentration and the hydrolysis trendline (Figure 1b and Supporting Information Figure S1). This experiment also indicated that the loss of fermentable sugar caused by *P. variotii* FN89 before reaching the end point of biodetoxification is negligible (below 2% of the total sugars). The wheat straw hydrolysate contained ~2−3 g/L of the total phenolic compounds, and *P. variotii* FN89 is capable of removing over 80% of the phenolic aldehydes during the biodetoxification.[18,32] The residual phenolics generate less toxicity to *P. variotii* FN89; thus, the prolonged biodetoxification on removing the residual phenolics causes the consumption of fermentable sugars. For the practical determination of the phenolics, each single phenolic component has a very low titer and it is difficult to have an accurate determination. Therefore, the biodetoxification process is monitored by following the changes in the three major inhibitors in this study.

An interesting phenomenon of pH value fluctuation was observed during submerged liquid biodetoxification. Before the end point (12.5 h), the pH value gradually increased from the initial 4.64 ± 0.05 to a peak value of 6.82 ± 0.01; after the end point, the pH value quickly decreased from the peak value of 6.82 ± 0.01 to 5.93 ± 0.01 within ~4.5 h (Figure 1c). This phenomenon was also observed at several submerged liquid biodetoxifications with varying initial pH value from 4.6 to 5.5, inoculation ratio from 1% (w/w) to 20% (w/w), agitation rate from 300 to 750 rpm, and aeration rate from 0.5 to 1.5 vvm (Supporting Information Figure S2).

The reason for pH fluctuation was investigated by semiquantitative measurement of acidic compound changes during submerged liquid biodetoxification (Figure 2). The biodetoxification strain *P. variotii* FN89 consumes the inhibitor substances (furfural, HMF, acetic acid, etc.) as the priority substrates compared to glucose and xylose.[8] In the Figure 2 case, before reaching the end point of detoxification (12.5 h), acetic acid was steadily consumed by *P. variotii* FN89 until a low threshold concentration (~1 g/L), resulting in the increase in pH values (Figure 2a). After reaching the end point, GC-MS analysis showed the generation of various organic acids during submerged liquid biodetoxification by *P. variotii* FN89, including furoic acid and benzoic acid, as well as several long chain fatty acids such as *n*-hexadecanoic acid and octadecanoic acid. These organic acids were only in trace amounts before the end point but increased quickly to the high threshold values shortly after the end point and became detectable. Clearly, the decrease of pH values of the wheat straw hydrolysate after the end point was due to the accumulation of these organic acids (Figure 2b). Therefore, the biodetoxification end point was easily identified by monitoring the online changes in pH values. The biorefinery chain could be easily transferred from the biodetoxification phase to the fermentation (or simultaneous saccharification and co-fermentation, SSCF) phase when the pH value reached its peak value. This method is applicable for the general scenario of inhibitor removal of lignocellulose hydrolysates because the principle does not depend on specific circumstances. Acetic acid generation is the natural outcome of acetyl group hydration from hemicellulose (the acetyl group in lignocellulose is up to 2−3% of the overall biomass weight), regardless of


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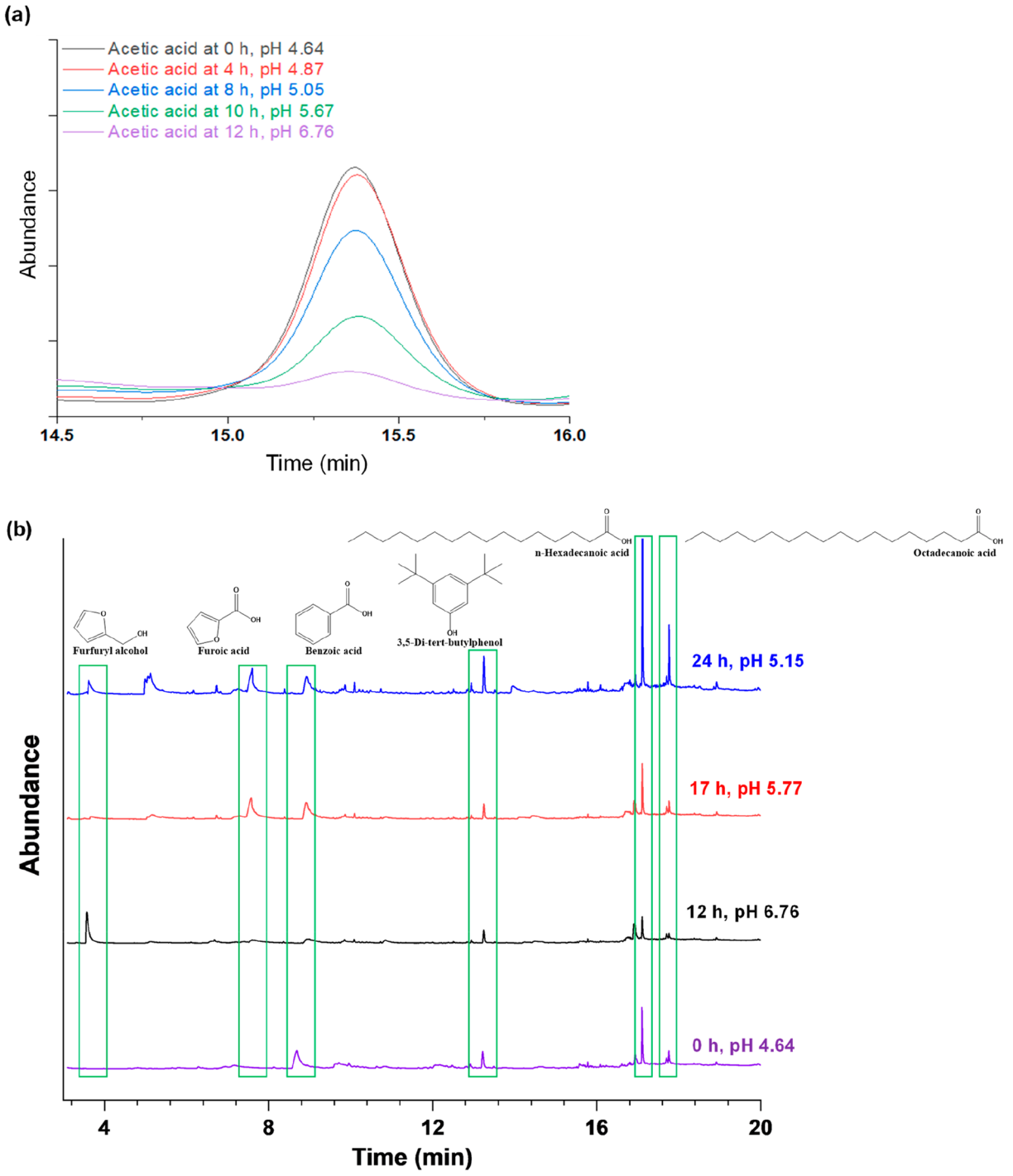


**Figure 2.** Acetic acid degradation and acidic component formation during the submerged liquid biodetoxification. (a) Acetic acid degradation during the biodetoxification determined by HPLC; (b) acidic acid compound formation after biodetoxification determined by GC-MS.

the acetic acid formation from furfural and HMF degradation. This property ensures the pH increase with acetic acid depletion during the early stage of biodetoxification by *P. variotii* FN89 in the lignocellulose hydrolysate. On the other hand, once the major inhibitors are degraded by *P. variotii* FN89 and the fermentable sugars are used as the carbohydrates for cell growth of *P. variotii* FN89, acidic compounds such as furoic acid and/or long-chain fatty acids will be generated and result in the decrease in pH value.

**3.2. Biodetoxification Parameter Optimization and Bioproduct Fermentation Verifications.** Biodetoxification of various aldehydes via multiple oxidoreductase-catalyzed pathways makes the submerged liquid biodetoxification an oxygen intensive process.[33] Although *P. variotii* FN89 grows at low oxygen levels and degrades inhibitors prior to the consumption of fermentable sugars,[8,12,34] the submerged liquid biodetoxification is hindered by its high solids loading and viscous nature of acid pretreated lignocellulose hydrolysate and result in poor transfer of dissolved oxygen.[35] To enhance the efficiency of submerged liquid biodetoxification, optimization was performed on cell culture and gas−liquid oxygen transfer


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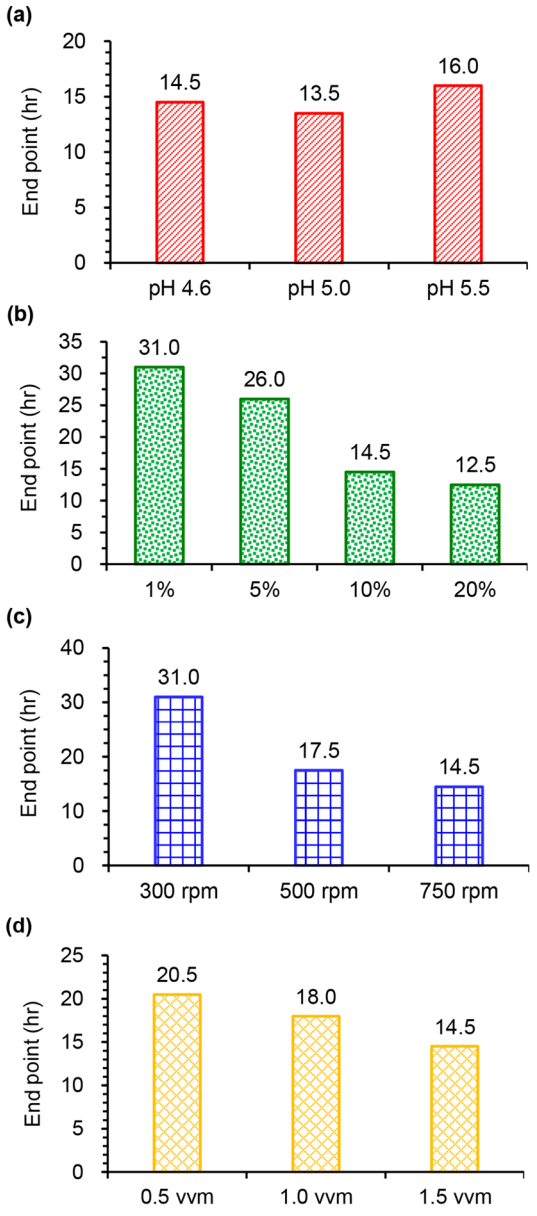


**Figure 3.** Acceleration of submerged liquid biodetoxification at varying operation parameters: (a) initial pH at 1.5 vvm, 750 rpm, 10% (w/w) inoculation; (b) inoculation at natural pH (~4.6), 1.5 vvm, 750 rpm; (c) agitation rate at 1.5 vvm, 10% (w/w) inoculation; and (d) aeration rate at 750 rpm, 10% (w/w) inoculation. Each case was at 37 °C and performed in triplicate. Some points in the panels have small errors; therefore, the corresponding error bars are not significantly shown. For details see Supporting Information (Figure S2).

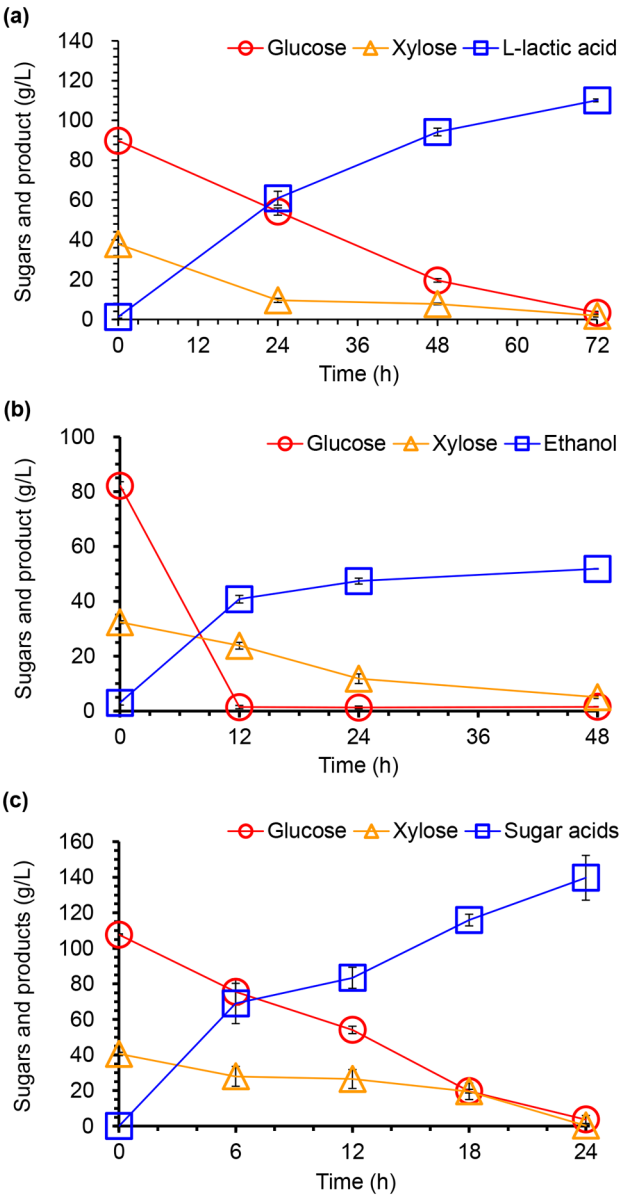


**Figure 4.** Biodetoxified wheat straw hydrolysates for production of (a) chiral L-lactic acid fermentation by *P. acidilactici* ZY271 at 10% (w/w) inoculum ratio, 42 °C, 300 rpm, pH 5.5; (b) ethanol by *S. cerevisiae* XH7 at 20% (w/w) inoculum ratio, 30 °C, 300 rpm, pH 5.5; (c) sugar acids including gluconic acid and xylonic acid by *G. oxydans* RM7 at 10% (w/w) inoculum ratio, 30 °C, 500 rpm, 2.4 vvm, pH 5.5. Each case was performed in triplicate.

parameters including initial pH value, inoculum size, agitation rate, and aeration rate, (Figure 3).

Figure 3a shows that the initial pH value had a limited effect on the biodetoxification rate. The shortest end point was at pH 5.0 (~13.5 h), and no inhibitor degradation occurred when the initial pH was below 4.0 (Supporting Information Figure S2). Figure 3b shows that the increased inoculum ratio of *P. variotii* FN89 accelerated the biodetoxification rate, but the practical inoculum ratio was still set at 10% (w/w) considering the cost and less dilution of the product titers. The energy cost of aerobic fermentation comes from aeration and agitation of the fermentation broth. The purpose of the two factors is to provide the sufficient dissolved oxygen for microbe growth and metabolism. The present submerged liquid biodetoxification uses a filamentous fungus *P. variotii* FN89 and a certain level of dissolved oxygen can be realized by proper aeration and agitation. Panels c and d of Figures 3 show the dissolved oxygen transfer enhancement by adjusting the aeration flow rate and agitation rate. The liquid wheat straw hydrolysate slurry is a viscous slurry containing a high amount of lignin solids particles. These properties make the oxygen transfer from air bubbles into the liquid rather difficult.[35] Increase of either aeration flow rate or agitation rate accelerated the biodetoxification rate significantly, indicating that the oxygen



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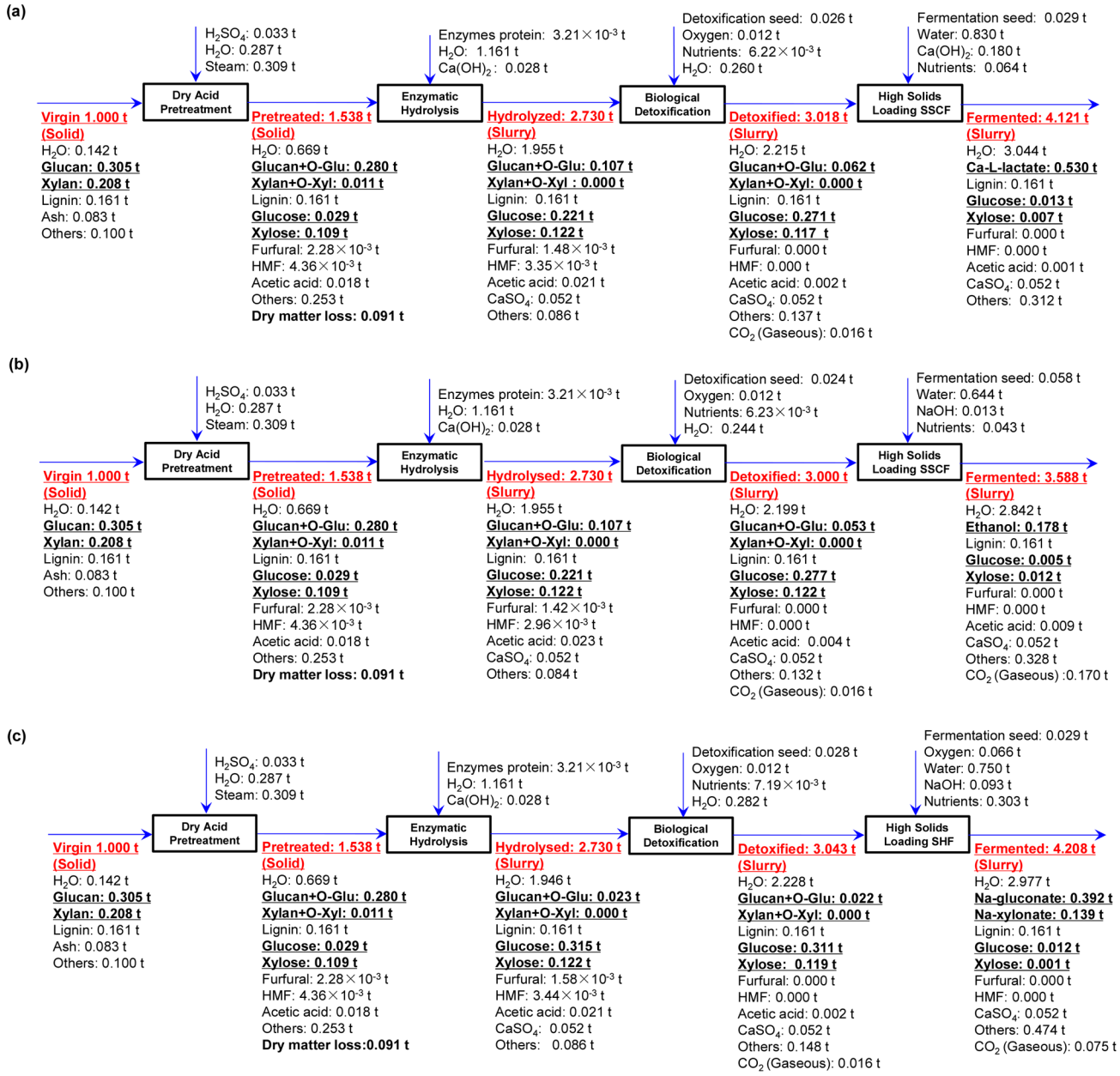


**Figure 5.** Mass balances of overall biorefining chain for production of cellulosic L-lactic acid (a), cellulosic bioethanol (b), and cellulosic sugar acids (c). Mass balances were started from 1.0 ton of virgin feedstock (wet weight) in order to calculate and demonstrate the conversion efficiency more intuitively, which contained 0.305 ton of cellulose and 0.208 ton of xylan, equivalent to 35.6% cellulose and 24.3% xylan on dry weight.

transfer was the determinative factor on the submerged liquid biodetoxification rate. A moderate aeration (~1.0 vvm) and agitation rate (500−750 rpm) corresponded to a gas−liquid oxygen transfer coefficient ($k_La$) in the range of 10−40 $h^{-1}$.[35] These conditions resulted in an acceptable and reasonable energy cost of aerobic fermentations during the biodetoxification (14.5−17.5 h) in practical applications.

The biodetoxified wheat straw hydrolysate was then used for the production of L-lactic acid, ethanol, or sugar acids to confirm the effectiveness of submerged liquid biodetoxification (Figure 4). Figure 4a shows the L-lactic production from wheat straw after dry acid pretreatment, enzymatic hydrolysis, submerged liquid biodetoxification, and SSCF. The final L-lactic acid titer reached 110.1 ± 0.7 g/L within 72 h of SSCF with the total residual sugars of 5.2 ± 0.7 g/L (3.3 ± 0.7 g/L glucose and 1.8 ± 0.6 g/L xylose) and the chiral purity of 99.5%. The production and chirality of L-lactic acid met the requirement of L-lactide synthesis.[18] Figure 4b shows that the ethanol production from wheat straw after the same biorefinery processing steps reached 51.9 ± 0.4 g/L within 48 h of SSCF. Figure 4c shows the sugar acids production (simultaneous production of gluconic acid and xylonic acid) from wheat straw after pretreatment, enzymatic hydrolysis, and submerged liquid biodetoxification to a detoxified hydrolysate containing 107.7 ± 0.5 g/L glucose and 40.6 ± 0.9 g/L xylose; then the sugar acid fermentation was conducted for 24 h to obtain 139.7 ± 12.6 g/L total sugar acids with the yield of 0.76 g/(g of total sugars). Several studies have shown that the generation of keto-gluconic acid (KGA) byproduct from gluconic acid by *G. oxydans* was inevitably accompanied by the decrease of sugar


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**Table 1. Production Performances of Lignocellulosic Biorefineries Involving Submerged Liquid- or Solid-State Biodetoxification**

| Biodetoxification method | Product and feedstock | Initial solids[a] (%, w/w) | Detoxification + fermentation (h) | Titer (g/L) | Yield[b] (g/g) | Productivity[c] ((g/L)/h) | Source |
|---|---|---|---|---|---|---|---|
| Submerged liquid by *P. variotii* FN89 | L-Lactic acid using wheat straw | ~25 | 90 (18 + 72) | 110.1 | 0.76 | 1.22 | This study |
| Solid state by *A. resinae* ZN1 | L-Lactic acid using wheat straw | ~27 | 144 (72 + 72) | 129.4 | 0.77 | 0.98 | 18 |
| Submerged liquid by *P. variotii* FN89 | Ethanol using wheat straw | ~23 | 66 (18 + 48) | 51.9 | 0.29 | 0.79 | This study |
| Solid-stateby *P. variotii* FN89 | Ethanol using corn stover | ~27 | 96 (48 + 48) | 56.5 | 0.28 | 0.59 | 8 |
| Submerged liquid by *P. variotii* FN89 | Sugar acids using wheat straw | ~25 | 42 (18 + 24) | 139.7 | 0.76 | 3.33 | This study |
| Solid state by *A. resinae* ZN1 | Sugar acids using corn stover | ~27 | 64 (32 + 32) | 148.4 | 0.78 | 2.31 | 23 |

[a]Solids loading was reduced due to inoculations of fermentation seed broth and/or biodetoxification seed broth. In submerged liquid biodetoxification, both seed broths were added; in solid-state biodetoxification, only fermentation seed broth was added. [b]Yield was calculated based on the produced lactic acid, ethanol, or sugar acids (grams) per gram of the sugars in the feedstocks. [c]Productivity was calculated based on the produced lactic acid, ethanol, or sugar acids (grams) per liter over the time period of detoxification and fermentation (or SSCF).

acids.[23,36] In the current study, no decrease in the concentration of sugar acids was observed (Figure 4c), indicating that KGA generation was negligible.

**3.3. Mass Balance and Process Evaluation of Submerged Liquid Biodetoxification.** The overall mass balances of dry biorefinery processing involving dry acid pretreatment, hydrolysis, submerged liquid biodetoxification, and fermentation were performed in order to show the overall sugar conservation and conversion efficiency of the dry biorefinery processing chain (Figure 5). The mass balances for production of L-lactic acid, ethanol, and sugar acids were calculated based on the experimental results. The mass balances used one metric ton of raw wheat straw as the basis, which contained 0.305 ton of glucan and 0.208 ton of xylan. In the dry acid pretreatment step, a majority of xylan was hydrolyzed, while the cellulose was well preserved, with the generation of 2.28 kg of furfural, 4.36 kg of HMF, and 18 kg of acetic acid. In the present dry acid pretreatment operation, no wastewater streams were generated and all of the inhibitory compounds were accumulated into the pretreated solid feedstocks. Although yeast strains are relatively tolerant to one or several inhibitors at low concentrations, the high concentrations of inhibitors harshly inhibit their cell growth and metabolisms in the scenario of dry acid pretreated wheat straw hydrolysate under high solids loading hydrolysis.[37,38] Therefore, the complete removal of inhibitors is a prerequisite and crucially important step for cell growth and the high fermentation performance of the consequent fermenting strains. The enzymatic hydrolysis was conducted to generate the liquid slurry of wheat straw hydrolysate for submerged liquid biodetoxification.[15] In the hydrolysis step, a 12 h hydrolysis produced 0.221 ton of free glucose (Figure 5a,b), and 48 h hydrolysis produced 0.311 ton of free glucose (Figure 5c). The consequent submerged liquid biodetoxification was conducted in the wheat straw hydrolysate, and all furfural and HMF and ~80% acetic acid were consumed with only 1.0% (w/w) (Figure 5a,b) and 1.7% (w/w) (Figure 5c) of fermentable sugars' loss. In the fermentation step, 0.530 ton of calcium lactate, 0.178 ton of ethanol, or 0.392 ton of sodium gluconate and 0.139 ton of sodium xylonate were produced.

Table 1 summarizes the performance of several biorefinery processes using submerged liquid biodetoxification and compares their performance to those using solid-state biodetoxification. The final titer, productivity, and yield of the target products using the submerged liquid biodetoxification method were close to those using the solid-state biodetoxification method. The overall bioconversion time (biodetoxification plus fermentation) was reduced by ~30% in the submerged liquid biodetoxification-based process compared with those using the solid-state biodetoxification. Correspondingly, the productivity was increased by 15−40%, although the product titers were diluted by the inoculation of biodetoxification seed culture in submerged liquid biodetoxification, in comparison with that in solid-state biodetoxification.

Submerged liquid biodetoxification offers several advantages over solid-state biodetoxification due to the liquid method's inherent properties and compatibility with the existing biorefinery chain. The pretreated lignocellulose feedstock can be hydrolyzed directly into high solids loading slurry, resulting in an increase in density from approximately 380 to around 1030 kg/m$^3$ and the equipment cost is reduced correspondingly.[39,40] The improved heat and mass transfer in the liquid slurry enable efficient mixing, cell growth, and inhibitor metabolism, resulting in reduced electricity consumption and shorter biodetoxification times compared to solid-state biodetoxification. Submerged liquid biodetoxification of typical dry acid pretreated lignocellulosic feedstock by *P. variotii* FN89 can be completed within 18.0 h under moderate aeration (1.0 vvm) with minimal loss of fermentable sugars (approximately 1−2%). Another significant advantage of submerged liquid biodetoxification is that it allows for the identification of a detectable end point of biodetoxification: the pH value peak. This feature solves the difficulties previously encountered in determining the end point of biodetoxification and high fermentable sugars loss in the detoxification step.

Unlike difficult discharge and transport of solid biomass in or after solid-state biodetoxification, the high solids loading hydrolysate has an apparent viscosity range of 0.11−0.56 Pa·s, which allows an efficient transportation through regular pumps, vessels, and pipelines.[41] By operating in closed vessels, pipelines, and pumps, microbial contamination of biodetoxified lignocellulosic biomass can also be effectively prevented.

In conclusion, the proposed submerged liquid biodetoxification offers significant advantages over solid-state biodetoxification in terms of equipment cost, efficiency, and


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controllability in the scenario of dry biorefining processing. It is expected that submerged liquid fermentation, instead of solid-state fermentation, will be the option for biodetoxification operations in future dry biorefinery processing.

## ■ ASSOCIATED CONTENT

### Data Availability Statement

The source data supporting the findings of this study are available within the article.

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.3c04986.

Submerged liquid biodetoxification; submerged liquid biodetoxification optimized parameters (PDF)

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T.H. and B.Z. equally contributed to this work. T.H. and B.Z. conducted the biorefinery and microbial fermentation experiments. H.Y. participated in the experiment. J.B., B.Z., and T.H. designed the study. J.B and X.L conceived the study. T.H., B.Z., and J.B. wrote the manuscript. All authors contributed to the revisions of the manuscript.

### Notes

The authors declare no competing financial interest.

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