Received: 11 September 2017 | Revised: 11 December 2017 | Accepted: 5 January 2018

DOI: 10.1002/bit.26541

ARTICLE

WILEY BIOTECHNOLOGY & BIOENGINEERING

Consolidated bioprocessing of lignocellulosic biomass to lactic acid by a synthetic fungal-bacterial consortium

Robert L. Shahab[1,2] | Jeremy S. Luterbacher[1] | Simone Brethauer[2] | Michael H. Studer[2]

[1] Laboratory of Sustainable and Catalytic Processing, Institute of Chemical Sciences and Engineering, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

[2] Laboratory of Biofuels and Biochemicals, School of Agricultural, Forest and Food Sciences, Bern University of Applied Sciences (BFH), Zollikofen, Switzerland

Correspondence
Michael Hans-Peter Studer, Laboratory of Biofuels and Biochemicals, School of Agricultural, Forest and Food Sciences, Bern University of Applied Sciences (BFH), CH–3052 Zollikofen, Switzerland.
Email: michael.studer1@bfh.ch

Funding information
Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Grant number: 407040-153868

Abstract

Consolidated bioprocessing (CBP) of lignocellulosic feedstocks to platform chemicals requires complex metabolic processes, which are commonly executed by single genetically engineered microorganisms. Alternatively, synthetic consortia can be employed to compartmentalize the required metabolic functions among different specialized microorganisms as demonstrated in this work for the direct production of lactic acid from lignocellulosic biomass. We composed an artificial cross-kingdom consortium and co-cultivated the aerobic fungus *Trichoderma reesei* for the secretion of cellulolytic enzymes with facultative anaerobic lactic acid bacteria. We engineered ecological niches to enable the formation of a spatially structured biofilm. Up to 34.7 gL^{-1} lactic acid could be produced from 5% (w/w) microcrystalline cellulose. Challenges in converting pretreated lignocellulosic biomass include the presence of inhibitors, the formation of acetic acid and carbon catabolite repression. In the CBP consortium hexoses and pentoses were simultaneously consumed and metabolic cross-feeding enabled the in situ degradation of acetic acid. As a result, superior product purities were achieved and 19.8 gL^{-1} (85.2% of the theoretical maximum) of lactic acid could be produced from non-detoxified steam-pretreated beech wood. These results demonstrate the potential of consortium-based CBP technologies for the production of high value chemicals from pretreated lignocellulosic biomass in a single step.

KEYWORDS

biofilm, consolidated bioprocessing (CBP), lactic acid, lignocellulose, synthetic microbial consortium, *Trichoderma reesei*

1 | INTRODUCTION

Lactic acid and its derivatives are versatile sustainable platform molecules for the food and chemical industries (Abdel-Rahman, Tashiro, & Sonomoto, 2013) and are mainly produced in anaerobic batch fermentations of feedstocks rich in starch or sugar. However, the high costs of these raw materials and their competitive use as food and feed ingredients remain major drawbacks of the fermentative production of lactic acid, which limits the large-scale development of poly-lactic acid as renewable alternative to fossil-based plastics. Alternatively, lignocellulosic biomass—the most abundant form of fixed renewable carbon on earth—is a promising feedstock for producing sustainable chemicals at low cost (Brethauer & Studer, 2015). Generally, the biotechnological processing of lignocellulose can be divided in four main steps: pretreatment to enhance enzymatic digestibility, production of saccharifying enzymes (cellulases and

hemicellulases), enzymatic hydrolysis to depolymerize cellulose, and hemicellulose to fermentable saccharides and fermentation of soluble saccharides to the target product (Brethauer & Studer, 2014; Minty et al., 2013). Central barriers of the industrial-scale conversion of lignocellulosic biomass to biochemicals are the high capital costs due to the complex process and the high cost of cellulolytic enzymes (Geddes, Nieves, & Ingram, 2011; Kawaguchi, Hasunuma, Ogino, & Kondo, 2016). The simplification of the process by integrating all biochemical process steps into one single unit operation, an approach termed consolidated bioprocessing (CBP), has attracted considerable attention for the production of biochemicals, due to its anticipated favorable economic performance (Kawaguchi et al., 2016; Lynd, Van Zyl, McBride, & Laser, 2005). The engineering and the optimization of multiple capabilities, for example, the production of cellulolytic enzymes, the co-fermentation of hexoses and pentoses and the tolerance to inhibitory degradation products derived from biomass pretreatement, in one single strain has proven to be challenging (den Haan, van Rensburg, Rose, Görgens, & van Zyl, 2015; Minty et al., 2013; Peng, Gilmore, & O'Malley, 2016). Thus, consortium-based bioprocesses, where different specialized microorganisms can efficiently combine various pathways and processes required for the degradation of complex substrates, are developed and investigated as an alternative approach for CBP (Peng et al., 2016).

Successful consortium-based conversions of cellulosic feedstocks to a variety of different compounds including ethanol (Brethauer & Studer, 2014; Xu & Tschirner, 2011; Zuroff, Barri Xiques, & Curtis, 2013), isobutanol (Minty et al., 2013), or acetone, butanol, and ethanol (Wen et al., 2014) have been reported. However, improved strategies to control intercellular interactions and to enable stable microbial communities are required (Agapakis, Boyle, & Silver, 2012; Johns, Blazejewski, Gomes, & Wang, 2016; Nadell, Drescher, & Foster, 2016). Furthermore, any biological production of lactic acid from lignocellulosic biomass is especially challenging as recently described due to three major limitations specifically associated with it's fermentation from heterogeneous saccharides (e.g., glucose, xylose): (i) The heterofermentation of xylose via the phosphoketolase (PK) pathway leads to the accumulation of high amounts of by-products such as acetic acid which increases the downstream cost and reduces the profitability; (ii) Carbon catabolite repression (CCR) of the non-favorable sugars such as xylose lowers the productivity of the process; and (iii) Lactic acid bacteria are sensitive to inhibitory compounds released during pretreatment of the biomass (Abdel-Rahman & Sonomoto, 2016). Table 1 summarizes recently published results for the production of lactic acid from lignocellulosic biomass. To the best of our knowledge, only simultaneous saccharification and fermentation (SSF) processes which require the addition of costly, externally produced cellulolytic enzymes were developed, but no consolidated bioprocesses targeting lactic acid were reported.

In this work, we designed and characterized artificial cross-kingdom microbial consortia of *Trichoderma reesei* and different *Lactobacilli* for the high-yield production of lactic acid from lignocellulosic biomass. We demonstrate their ability to directly convert non-detoxified pretreated biomass containing C_5 and C_6 sugars to lactic acid without carbon catabolite repression and to concomitantly remove the unwanted side product acetic acid.

2 | RESULTS AND DISCUSSION

2.1 | Design and implementation of a microbial consortium for CBP of lignocellulose to lactic acid

Consortium-based CBP of lignocellulosic biomass to lactic acid requires at least one microorganism for the production of cellulolytic enzymes and one strain that converts the sugars released to lactic acid. Many lactic acid bacteria (LAB) are known for microaerophilic or anaerobic fermentations (Tian et al., 2015). In contrast aerobic fungi such as *T. reesei* are primarily used for the industrial production of cellulolytic enzymes (Geddes et al., 2011; Sharma, Tewari, Rana, Soni, & Soni, 2016). In order to concomitantly meet these contrary oxygen requirements in one reactor, we developed a membrane reactor that enables locally defined aeration through an oxygen permeable membrane. Here, the aerobic fungus *T. reesei* forms a biofilm directly on the surface of the tubular membrane, which is continuously flushed with air (Supplementary Figure S1). Oxygen diffuses through the membrane into the fungal biofilm that produces and secretes cellulolytic enzymes into the fermentation broth. As all oxygen is consumed in the biofilm, anaerobic conditions prevail in the bulk phase where the enzymatic hydrolysis is taking place and LAB ferment the saccharides released to the target product lactic acid (Figure 1). Taken together, the process concept enables the compartmentalization of the metabolic functions between different microorganisms and the creation of an ecological niche for each member the consortium by spatial structuring of the bioreactor.

2.2 | Conversion of cellulose to lactic acid in batch processes

In order to prove the functionality of the synthetic consortia, we first studied the conversion of different amounts and types of carbon sources (microcrystalline cellulose [Avicel] and steam-pretreated beech wood) to lactic acid by a co-culture of *T. reesei* and *L. pentosus*. A sequential inoculation scheme with a two-day delay between the fungal and the bacteria inoculation was applied to enable the formation of a fungal biofilm and to ensure anaerobic conditions in the bulk phase at the time of inoculation with the product forming strain. In effect, the dissolved oxygen concentration dropped below the detection limit 12 hr after fungal inoculation indicating that growth of *T. reesei* was oxygen limited and not nutrient limited (Supplementary Figure S2). With microcrystalline cellulose as the substrate, the maximum lactic acid yield increased from 0.45 gg^{-1} (45.9% of the theoretical maximum) at 1.75% (w/w) Avicel to 0.62 gg^{-1} (62.4%) at 5.00% (w/w) Avicel (Table 2). The latter corresponds to a maximum lactic acid concentration of 34.7 ± 0.2 gL^{-1} (Figure 2a) and a productivity of 0.16 $gL^{-1}\,h^{-1}$. Due to the chosen sequential inoculation scheme, *T. reesei* had during the first two days exclusive access to the substrate. The increasing lactic acid yield suggested that the amount of the

TABLE 1 Reported and own data on the production of lactic acid from pretreated lignocellulosic material or crystalline cellulose either in SSF processes or by CBP

Microorganisms	Feedstock	Process	$C_{LA,max}$ (gL^{-1})	Productivity ($gL^{-1}\,h^{-1}$)	Reference
L. rhamnosus/*L. brevis*	Corn stover	SSF	20.95	0.58	Cui, Li, and Wan (2011)
B. coagulans	Solka Floc crystalline cellulose	SSF	80	0.30	Ou, Ingram, and Shanmugam (2011)
L. rhamnosus	Cellulosic biosludge	SSF	39.4	0.82	Romaní, Yáñez, Garrote, and Alonso (2008)
B. coagulans	Sugarcane bagasse	SSF	58.7	1.33	van der Pol, Eggink, and Weusthuis (2016)
B. coagulans	Wheat straw	SSF	40.7	0.74	Maas et al. (2008)
T. reesei/*L. pentosus*	Microcrystalline cellulose	CBP	34.7	0.16	This article
T. reesei/*L. pentosus*	Beech wood	CBP	19.8	0.10	This article

carbon source that was consumed for fungal growth remained constant and was independent of the solid loading, that is, the fraction of carbon source that remained for the conversion to lactic acid increased with increasing carbon loading. Next, we switched to one-stage steam-pretreated washed beech wood (containing mainly cellulose and lignin) as a substrate and investigated three different solid loadings (1.93, 2.21, and 3.86% [w/w]). Due to stirring limitations in the reactor, higher solid loadings could not be tested. Up to 15.1 gL^{-1} lactic acid (65.6% yield) were produced after 134 hr without any detectable amounts of acetic acid as a side product. The highest yield of 78.1% lactic acid (10.3 gL^{-1}) was reached with an initial solid loading of 2.21% (w/w) (Figure 2c, Table 2).

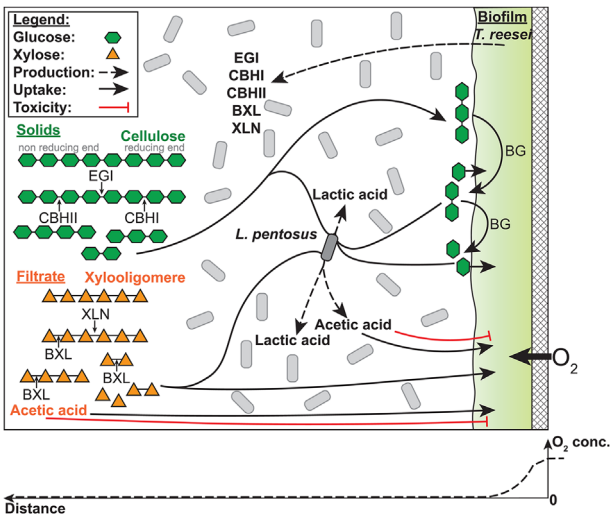

FIGURE 1 Schematic representation of the *T. reesei*/*L. pentosus* consortium. *T. reesei* grows immobilized in an aerobic biofilm directly on the surface of an oxygen permeable, dense membrane. The locally defined aeration through the membrane enables the production of cellulases and hemicellulases (EGI: endoglucanase I, CBHI: cellobiohydrolase I, CBHII: cellobiohydrolase II, BXL: beta-xylosidase, XLN: beta-endoxylanase) under aerobic conditions. The enzymes are secreted to the fermentation slurry where the hydrolysis of cellulose and xylooligomeres to soluble saccharides is taking place. *L. pentosus* produces lactic and acetic acid under anaerobic conditions, whereby, the latter inhibits the growth of *T. reesei* (amensalism). Acetic acid, however, can be degraded by *T. reesei* (commensalism), with the effect that lactic acid is purified in situ in the mixed culture. The dissolved oxygen concentration in the reactor is depicted in the lower part of the illustration

The activity of cellobiohydrolase (CBH), beta-glucosidase (BG) and endoglucanase (EG) in the supernatant of the fermentation slurry during various stages of the fermentation is shown for 1.75% (w/w) Avicel in Figure 2b and different pretreated beech wood loadings in Figure 2d. The enzymatic activity of CBH and EG on Avicel increased over two days after inoculation of *L. pentosus* and reached 289 mIU ml^{-1} and 9.48 IU ml^{-1}, respectively. In comparison to the CBP on Avicel the enzymatic activities in the supernatant using pretreated beech wood were lower and reached a maximal CBH activity of 127 mIU ml^{-1} (Figure 2d). Although the initial lactic acid accumulation was similar for all tested beech wood loadings which indicates comparative enzymatic hydrolysis rates in the sugar-limited system the CBH activity was reduced with increasing solid loading. This was presumably caused by a higher non-productive binding of CBH with increasing lignin content in the reactor (Rahikainen et al., 2013) and might explain the lower yield in comparison to 2.21% (w/w).

We also studied the reuse of the immobilized fungal cells in a semi continuous experiment and exchanged every 72 hr one quarter of the fermentation slurry (Supplementary Figure S7). Although we measured a decrease of CBH and EG activity during the course of the experiment, the immobilization of the fungal biomass in the biofilm prevented its washout. An increase of CBH and EG after the dilution indicates the possible reuse and stability of the fungal biofilm.

2.3 | Co-fermentation of C_5 and C_6-sugars and in situ acetic acid degradation by the synthetic consortium

The co-fermentation of hexoses and pentoses is a prerequisite for the cost-effective conversion process of heterogeneous lignocellulosic feedstocks containing both types of sugars in polymeric form. Generally, several LAB can consume C_5 sugars that are converted by hetero-fermentation via the PK pathway to lactic acid, acetic acid and ethanol with a theoretical lactic acid yield of 0.6 gg^{-1} xylose (Abdel-Rahman et al., 2011). Furthermore, it is known that *L. pentosus* controls the utilization of carbohydrates by CCR (Mahr, Hillen, & Titgemeyer, 2000).

TABLE 2 CBP of different substrates in batch and fed-batch mode by *T. reesei* and different LAB

Experiment	Microorganisms	Solid (% [w/w])	Xylose (gL^{-1})	$C_{LA,max}$ (gL^{-1})	Time[a] (h)	$Y_{P/S}$[b] (gg^{-1})	Y_{LA}[c] (%)	C_{AA}[a] (gL^{-1})
Avicel (batch)	*T. reesei*/*L. pentosus*	1.75	–	8.9 ± 0.3	109.8	0.46	45.9	–
		3.50	–	23.0 ± 0.2	145.0	0.59	59.1	0.1 ± 0.1
		5.00	–	34.7 ± 0.2	215.4	0.62	62.4	0.1 ± 0.1
Avicel + xylose (batch)	*T. reesei*/*L. pentosus*	1.75	9.32	11.3 ± 0.3	116.5	0.39	45.0	0.4 ± 0.3
	T. reesei/*L. brevis*			9.3 ± 0.1	184.8	0.32	60.4	4.4 ± 0.3
Washed steam-pretreated beech wood solids (batch)	*T. reesei*/*L. pentosus*	1.93	–	8.6	60.7	0.75	74.6	0.5
		2.21	–	10.3	91.9	0.78	78.1	–
		3.86	–	15.1	133.8	0.66	65.6	–
Avicel (batch) + xylose (fed-batch, 100 h)	*T. reesei*/*L. pentosus*	1.75	9.32	14.5 ± 0.7	146.7	0.50	57.9	0.8 ± 0.2
Avicel (batch) + xylose (fed-batch, 200 h)		5.00	26.63	54.6 ± 2.1	398.9	0.66	76.3	4.2 ± 0.9
One-stage pretreated beech wood solids (batch) + prehydrolyzate (fed-batch, 150 h)	*T. reesei*/*L. pentosus*	3.86	–	15.7 ± 0.2	250.3	0.68	68.1	0.1 ± 0.1
Two-stage pretreated beech wood solids (batch) + first stage prehydrolyzate (fed-batch, 150 h)	*T. reesei*/*L. pentosus*	3.86	5.01[d]	19.8 ± 0.7	200.5	0.78	85.2	0.5 ± 0.1

[a]At maximum lactic acid concentration.
[b]Yield coefficient expressed as mass of product formed per mass of fermentable carbohydrates at maximum lactic acid concentration. Glucan and xylan concentrations were calculated to corresponding glucose and xylose concentrations using a conversion factor of 1.11 and 1.136, respectively.
[c]The theoretical yields for *L. pentosus* are based on pentose assimilation through the PK pathway and hexose assimilation through the Embden-Meyerhof-Parnas pathway. The theoretical yields for *L. brevis* are based on the assimilation of both hexoses and pentoses through the PK pathway.
[d]Recalculated from xylooligosaccharides and xylose fed with the prehydrolyzate.

Glucose represses the *xylAB* operon encoding D-xylose isomerase and D-xylulose kinase which are both required for xylose fermentation (Lokman et al., 1997). To study the co-fermentation abilities of the consortium and the possible presence of CCR, mixtures of Avicel and xylose were used as the carbon source. A co-culture of *T. reesei* and *L. pentosus*—a facultative heterofermentative strain—on 1.75% (w/w) Avicel and 9.32 gL^{-1} xylose resulted in 11.3 ± 0.3 gL^{-1} lactic acid (45.0% yield) and 0.4 ± 0.3 gL^{-1} acetic acid after 117 hr. Xylose was consumed from the beginning of the fermentation, while more acetic acid was accumulating compared to when only Avicel was fermented. This indicated that Avicel and xylose were simultaneously co-degraded without active CCR. Generally, the amounts of acetic acid measured in the co-cultures were very low, up to ten times lower than the values detected in monoseptic cultures of only *L. pentosus* (Table 2, Supplementary Figure S5) were detected. Furthermore, acetic acid degradation was observed in some experiments (see e.g., Figure 3a, where only up to 1.0 gL^{-1} acetic acid accumulated temporarily). Thus, we investigated the apparent in situ acetic acid degradation of the consortia in more detail. Degradation experiments confirmed the ability of *T. reesei* to co-degrade acetic acid, glucose and xylose (Supplementary Figure S4). However, acetic acid mineralization to presumably carbon dioxide was not associated with growth (Jourdier, Poughon, Larroche, & Ben Chaabane, 2013) and above 0.3 gL^{-1}, acetic acid inhibited the metabolic activity of *T. reesei*. In order to test the limits of the in situ acetic acid degradation, *L. pentosus* was replaced with *L. brevis*—an obligate heterofermenative strain that utilizes pentoses and hexoses exclusively via the PK pathways—which leads to a higher production of acetic acid. Under these conditions, 9.3 gL^{-1} lactic acid (60.4% yield) and 4.4 ± 0.3 gL^{-1} acetic acid were measured after 185 hr (Figure 3b, Table 2). The high accumulation of acetic acid indicated that the purification potential of *T. reesei* was exceeded in this case. As the degradation of acetic acid required oxygen, the oxygen transfer rate presumably determined the limits of the in situ purification rate. This assumption was confirmed by a mono-culture of *T. reesei* in the biofilm-membrane reactor with acetic acid as the sole carbon source, which showed a constant acetic acid degradation rate of 0.01 $gL^{-1}\,h^{-1}$ (data not shown). In order to simulate the continuous release of xylose from the enzymatic hydrolysis of soluble xylooligosaccharides, we also performed fed-batch experiments with Avicel and xylose as model substrates. While 1.75% (w/w) Avicel was added all at once, xylose was fed linearly with a constant feeding rate to the reactor to an accumulated theoretical concentration of 9.32 gL^{-1}. This led to a lactic acid concentration of 14.5 ± 0.7 gL^{-1} after 146 hr (Figure 4a, Table 2). The highest xylose concentration of 1.1 gL^{-1} was measured after 103 hr and dropped below the detection limit after 139 hr. The amount of xylose measured at any sampling point in the fed-batch experiments was below the theoretical amount calculated from the feeding rate. The highest acetic acid concentration of 1.1 gL^{-1} was measured after 126 hr fermentation time and dropped below the detection limit after 215 hr, due to in situ acetic acid degradation by *T. reesei*.

The lactic acid yield in the fed-batch experiments was 28% higher than in the batch experiment at identical substrate levels (Figure 3a, Table 2). This could be explained by the exclusive access of *T. reesei* to xylose prior to inoculation of LAB in the batch experiment. Feeding is known to influence the microbial interactions and can increase the stability of the microbial community (Coyte, Schluter, & Foster, 2015). We reduced the interactions between the species by feeding xylose as an additional alternative carbon source. While *L. pentosus*—as

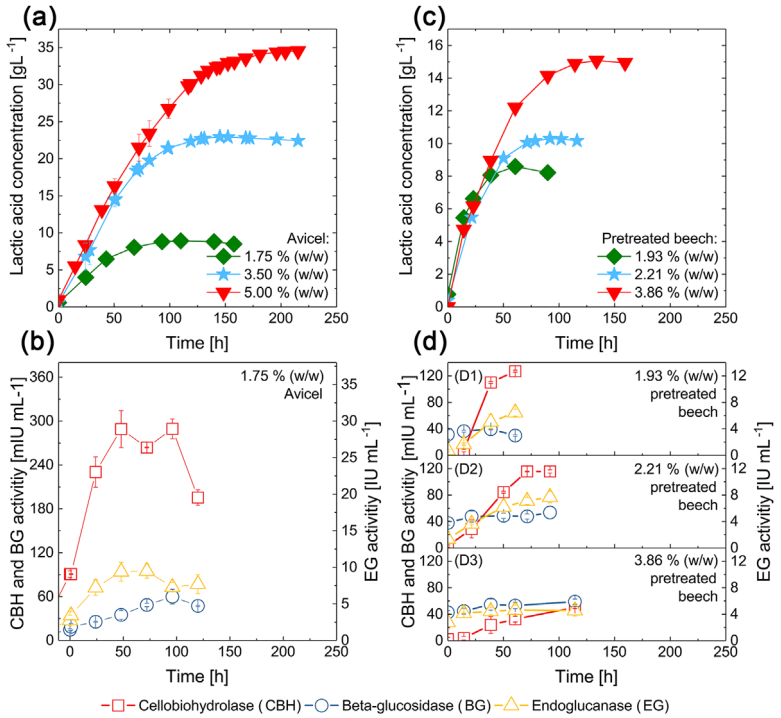

FIGURE 2 Batch CBP of lignocellulosic biomass to lactic acid using a synthetic microbial consortium of *T. reesei* and a *Lactobacillus* strain. Conversion of (a) microcrystalline cellulose (Avicel) and (c) one-stage steam-pretreated washed beech wood to lactic acid using *L. pentosus* as the fermenting strain. Error bars represent the standard deviation from two independent batch experiments. (b,d) Corresponding activities of cellobiohydrolase (CBH), beta-glucosidase (BG), and endoglucanase (EG) in the supernatant of the fermentation slurry during various stages of the fermentation of B) Avicel (as shown in a) or (c) different solid loadings of pretreated beech wood (as shown in c). Error bars represent the standard deviation from duplicates

non-cellulolytic bacterium—was previously on Avicel as sole carbon source dependent on the secretome of *T. reesei*, the additional xylose feed reduced this level of dependency because *L. pentosus* was able to metabolize xylose directly. Therefore, *L. pentosus* was only extrinsically limited by the feed and not exclusively dependent on the secretome of *T. reesei*, which might explain the higher lactic acid yields in fed-batch mode. In order to converge to industrially achievable lactic acid concentrations, the substrate loading was increased to 5.00% (w/w) Avicel and a feed of accumulated 26.7 gL^{-1} xylose over a period of 200 hr. Under these conditions, a concentration of 54.6 ± 2.1 gL^{-1} lactic acid was measured after 398 hr (Figure 4b, Table 2).

2.4 | Conversion of whole-slurry pretreated beech wood in fed-batch experiments

Finally, we aimed to directly convert whole-slurry steam-pretreated beech wood (i.e., the unprocessed liquid and the solid phase after pretreatment) to lactic acid. In the pretreatment process, a liquid phase—called

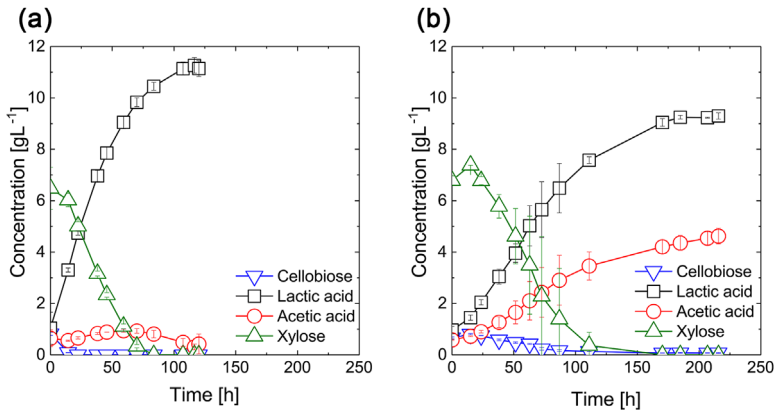

FIGURE 3 Batch CBP of Avicel and xylose to lactic acid using a synthetic microbial consortium of *T. reesei* and a *Lactobacillus* strain. 1.75% (w/w) Avicel and 9.32 gL^{-1} xylose were co-fermented by *T. reesei* and (a) *L. pentosus* or (b) *L. brevis*. Error bars represent the standard deviation from two independent batch experiments

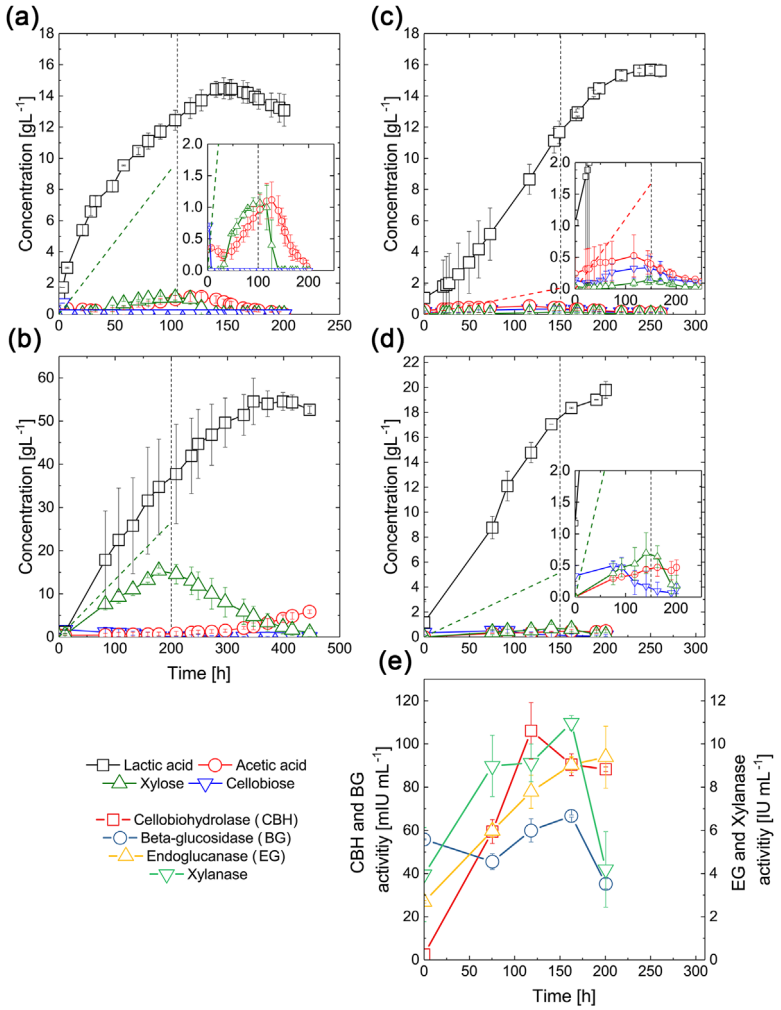

FIGURE 4 Fed-batch CBP of heterogenous substrates containing hexose and pentose to lactic acid by a consortium of *T. reesei* and *L. pentosus*. (a) Co-fermentation of 1.75% (w/w) Avicel added at $t = 0$ and xylose fed over a period of 100 hr to a total concentration of 9.32 gL^{-1}. (b) Co-fermentation of 5.0% (w/w) Avicel added at $t = 0$ and xylose fed over a period of 200 hr to a total concentration of 26.63 gL^{-1}. (c) Conversion of 3.86% (w/w) one-stage steam-pretreated beech wood solids with a feed of the prehydrolyzate over a period of 100 hr. The dashed red line shows the amount of acetic acid added through the prehydrolyzate feed. (d) Co-fermentation of 3.86% (w/w) two-stage steam-pretreated beech wood solids added at $t = 0$ and xylooligomer containing first stage prehydrolyzate fed over a period of 100 hr. The dashed green lines show the amount of xylose (A+B) or xylose recalculated from xylooligomers (d), respectively, added through the substrate feed. Error bars represent standard deviation from two independent fed-batch experiments. (e) Activity of cellobiohydrolase (CBH), beta-glucosidase (BG), endoglucanase (EG), and xylanase in the supernatant of the fermentation slurry during various stages of the fermentation shown in Figure 4d. Error bars represent the standard deviation from duplicates

prehydrolyzate—is formed by condensation of steam, which contains soluble xylooligosaccharides, acetic acid derived from deacetylation of the hemicellulose and other inhibitory substances such as formic acid, phenolics, furfural, and hydroxymethylfurfural (Jönsson & Martín, 2016; Stephanopoulos, 2007). The composition of the prehydrolyzate depends on the pretreatment conditions. When beech wood was steam-pretreated at 230 °C for 15 min (the conditions that allowed maximal glucose release by enzymatic hydrolysis with commercial cellulase cocktails, but led to complete degradation of xylan [data not shown]), the resulting slurry could not directly be converted to lactic acid by the consortium, primarily due to the high concentration of acetic acid (Supplementary Figure S6), which inhibited *T. reesei*. Thus, in order to enable growth on the inhibitory substrates and to benefit from the in situ detoxification potential of *T. reesei* at moderate acetic acid concentrations, we chose to feed the prehydrolyzate to the membrane biofilm reactor, which initially only contained the pretreated beech wood solids. As acetic acid degradation occurs at a constant rate due to being limited by the oxygen transfer rate, we chose a constant feeding profile. Under these conditions, whole-slurry pretreated beech wood could successfully be converted to lactic acid resulting in a concentration of 15.7 ± 0.2 gL^{-1} after 250 hr (Figure 4c, Table 2). This amount corresponded to a similar lactic acid yield compared to the experiment where only pretreated solids were used.

In order to investigate the co-fermentation abilities of the consortium in the presence of inhibitors, we used two-stage pretreated

beech wood as the substrate. Here, beech wood was first pretreated under moderate conditions (180 °C, 25 min) that were optimized for maximal hemicellulose recovery in the liquid phase. After removal of the liquid phase, a second pretreatment of the remaining solids was performed under harsher conditions that allowed maximal glucose release by subsequent enzymatic hydrolysis. Fed-batch experiments on two-stage pretreated beech wood were performed with a linear feed of the 180 °C prehydrolyzate which contained, among others, 25.8 gL^{-1} of soluble xylooligosaccharides. The feeding of soluble xylooligosaccharides, instead of monomeric saccharides such as xylose, changed the metabolic dependencies in the consortium.

Although putative orthologs of the xylosidase *xynB2* of *L. brevis* were found in the genome of *L. pentosus*, which has a high catalytic efficiency for xylobiose, LAB depend on the secretome of *T. reesei* for both longer xylooligomers and cellulose conversion (Michlmayr et al., 2013). The feeding of the prehydrolyzate seems to be beneficial for fungal enzyme production as it resulted in an increase of the CBH and EG activities by a factor of two (Figure 4e) compared to the experiment where only pretreated solids were used (Figure 2d). Despite the heterogeneous and metabolically challenging mixture only $0.5 \pm 0.1\ gL^{-1}$ acetic acid accumulated and $19.8 \pm 0.7\ gL^{-1}$ lactic acid was produced after 200 hr (Figure 4d, Table 2). This corresponds to a productivity of 0.1 $gL^{-1}\,h^{-1}$ (Table1) and a yield of 85.2%, which is the highest yield achieved with the synthetic microbial consortium. Future work will include the optimization of the in situ enzyme production in order to increase lactic acid productivity to a level comparable to state of the art SSF approaches (Table 1).

Taken together, the fed batch experiments showed the potential of the synthetic consortium to utilize unprocessed inhibitory process streams that are detoxified in situ without any detectable carbon loss. In contrast, significant carbon loss has been reported, when separate biological detoxification was performed (Palmqvist, Hahn-Hägerdal, Szengyel, Zacchi, & Rèczey, 1997).

2.5 | Interactions between the members of the consortium

The results presented show that the synthetic consortium of *T. reesei* and *L. pentosus* is highly functional. The stable coexistence of the two strains is mainly based on competitive cheater and cooperator interactions. Secretion of cellulases by *T. reesei* is a cooperative feature because it is metabolically costly and the sugars released by enzymatic hydrolysis are available as public goods for both strains. *L. pentosus* consumes the released soluble saccharides as a cheater without contributing energy to enzyme production. However, *T. reesei* also benefits from the presence of *L. pentosus*. *T. reesei* is deficient in beta-glucosidase production (Tangnu, Blanch, & Wilke, 1981), which leads to an accumulation of cellobiose-a potent inhibitor of endoglucanase and of cellobiohydrolases I and II (Teugjas & Väljamäe, 2013). For example, in a monoseptic cultivation of *T. reesei* growing on microcrystalline cellulose, a cellobiose concentration above 0.7 gL^{-1} was measured after 48 hr throughout the experiment (Supplementary Figure S3) which is in the inhibitory concentration range of several cellulases, including CBHI (Jalak, Kurasin, Teugjas, & Valjamae, 2012; Teugjas & Väljamäe, 2013). Upon inoculation with *L. pentosus*, the cellobiose concentration dropped below the detection limit (Supplementary Figure S3), because the LAB were able to metabolize the disaccharide (Michlmayr & Kneifel, 2014). Thus, *L. pentosus* reduces the negative product inhibition on the cellulolytic system and facilitates the faster release of glucose. Furthermore, *L. pentosus* produces acetic acid as a by-product, which can be consumed by *T. reesei* by metabolic cross feeding (commensalism). These beneficial effects of LAB for the fungi lead to microbial mutualism. Simultaneously, an inhibitory effect of acetic acid on the metabolic activity of *T. reesei* was observed (amensalism). It has been suggested that such unilateral interactions greatly enhance community stability (Mougi, 2016).

3 | CONCLUSIONS

We successfully established a consolidated bioprocess for the direct production of lactic acid from lignocellulosic biomass using a synthetic consortium of *T. reesei* and a lactic acid bacterium growing in a membrane-aerated biofilm reactor. In particular, we demonstrated the ability of the consortium to co-ferment hexoses and pentoses from non-detoxified whole-slurry pretreated beech wood without carbon catabolite repression. Furthermore, superior product purities were achieved by in situ acetic acid degradation by *T. reesei*. We applied metabolic compartmentalization and spatial structuring to stabilize the cooperator-cheater community. Our results establish the potential of engineering microbial consortia for the CBP of lignocellulosic biomass to high value biochemicals.

4 | MATERIALS AND METHODS

4.1 | Strains and media

T. reesei Rut-C30 was used as a producer of cellulolytic and hemicellulolytic enzymes. *L. pentosus* and *L. brevis* were used as lactic and acetic acid forming microorganisms. Details on media composition and the inoculation procedure can be found in the supplementary.

4.2 | Biofilm membrane reactor

Commercially available Multifors 2 (Infors HT, Switzerland) stirred-tank reactors with a working volume of 500 ml were adapted to hold a membrane. To this end, a custom-made quadrangular frame structure was installed in the middle of the reactor. A tubular, dense polydimethylsiloxane (PDMS) membrane (Mono-Lumen Tubing, ID 0.64 x OD 1.19; 50VMQ Q7-4750, Dow Corning, Midland, MI) was wrapped round the frame. The membrane area to volume ratio was set to 0.31 $cm^2\,ml^{-1}$. The membrane was continuously flushed with air (80 ml min^{-1}) controlled by a mass flow controller (red-y compact, Voegtlin Instruments AG, Switzerland). The fermentation slurry was mixed by a central rushton turbine or a custom-made helical PEEK stirrer rotating at 250 rpm. A custom-made sampling device enabled the sterile collection of samples using a UV lamp as a contamination barrier.

4.3 | Batch and fed-batch fermentations

For batch experiments the reactor containing the solid substrate and water was autoclaved for 20 min at 121 °C. The remaining medium ingredients were added and the reactor was inoculated with 5% (v/v) *T. reesei* preculture. LAB were inoculated 48 hr after fungal inoculation to reach an optical density (OD_{600}) of 0.5.

For fed-batch fermentations, all feed solutions were sterile filtered. To calculate the liquid volume of the prehydrolyzate feed, the same ratio of solids to prehydrolyzate as obtained after pretreatment was used. The linear feed was started simultaneously with the inoculation of the LAB. All fermentations were performed at a temperature of 30 °C and at a pH value of 5.0 maintained by addition of 4% (w/w) hydrochloric acid or 4 M sodium hydroxide, respectively.

4.4 | Steam pretreatment of beech wood

Air-dried beech wood chips *Fagus sylvatica* from local forests were milled to a particle size of 1.5 mm and pretreated by steam explosion with a custom-built steam gun (Industrieanlagen Planungsgesellschaft m.b.H., Austria). Two different pretreatment conditions were applied. For one-stage steam pretreatment, the biomass was heated by direct steam injection to 230 °C and the pressure was explosively released after 14.9 min. In the tested temperature range of 160–230 °C this condition was shown to allow maximum glucose yields in enzymatic hydrolysis of the solid fraction using 15 FPU/g cellulose Accellerase 1500 (Accellerase® 1500, DuPont, Wilmington, DE) at 50 °C in an orbital shaker at 150 rpm. The enzymatic hydrolysis was performed in 50 ml screw-top flasks for seven days at pH 4.8 using a 50 mM citrate buffer in the presence of 0.001% (w/v) sodium azide. One-stage pretreated beech wood solids contained 53.7 ± 0.6% (w/w) glucan and no xylan, while the prehydrolyzate contained (in gL^{-1}) 8.6 acetic acid, 2.3 formic acid and 0.8 xylose. In order to recover more xylan in the form of solubilized xylooligomers, a two-stage steam pretreatment procedure was applied. Beech wood was heated to 180 °C in the steam gun and the pressure was slowly released after a residence time of 24.8 min. After separation of the prehydrolyzate, the solid fraction was pretreated in the second stage at 230 °C for 14.1 min. The resulting solids contained 47.2 ± 0.7% (w/w) glucan and no xylan. The first stage prehydrolyzate contained (in gL^{-1}): 1.9 acetic acid, 0.5 formic acid, 0.8 xylose and 25.8 xylooligosaccharides.

4.5 | Enzyme assays

EG, BG, CBH, and xylanase activity assays were performed in 50 mM citrate buffer at pH 5.0 in the presence of 0.02% (w/v) sodium azide at 50 °C in duplicates. Enzymatic activity was expressed in international units (IU) where one unit is defined as the amount of enzyme that catalyzes the conversion of one micro-mole of substrate per minute. EG, BG, and CBH assays were performed as described previously by (Xiros & Studer, 2017). The substrates were ultra low viscosity carboxymethylcellulose (2% [w/w]) for EG, Avicel (2% [w/w]) for CBH and 4-Nitrophenyl α-D-glucopyranoside (1 mM) for BG. Xylanase activity was measured using birch wood xylan (1% [w/w]) as substrate (12.5 µL sample in 125 µL total reaction volume). Reducing sugars were quantified using the 3,5-Dinitrosalicylic acid (DNS) method (Miller, 1959).

4.6 | Analytical methods

Lactic acid, acetic acid, formic acid, glucose, xylose and cellobiose were quantified by high-performance liquid chromatography (Waters 2695 Separation Module, Waters Corporation, Milford, MA) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 65 °C with 5 mM H_2SO_4 as the mobile phase flowing at 0.6 ml min^{-1} and a refractive index detector (Waters 410) at 40 °C. The detection limit was 0.05 gL^{-1}. Structural carbohydrates, soluble xylooligosaccharides, and lignin content were analyzed according to standardized methods (Sluiter et al., 2008).

ACKNOWLEDGMENTS

This work was supported by the Swiss National Science Foundation in the framework of the National Research Program NRP 70 Energy turnaround (grant number 407040–153868). We are grateful to Robert Balan for his help on the steam pretreatment of beech wood and Charilaos Xiros for his help on the measurements of the enzyme activities.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ORCID

Robert L. Shahab http://orcid.org/0000-0002-2954-5210

REFERENCES

Abdel-Rahman, M. A., & Sonomoto, K. (2016). Opportunities to overcome the current limitations and challenges for efficient microbial production of optically pure lactic acid. *Journal of Biotechnology*, *236*, 176–192.

Abdel-Rahman, M. A., Tashiro, Y., & Sonomoto, K. (2013). Recent advances in lactic acid production by microbial fermentation processes. *Biotechnology Advances*, *31*, 877–902.

Abdel-Rahman, M. A., Tashiro, Y., Zendo, T., Hanada, K., Shibata, K., & Sonomoto, K. (2011). Efficient homofermentative L-(+)-Lactic acid production from xylose by a novel lactic acid bacterium, *Enterococcus mundtii* QU 25. *Applied and Environmental Microbiology*, *77*, 1892–1895.

Agapakis, C. M., Boyle, P. M., & Silver, P. A. (2012). Natural strategies for the spatial optimization of metabolism in synthetic biology. *Nature Chemical Biology*, *8*, 527–535.

Brethauer, S., & Studer, M. H. (2015). Biochemical conversion processes of lignocellulosic biomass to fuels and chemicals–a review. *International Journal of Chemistry*, *69*, 572–581.

Brethauer, S., & Studer, M. H. (2014). Consolidated bioprocessing of lignocellulose by a microbial consortium. *Energy Environmental Science*, *7*, 1446.

Coyte, K. Z., Schluter, J., & Foster, K. R. (2015). The ecology of the microbiome: Networks, competition, and stability. *Science*, *350*, 663–666.

Cui, F., Li, Y., & Wan, C. (2011). Lactic acid production from corn stover using mixed cultures of Lactobacillus rhamnosus and Lactobacillus brevis. *Bioresource Technology*, *102*, 1831–1836.

den Haan, R., van Rensburg, E., Rose, S. H., Görgens, J. F., & van Zyl, W. H. (2015). Progress and challenges in the engineering of non-cellulolytic microorganisms for consolidated bioprocessing. *Current Opinion in Biotechnology*, *33*, 32–38.

Geddes, C. C., Nieves, I. U., & Ingram, L. O. (2011). Advances in ethanol production. *Current Opinion in Biotechnology.*, *22*, 312–319.

Jalak, J., Kurasin, M., Teugjas, H., & Valjamae, P. (2012). Endo-exo synergism in cellulose hydrolysis revisited. *The Journal of Biological Chemistry*, *287*, 28802–28815.

Johns, N. I., Blazejewski, T., Gomes, A. L., & Wang, H. H. (2016). Principles for designing synthetic microbial communities. *Current Opinion in Microbiology*, *31*, 146–153.

Jönsson, L. J., & Martín, C. (2016). Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology*, *199*, 103–112.

Jourdier, E., Poughon, L., Larroche, C., & Ben Chaabane, F. (2013). Comprehensive Study and Modeling of Acetic Acid Effect on *Trichoderma reesei* Growth. *Industrial Biotechnology*, *9*, 132–138.

Kawaguchi, H., Hasunuma, T., Ogino, C., & Kondo, A. (2016). Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. *Current Opinion in Biotechnology*, *42*, 30–39.

Lokman, B. C., Heerikhuisen, M., Leer, R. J., van den Broek, A., Borsboom, Y., Chaillou, S., . . . Pouwels, P. H. (1997). Regulation of expression of the *Lactobacillus pentosus xylAB* operon. *Journal of Bacteriology*, *179*, 5391–5397.

Lynd, L. R., Van Zyl, W. H., McBride, J. E., & Laser, M. (2005). Consolidated bioprocessing of cellulosic biomass: An update. *Current Opinion in Biotechnology*, *16*, 577–583.

Maas, R. H. W., Bakker, R. R., Jansen, M. L. A., Visser, D., De Jong, E., Eggink, G., & Weusthuis, R. A. (2008). Lactic acid production from lime-treated wheat straw by *Bacillus coagulans*: Neutralization of acid by fed-batch addition of alkaline substrate. *Applied Microbiology and Biotechnology*, *78*, 751–758.

Mahr, K., Hillen, W., & Titgemeyer, F. (2000). Carbon catabolite repression in *Lactobacillus pentosus*: analysis of the ccpA region. *Applied and Environmental Microbiology*, *66*, 277–283.

Michlmayr, H., Hell, J., Lorenz, C., Bohmdorfer, S., Rosenau, T., & Kneifel, W. (2013). Arabinoxylan oligosaccharide hydrolysis by family 43 and 51 glycosidases from *Lactobacillus brevis* DSM 20054. *Applied and Environmental Microbiology*, *79*, 6747–6754.

Michlmayr, H., & Kneifel, W. (2014). β-Glucosidase activities of lactic acid bacteria: Mechanisms, impact on fermented food and human health. *FEMS Microbiology Letters*, *352*, 1–10.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing Sugar. *Analytical Chemistry*, *31*, 426–428.

Minty, J. J., Singer, M. E., Scholz, S. A., Bae, C.-H., Ahn, J.-H., Foster, C. E., . . . Lin, X. N. (2013). Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. *Proceedings of the National Academy of Sciences of the United States of America*, *110*, 14592–14597.

Mougi, A. (2016). The roles of amensalistic and commensalistic interactions in large ecological network stability. *Scientific Reports*, *6*, 29929.

Nadell, C. D., Drescher, K., & Foster, K. R. (2016). Spatial structure, cooperation and competition in biofilms. *Nature Reviews Microbiology*, *14*, 589–600.

Ou, M. S., Ingram, L. O., & Shanmugam, K. T. (2011). L(+)-Lactic acid production from non-food carbohydrates by thermotolerant *Bacillus coagulans*. *Journal of Industrial Microbiology & Biotechnology*, *38*, 599–605.

Palmqvist, E., Hahn-Hägerdal, B., Szengyel, Z., Zacchi, G., & Rèczey, K. (1997). Simultaneous detoxification and enzyme production of hemicellulose hydrolysates obtained after steam pretreatment. *Enzyme and Microbial Technology*, *20*, 286–293.

Peng, X. N., Gilmore, S. P., & O'Malley, M. A. (2016). Microbial communities for bioprocessing: Lessons learned from nature. *Current Opinion in Chemical Engineering*, *14*, 103–109.

van der Pol, E. C., Eggink, G., & Weusthuis, R. A. (2016). Production of l(+)-lactic acid from acid pretreated sugarcane bagasse using Bacillus coagulans DSM2314 in a simultaneous saccharification and fermentation strategy. *Biotechnology for Biofuels*, *9*, 248.

Rahikainen, J. L., Evans, J. D., Mikander, S., Kalliola, A., Puranen, T., Tamminen, T., . . . Kruus, K. (2013). Cellulase-lignin interactions-The role of carbohydrate-binding module and pH in non-productive binding. *Enzyme and Microbial Technology*, *53*, 315–321.

Romaní, A., Yáñez, R., Garrote, G., & Alonso, J. L. (2008). SSF production of lactic acid from cellulosic biosludges. *Bioresource Technology*, *99*, 4247–4254.

Sharma, A., Tewari, R., Rana, S. S., Soni, R., & Soni, S. K. (2016). Cellulases: Classification, methods of determination and industrial applications. *Applied Biochemistry and Biotechnology*, *179*, 1346–1380.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). Laboratory Analytical Procedure (LAP). Determination of structural carbohydrates and lignin in biomass. *Technical Report*, http://www.nrel.gov/biomass/analytical_procedures.html.

Stephanopoulos, G. (2007). Challenges in engineering microbes for biofuels production. *Science*, *315*, 801–804.

Tangnu, S. K., Blanch, H. W., & Wilke, C. R. (1981). Enhanced production of cellulase, hemicellulase, and beta-glucosidase by *Trichoderma reesei* (Rut C-30). *Biotechnology and Bioengineering*, *23*, 1837–1849.

Teugjas, H., & Väljamäe, P. (2013). Product inhibition of cellulases studied with ^{14}C-labeled cellulose substrates. *Biotechnology for Biofuels*, *6*, 104.

Tian, X., Zhang, N., Yang, Y., Wang, Y., Chu, J., Zhuang, Y., & Zhang, S. (2015). The effect of redox environment on l-lactic acid production by *Lactobacillus paracasei*–A proof by genetically encoded in vivo NADH biosensor. *Process Biochemistry*, *50*, 2029–2034.

Wen, Z., Wu, M., Lin, Y., Yang, L., Lin, J., & Cen, P. (2014). Artificial symbiosis for acetone-butanol-ethanol (ABE) fermentation from alkali extracted deshelled corn cobs by co-culture of *Clostridium beijerinckii* and *Clostridium cellulovorans*. *Microbial Cell Factories*, *13*, 92.

Xiros, C., & Studer, M. H. (2017). A multispecies fungal biofilm approach to enhance the celluloyltic efficiency of membrane reactors for consolidated bioprocessing of plant biomass. *Frontiers in Microbiology*, *8*, 1–14.

Xu, L., & Tschirner, U. (2011). Improved ethanol production from various carbohydrates through anaerobic thermophilic co-culture. *Bioresource Technology*, *102*, 10065–10071.

Zuroff, T. R., Barri Xiques, S., & Curtis, W. R. (2013). Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic *Clostridium phytofermentans*/yeast co-culture. *Biotechnology for Biofuels*, *6*, 59.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Shahab RL, Luterbacher JS, Brethauer S, Studer MH. Consolidated bioprocessing of lignocellulosic biomass to lactic acid by a synthetic fungal-bacterial consortium. *Biotechnology and Bioengineering*. 2018;115:1207–1215. https://doi.org/10.1002/bit.26541