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Co-valorization of paper mill sludge and corn steep liquor for enhanced *n*-butanol production with *Clostridium tyrobutyricum* Δ*cat1::adhE2*

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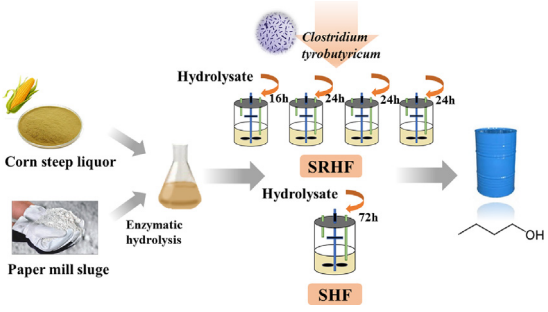
GRAPHICAL ABSTRACT



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ABSTRACT

In this study, hyper-butanol producing *Clostridium tyrobutyricum* Δ*cat1::adhE2* was used for butanol production from paper mill sludge (PMS) and corn steep liquor (CSL). Our results demonstrated that CSL can not only serve as a cheap nitrogen source, but also provide lactic acid that can be assimilated by *C. tyrobutyricum* for enhanced butanol production. Through a separate hydrolysis and fermentation, 16.5 g/L butanol with a yield of 0.26 g/g was obtained from PMS hydrolysates supplemented with 5% CSL. Further, a separate repeated hydrolysis was conducted to improve PMS hydrolysis rate and enhance sugar yield. Fermentation using hydrolysates from such process also generated high-level butanol with high yield. Our results suggested an innovative bioprocess for efficient biobutanol production from low-value waste streams.

1. Introduction

n-Butanol (butanol hereafter) is an important platform chemical with a wide spectrum of applications, such as being used as a solvent, precursor for paints and polymers, and biofuel. Especially, butanol as a fuel additive has gained great interests in recent years due to its high energy content, low emissions and good compatibility with engine (Dürre, 2007; Lee et al., 2008; Pospíšil et al., 2014). Butanol has been

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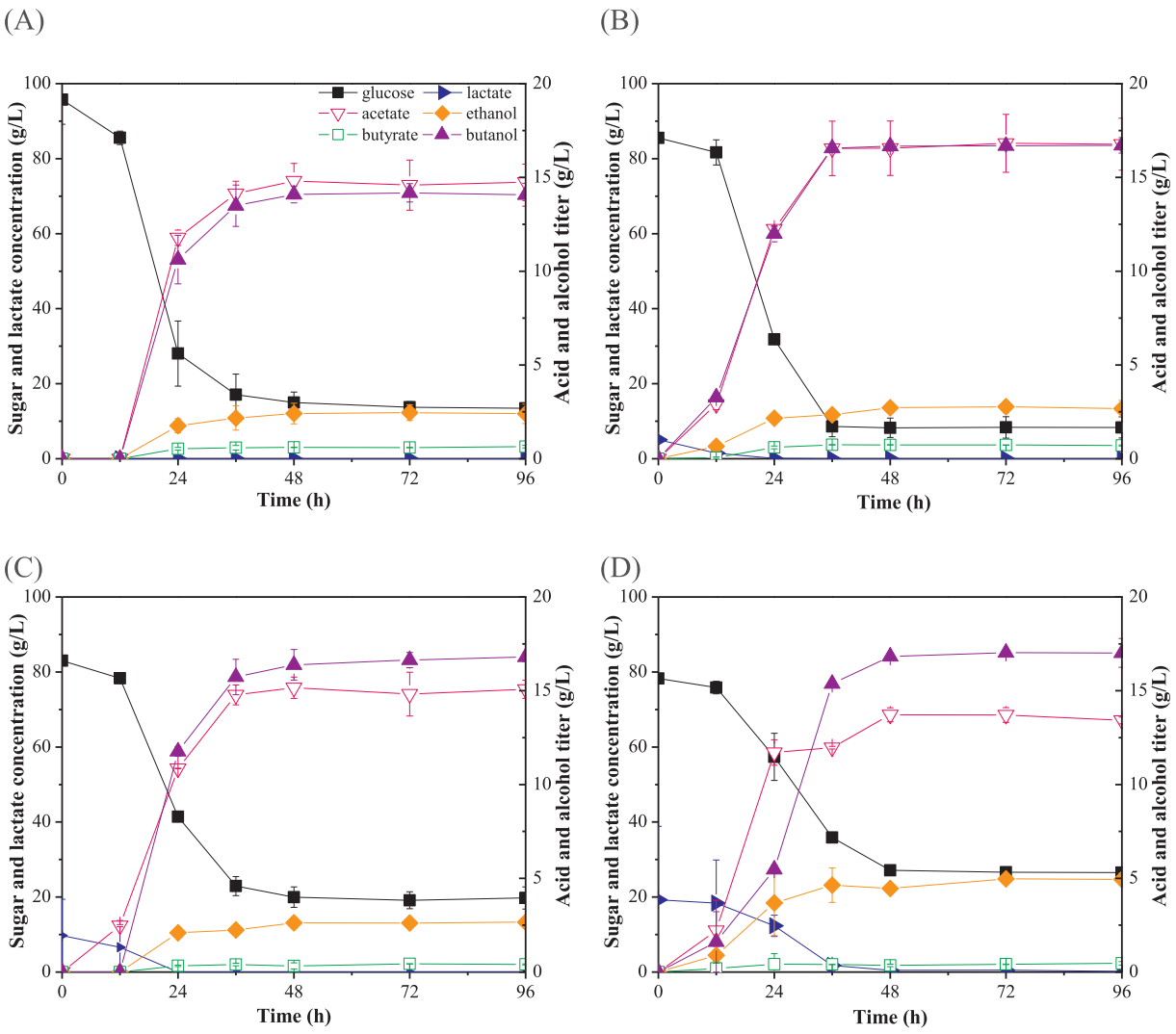

Fig. 1. Effects of lactic acid (LA) supplementation on butanol production in batch fermentation with *C. tyrobutyricum* Δ*cat1::adhE2*. (A) 0 g/L LA (glucose as the sole carbon source); (B) 5 g/L LA; (C) 10 g/L LA; (D) 20 g/L LA.

traditionally produced from petroleum resources via hydrogenation of butanal derived from hydroformylation of propene, but the escalating depletion of petroleum conservation has driven people for the exploration of more renewable and sustainable pathways for butanol production. The conventional acetone, butanol, ethanol (ABE) fermentation with lignocellulosic biomass derived sugars as carbon sources is a well-known biological route for renewable production of butanol. Although ABE process has the great potential to be successfully deployed at industrial scale, many challenges including low solvent titer, yield, productivity and high feedstock cost, remain to be overcome (Jang et al., 2012; Jones & Woods, 1986; Wang & Chen, 2011). Metabolic engineering of butanol-producing strains using different strategies, including chemical mutagenesis, adaptive evolution, and gene deletion, has been explored in the past several decades. Although decent progresses have been achieved, overall this is still a very slow process (Formanek et al., 1997; Fu et al., 2017; Xue et al., 2012). Recently, the advent of advanced genome-editing technology has enabled versatile genome engineering purposes in butanol-producing clostridial strains, and in turn, improved their performance for butanol production at a fast pace (Ahn et al., 2011; Yu et al., 2015). Recently, our group have developed a hyper-butanol producing *Clostridium tyrobutyricum* Δ*cat1::adhE2* strain through genome engineering using the native CRISPR-Cas system in the host, with which a record high butanol production of 26.2 g/L was achieved in a batch fermentation (Zhang et al., 2018). Although this strain showed great potential for high-level butanol production, it is still necessary to lower the overall cost associated with butanol production from the bioprocess aspects.

Lignocellulosic biomass is an abundant and inexpensive feedstock for biofuel production. However, due to its intrinsic recalcitrance, severe pretreatment is often needed, which accounts for a large portion of the process cost (Mosier et al., 2005). Paper mill sludge (PMS) is a solid by-product produced in the pulp and paper industry. The US Pulp and paper industry produces ~8.5 million dry tons/year of PMS (American Forest & Paper Association, 2010). Traditional landfill for PMS is becoming increasingly difficult and costly to construct and operate because of more stringent regulations, diminishing land availability, and public opposition. On the other hand, PMS can be used as an attractive feedstock for bioproducts production. Being a waste, it carries zero or, in most cases, negative cost of $20–50/ton (the disposal cost) (John et al., 2007). In addition, PMS has low content of lignin but a high content of cellulose, which can be hydrolyzed facilely into glucose without any pretreatment (Ioelovich, 2014), avoiding one major cost in bioconversion of lignocellulosic material. Given the high cost for disposing PMS and meanwhile the demand for low-cost feedstock for biobutanol production, valorization of PMS into butanol provides a 'two birds with one stone' strategy by addressing two key issues relevant to pulp and paper industry and biochemical production process.

While using PMS as the feedstock for butanol production could

Table 1
Summary of butanol production results with *C. tyrobutyricum* Δ*cat1*::*adhE2* using different carbon resources and nitrogen sources.

Carbon source	Nitrogen source	Sugar utilized (g/L)	LA utilized[a] (g/L)	Acetate (g/L)	Ethanol (g/L)	Butyrate (g/L)	Butanol (g/L)	Butanol yield		Butanol productivity (g/L/h)
								g/g	C-mol/C-mol	
Glucose	YTN[b]	78.6 ± 1.1	–	14.8 ± 0.9	2.4 ± 0.4	0.6 ± 0.0	14.1 ± 0.4	0.17	0.28	0.29
Glucose + 5 g/LA	YTN	76.9 ± 3.3	5.1 ± 0.0	16.5 ± 1.4	2.7 ± 0.3	0.7 ± 0.0	16.6 ± 0.2	0.20	0.33	0.46
Glucose + 10 g/LA	YTN	63.0 ± 5.1	9.7 ± 0.0	15.5 ± 0.6	2.6 ± 0.3	0.3 ± 0.1	16.4 ± 0.8	0.23	0.37	0.34
Glucose + 20 g/LA	YTN	51.1 ± 1.8	19.0 ± 0.3	13.7 ± 0.4	4.5 ± 0.1	0.4 ± 0.1	16.8 ± 0.2	0.24	0.39	0.35
Glucose	2.5% CSL[c]	66.3 ± 1.6	3.0 ± 0.1	10.3 ± 0.3	1.3 ± 0.0	2.5 ± 0.4	14.4 ± 0.1	0.21	0.34	0.30
Glucose	5% CSL	70.0 ± 0.6	5.9 ± 0.1	14.4 ± 0.8	1.4 ± 0.1	1.0 ± 0.0	16.1 ± 0.0	0.21	0.34	0.45
Glucose	10% CSL	58.9 ± 0.3	12.2 ± 0.8	13.6 ± 0.2	1.8 ± 0.1	0.2 ± 0.1	16.3 ± 0.1	0.23	0.37	0.45
PMS hydrolysates[d]	5% CSL	64.1 ± 1.0	7.1 ± 0.2	19.8 ± 0.6	1.5 ± 0.2	0.5 ± 0.2	16.5 ± 0.2	0.26	0.38	0.34
PMS hydrolysates-1st cycle of SRHF[e]	5% CSL	67.1 ± 4.7	6.3 ± 0.2	11.6 ± 0.6	0.8 ± 0.2	0.3 ± 0.1	18.1 ± 0.4	0.25	0.40	0.50
PMS hydrolysates-2nd cycle of SRHF	5% CSL	66.6 ± 0.1	6.4 ± 0.1	12.1 ± 0.3	0.9 ± 0.0	1.4 ± 0.0	16.4 ± 0.4	0.22	0.36	0.44
PMS hydrolysate-3rd cycle of SRHF	5% CSL	66.9 ± 0.0	7.1 ± 0.0	12.6 ± 0.2	0.6 ± 0.0	1.2 ± 0.2	16.2 ± 0.3	0.22	0.35	0.45
PMS hydrolysate-4th cycle of SRHF	5% CSL	64.6 ± 0.3	6.4 ± 0.1	12.4 ± 0.1	0.7 ± 0.0	1.2 ± 0.1	16.0 ± 0.3	0.23	0.37	0.45

[a] LA: lactic acid
[b] Yeast extract (5 g/L) + Tryptone (5 g/L) + $(NH_4)_2SO_4$ (3 g/L).
[c] CSL: corn steep liquor.
[d] PMS: paper mill sludge.
[e] SRHF: separate repeated hydrolysis and fermentation.

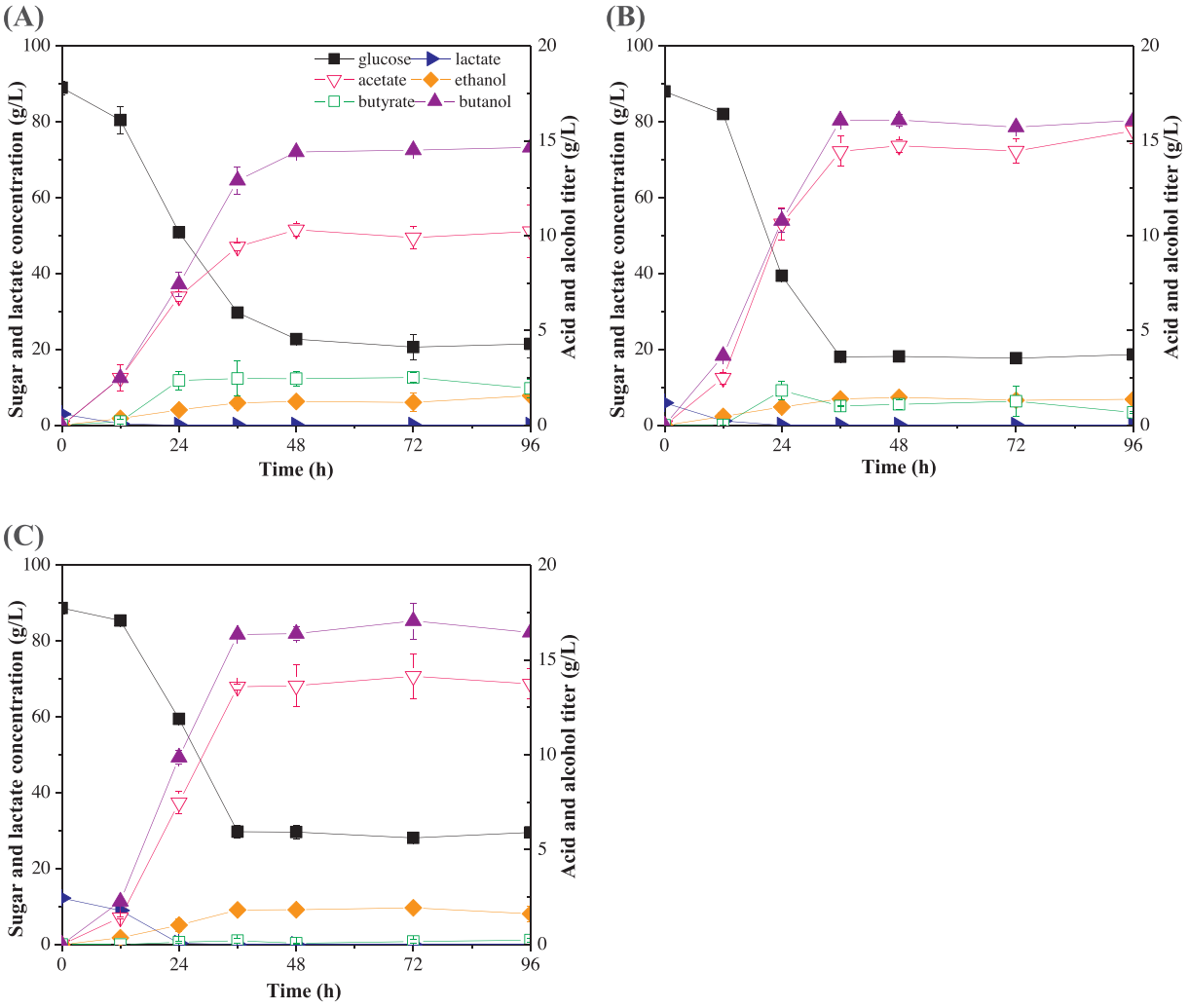

Fig. 2. Effects of different levels of corn steep liquor (CSL) on butanol production. (A) 2.5% (w/v) CSL; (B) 5% (w/v) CSL; and (C)10% (w/v) CSL.

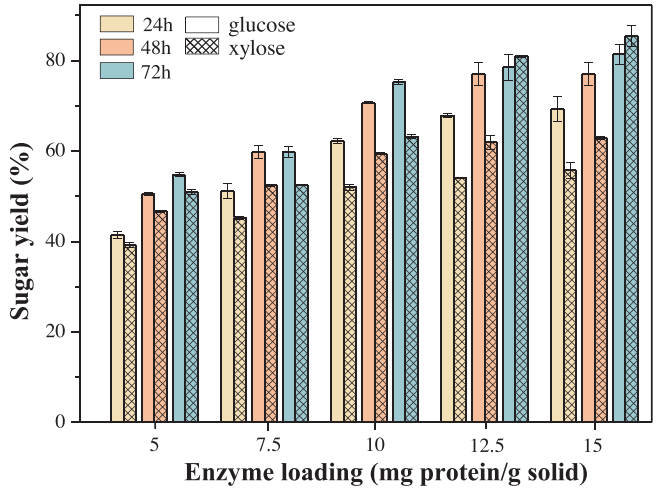

Fig. 3. Enzymatic hydrolysis of paper mill sludge at different enzyme loadings (5, 7.5, 10, 12.5 and 15 mg protein/g solid) with 12% (w/v) solid loading.

potentially reduce the cost of carbon sources, the cost of nutrient sources for the fermentation is still significant. Corn steep liquor (CSL) is a by-product from the corn starch production process (Sharma et al., 2013). Due to its high content of amino acids and other nutrients, it could serve as an excellent nitrogen source for the fermentation to replace the expensive counterparts (such as yeast extract and tryptone) (Mirza & Mushtaq, 2006). Moreover, CSL also contains a high level of lactic acid (LA) (Gao and Yuan, 2011). Previous studies have demonstrated that LA could be assimilated by other clostridial strains to enhance butanol production (Ahn et al., 2011; Zhang et al., 2018). However, it remains un-elucidated whether LA can play the same role in *C. tyrobutyricum*. It is noticed that *C. tyrobutyricum* harbors multiple lactate dehydrogenase genes in the genome (Lee et al., 2016). We thus hypothesized that this pathway could be possibly harnessed for LA assimilation (besides LA production) to improve butanol production in *C. tyrobutyricum* Δ*cat1::adhE2*.

Therefore, in this study, we first investigated the capability of *C. tyrobutyricum* for assimilating LA and the effects of such metabolism on butanol production. Furthermore, PMS and CSL were evaluated as carbon source and nitrogen source (meanwhile as an additional LA supplementation) respectively for efficient butanol production using *C. tyrobutyricum* Δ*cat1::adhE2*. The loading of CSL was first optimized. Then both separate hydrolysis and fermentation (SHF) and separate repeated hydrolysis and fermentation (SRHF) were employed for butanol production from PMS hydrolysates supplemented with CSL. Overall, we demonstrated an innovative bioprocess for efficient high-level butanol production through co-valorization of two significant waste streams.

2. Materials and methods

2.1. Feedstock and bacterial strain

The PMS was from the tissue production process from a paper mill in USA. The PMS contains 65.3 ± 1.7% glucan, 13.5 ± 1.2% xylan, 0.9 ± 0.2% galactan, 0.5 ± 0.2% arabinan, 2.2 ± 0.2% mannan, 11.9 ± 0.0% lignin and 5.8 ± 0.1% ash. Cellulase (Novozymes® Ctec 2) and CSL were purchased from Sigma-Aldrich (St. Louis, MO, USA). The protein concentration in Ctec 2 is 150 mg/mL. CSL contains 14.8% lactic acid and 7–8% (w/w) nitrogen. The types of nitrogen were in the form of amino acids and polypeptides. *C. tyrobutyricum* Δ*cat1::adhE2* used for butanol production was constructed as reported in our previous study (Zhang et al., 2018).

2.2. Enzymatic hydrolysis of PMS

Enzymatic hydrolysis of PMS was conducted in sodium acetate buffer (pH 5.0) with a solid loading of 12% (w/v) at 50 °C, 200 rpm for 72 h. The enzyme loading was optimized by varying the enzyme concentration from 5 to 15 mg protein/g solid. The hydrolysates generated for butanol fermentation (see below) was prepared following the same procedure as above but using the optimized enzyme loading.

2.3. Batch fermentations

All the batch fermentations were carried out in 500 ml bioreactors (GS-MFC, Shanghai Gu Xin Biological Technology Co., Shanghai, China) with a working volume of 250 ml. The basal medium for the fermentation was comprised (g/L): K_2HPO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.6; $FeSO_4{\cdot}7H_2O$, 0.03 (Zhang et al., 2017). For batch fermentations, either glucose (100 g/L) or PMS hydrolysates was used as the carbon source, while the nitrogen source was either the combination of yeast extract (5 g/L), tryptone (5 g/L) and $(NH_4)_2SO_4$ (3 g/L) or CSL. The fermentation medium was prepared and autoclaved at 121 °C for 15 min. In order to generate an anaerobic condition, the fermentation medium was thoroughly flushed with high purity nitrogen prior to the inoculation until the fermentation culture started to generate its own gases. For all the fermentations, the preculture was prepared by growing the strain in Tryptone-Glucose-Yeast (TGY) medium (Wang et al., 2013), at 37 °C in an anaerobic chamber. When the OD_{600} reached ~1.0, the active seed culture would be inoculated into the bioreactor at a ratio of 5% (v/v) to initiate the fermentation. Fermentations were carried out at 37 °C with pH controlled ≥6.0 using 4 M NaOH.

To investigate the effects of LA on butanol production, LA with an initial concentration of 5, 10, or 20 g/L was added into the fermentation medium. In order to completely replace the combination of yeast extract, tryptone and $(NH_4)_2SO_4$ with CSL as an inexpensive nitrogen source while do not compromise butanol production, the loading of CSL was optimized by testing the CSL concentration at levels of 2.5%, 5% or 10% (w/v) for the fermentation. All other fermentation parameters were kept the same as described above.

2.4. Separate repeated hydrolysis and fermentation (SRHF)

In the process of separation and repeated hydrolysis, there are four cycles. In the first cycle (Cycle 1), PMS (a loading of 12%, w/v) was hydrolyzed with 25 mg protein/g solid for 16 h at 200 rpm and 50 °C. The solid and liquid were then separated by centrifugation. The liquid portion was saved for fermentation, while the solid residue was mixed with the fresh PMS (12% w/v) and enzyme (8.3 mg protein/g fresh solid), and hydrolyzed at 50 °C and 200 rpm for 24 h. For the following two cycles (Cycles 3–4), the same procedure was employed as that for Cycle 2.

2.5. Analytical methods

Concentrations of sugars, butanol and other endproducts from the fermentation were determined using an HPLC (Agilent 1260 series, Agilent Technologies, Santa Clara, CA, USA) equipped with an HPX-87H column (Bio-Rad, Hercules, CA, USA) and refractive index detector. 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 ml/min.

3. Results and discussion

3.1. Effects of LA supplementation on butanol production

To investigate effects of LA on butanol fermentation, batch fermentation was carried out with *C. tyrobutyricum* Δ*cat1::adhE2* using glucose as the carbon source supplemented with various concentrations

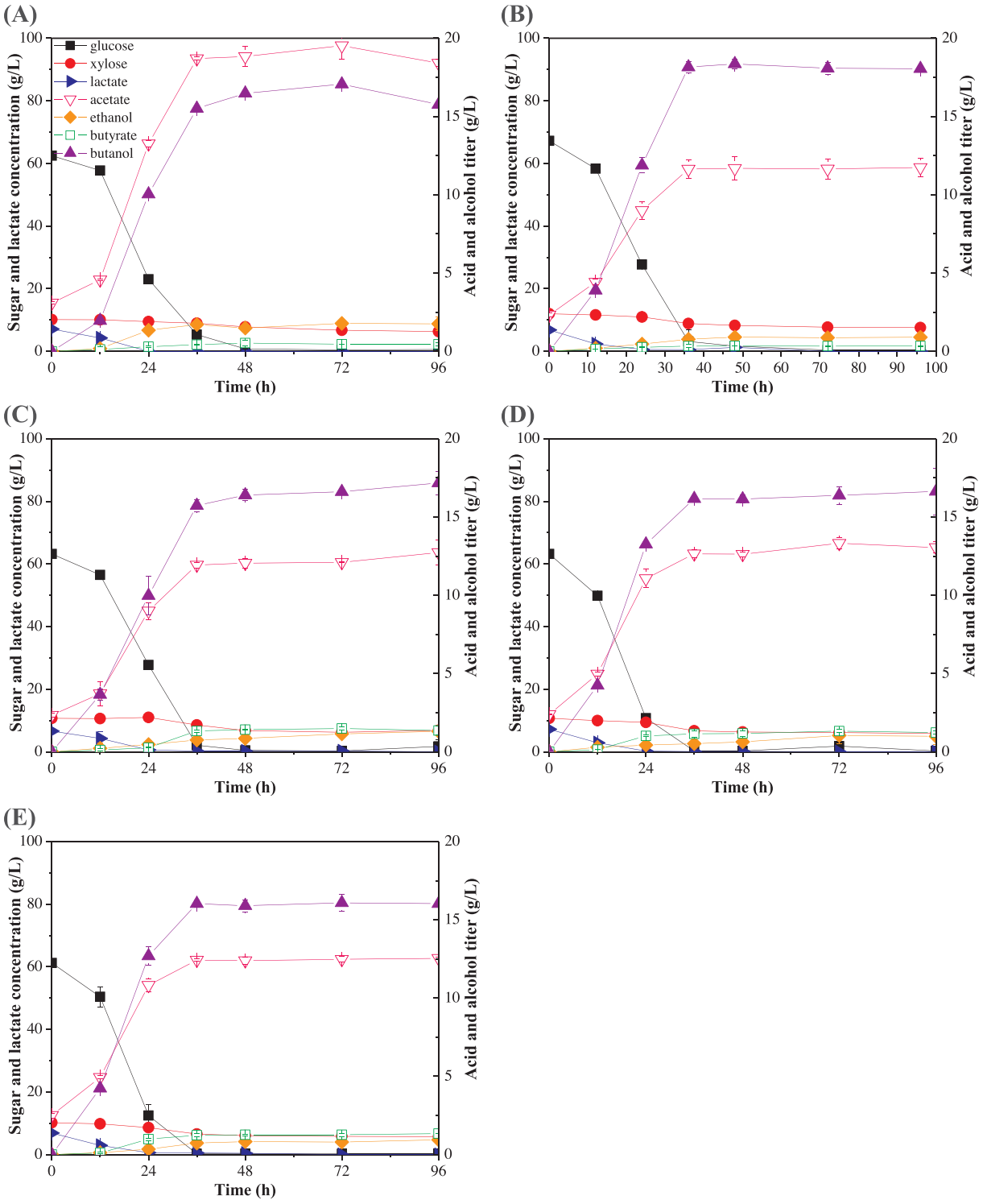

Fig. 4. Butanol fermentation with paper mill sludge (PMS) hydrolysates supplemented with 5% (w/v) CSL. (A) hydrolysates from the single batch enzymatic hydrolysis (SHF); (B) hydrolysates from the first cycle of separate repeated hydrolysis and fermentation (SRHF); (C) hydrolysates from the second cycle of SRHF; (D) hydrolysates from the third cycle of SRHF; (E) hydrolysates from the fourth cycle of SRHF.

of LA (5, 10, and 20 g/L). As shown in Fig. 1, all the supplemented LA was quickly assimilated (within 24 h for 5 or 10 g/L, and 48 h for 20 g/L). Compared to the control (without LA supplementation), the LA addition at levels of 5, 10, or 20 g/L all increased the butanol titer by about 20% (reached ~17.0 g/L vs 14.1 g/L in the control) (Fig. 1 and Table 1). Along with the production of butanol, low level of ethanol has also been produced, at 2.7 and 2.6 g/L when 5 or 10 g/L LA was supplemented respectively which was comparable to that in the control. While when 20 g/L LA was supplemented, the ethanol production reached 4.5 g/L (Fig. 1**D**). In addition, under all conditions, high-level of acetate was generated, which was a normal feature for *C. tyrobutyricum* Δ*cat1::adhE2* as we described previously (Zhang et al., 2018). In the control, the acetate production was even higher than butanol. While with the supplementation of 5 g/L LA, acetate production was at about the same level as butanol (Fig. 1**B** and Table 1). With the further increase of LA, the production of acetate became lower than

butanol (Fig. 1**C and D**). Overall, the production of butyrate was very low in all the fermentations (less than 0.8 g/L). This is because the natural butyrate production pathway has been blocked in *C. tyrobutyricum* Δ*cat1::adhE2*, and the negligible level of butyrate could have been produced from some unspecific pathways (Zhang et al., 2018).

Interestingly, when LA was supplemented, the glucose consumption was decreased in all the fermentations (76.9, 63.0 and 51.1 g/L glucose was consumed when 5, 10 and 20 g/L LA was supplemented while 78.6 g/L glucose was utilized in the control; Table 1). When LA was provided, additional carbon source (besides glucose) was assimilated for butanol production. While ~17 g/L butanol by the end of the fermentation is probably approximate to the maximum level that *C. tyrobutyricum* Δ*cat1::adhE2* could produce in a batch fermentation due to the limited tolerance to butanol of the cells. Therefore, more glucose was left unconsumed when high levels of LA were supplemented. With LA supplementation, the C-mol yield of butanol production from the substrates (considering both glucose and LA) increased by 10.0% (with 5 g/L LA), 19.0% (with 10 g/L LA) and 38.1% (with 20 g/L LA), respectively, compared to the control (Table 1). These results were in consistence with those from previous studies, which demonstrated that LA supplementation plays a positive role in enhancing butanol production for clostridia (Ahn et al., 2011; Oshiro et al., 2010; Yoshida et al., 2012; Zhang et al., 2018). Based on a kinetic modeling approach, Zhou et al. (2018) demonstrated that LA addition into butanol fermentation by *C. saccharoperbutylacetonicum* could increase the conversion rate of LA to pyruvate, as well as glyceraldehyde-3-P to pyruvate. Due to the enhancement of these two pathways, more NADH and ATP would be available for butanol production in *C. saccharoperbutylacetonicum*. It is well known that elevated levels of intracellular NADH and ATP would benefit butanol production in solventogenic clostridia (Meyer & Papoutsakis, 1989). In *C. tyrobutyricum* Δ*cat1::adhE2*, LA could be assimilated and converted into pyruvate by lactate dehydrogenase, during which 1 molecule NADH (per molecule of LA) will be generated (Diez-Gonzalez et al., 1995; Wang et al., 2013). Thus, butanol production would be enhanced. However, it should be noticed that when up to 20 g/L LA was added, glucose uptake rate and butanol production rate were both slightly decreased (compared to the control or when lower LA was supplemented; Fig. 1**D**), suggesting the inhibitory effects of LA on *C. tyrobutyricum*. Despite this, this strain is still much more robust and more tolerant to carboxylic acids than other butanol-producing strains, such as *C. saccharoperbutylacetonicum*. It has been reported that no cell growth was observed when 7.5 g/L LA was added to a batch fermentation for butanol production with *C. saccharoperbutylacetonicum* (Zhang et al., 2018).

3.2. Effects of CSL on butanol production

Although LA showed very positive effects on butanol production, supplementation of pure LA to the medium would inevitably increase the process cost. Thus, it would be desirable to find an inexpensive and easily available LA source for butanol fermentation. CSL is a by-product from corn starch production, and often contains a remarkable amount of LA due to fermentation by lactic-acid-producing organisms during the processing and storage stages (Noro et al., 2004; Xi et al., 2013). Moreover, CSL could serve as a cheap alternative nitrogen source to replace the expensive counterparts; it has been estimated that the cost of nitrogen source (yeast extract and tryptone) for commercial biofuel production could account for up to 50% of the fermentation medium cost (Edwinoliver et al., 2009). In this study, we evaluated the effects of various levels of CSL (2.5%, 5% and 10%, w/v) replacing other nitrogen sources (i.e., yeast extract, tryptone, and $(NH_4)_2SO_4$) on butanol fermentation with *C. tyrobutyricum* Δ*cat1::adhE2*.

As shown in Fig. 2**A**, With 2.5% (w/v) CSL, 14.4 g/L butanol was produced after 48 h of fermentation, which was comparable to the control in which the combination of yeast extract (5 g/L), tryptone (5 g/L) and $(NH_4)_2SO_4$ (3 g/L) was used as the nitrogen source. However, within the fermentation, less glucose was consumed (66.3 g/L vs. 78.6 g/L in the control), leading to decreased acetate production (10.3 g/L vs 14.8 g/L in the control). This is because the LA in CSL has been supplemented as the carbon source (besides glucose) for butanol production. Therefore, this led to elevated butanol yield (g/g) by 23.5% compared to the control (Table 1). When CSL was further increased to 5% or 10%, the butanol production was elevated to 16.1 g/L and 16.3 g/L, respectively, and the fermentation could be completed within 36 h (12 h shorter than the fermentation with 2.5% CSL or the control), leading to a high butanol productivity of 0.45 g/L/h (Table 1). Overall, these results demonstrated that CSL could serve as an inexpensive nitrogen source and meanwhile supplement LA for enhanced butanol production (titer, yield and productivity) with *C. tyrobutyricum*. Given that 5% and 10% CSL supplementation led to roughly similar butanol production performance, 5% CSL was used for the fermentation in the following steps.

3.3. Butanol production from PMS hydrolysates

In order to further decrease the cost of biobutanol production, we investigated the use of PMS as a feedstock for fermentation with *C. tyrobutyricum* Δ*cat1::adhE2*. First of all, we optimized the enzyme dosages for the sugar yield from PMS hydrolysis. With a solid loading of 12% (w/v), various enzyme dosages (5, 7.5, 10, 12.5, and 15 mg protein/g solid) were applied. As shown in Fig. 3, at 24 h, glucose yield was 41.5% for 5 mg protein/g solid, and was boosted to 67.8% and 69.3% when the enzyme loading was raised to 12.5 and 15 mg protein/g solid, respectively. The xylose yield followed a similar trend where a yield of 39.2% was obtained for 5 mg protein/g solid, and increased to 55.8% for 15 mg protein/g solid. Generally, with the extension of the hydrolysis time, the sugar yield (for both glucose and xylose) was further improved. At 72 h, with an enzyme loading of 12.5 and 15 mg protein/g solid, similar glucose yields (78.5% and 81.3% respectively) were obtained, which were much higher than those with lower enzyme loadings (Fig. 3). Therefore, we decided to use a loading of 12.5 mg protein/g solid for the enzymatic hydrolysis of PMS for the following fermentation process. With this optimized enzyme loading, hydrolysates containing 72.1 g/L glucose and 12.5 g/L xylose was obtained. It is noteworthy that the glucose concentration in the hydrolysates is much higher than xylose, which is highly suitable for butanol fermentation with *C. tyrobutyricum* Δ*cat1::adhE2* because glucose is a more preferable carbon source for this strain (Fu et al., 2017; Yu et al., 2015).

Fermentation was conducted with the PMS derived hydrolysates supplemented with 5% (w/v) CSL and other mineral salts. After 48 h of fermentation, the glucose in the medium was nearly completely depleted, and the butanol titer reached 16.8 g/L (Fig. 4**A**). Compared with glucose, xylose was consumed at a much lower rate, and only 50% of xylose was consumed by the end of the fermentation. In comparison to the fermentation with pure glucose as the substrate (along with 5% CSL; Fig. 2**B**), fermentation with PMS hydrolysates showed a better performance with an 11.8% increase in the C-mol/C-mol yield of butanol (Table 1). Overall, our results suggested that PMS hydrolysates is a desirable carbon source for biobutanol production with *C. tyrobutyricum* Δ*cat1::adhE2*. However, it was meanwhile observed that the capability of *C. tyrobutyricum* Δ*cat1::adhE2* for xylose consumption is rather limited (Fig. 4). This should be further enhanced through genome engineering strategies. Relevant work is currently underway in our lab.

Although decent butanol production was obtained using the separated hydrolysis and fermentation (SHF) strategy with PMS as the feedstock, the overall cost of the process is still high. It has been reported by many researchers that, after enzymatic hydrolysis of the lignocellulosic biomass, a large portion of cellulase would bind to the solid residue which could be recycled and reused (Jin et al., 2012; Yuan et al., 2018). By recycling the enzyme on solid residue into a new enzymatic hydrolysis cycle, the time needed for the enzymatic hydrolysis for the new cycle could be shortened while similar sugar yield (to that

from the regular single batch enzymatic hydrolysis process) could still be achieved. Thus, in this study, we further implemented such a separate repeated hydrolysis and fermentation (SRHF) strategy for butanol production from PMS hydrolysates. The whole process has been designed for four cycles. Based on the enzyme loading of 12.5 mg protein/g solid as we determined above, a total enzyme loading of 50 mg protein/g solid (corresponding to the amount of solid in one cycle; it is thus equivalent to 12.5 mg protein/g solid in each cycle for totally four cycles) would be needed. We designed to add 50% of this total loading (that is, 25 mg protein/g solid) for the first cycle of enzymatic hydrolysis, and then recycled the enzyme (along with the solid residue to which the cellulase enzyme bound) to be used for the next cycle (Cycle 2) in which 8.3 mg protein/g solid additional enzyme and the same solid loading (12%) of PMS as the previous cycle (Cycle 1) were added. The same procedure was employed for the Cycle 3 and Cycle 4 (see Section 2.4). With such a procedure, with a high loading of enzyme for the first cycle, high yields of glucose and xylose of 81.9% and 68.0% respectively were obtained within 16 h of hydrolysis. Meanwhile, slightly higher levels of sugars (74.7 g/L glucose and 13.2 g/L xylose) than that were obtained in a regular single batch enzymatic hydrolysis process as described above could be generated. For the following cycles, comparable concentrations and yields (as Cycle 1) of glucose and xylose could be achieved, although a slightly longer time (24 h) was needed for the hydrolysis. However, overall, the time needed for preparing the hydrolysates was much shorter compared with the single batch enzymatic hydrolysis (16 h or 24 h vs 72 h), which could greatly reduce the cost associated with energy consumption and operation.

The hydrolysates derived from each cycle was used for butanol fermentation with *C. tyrobutyricum* Δ*cat1::adhE2*. Not surprisingly, comparable levels (16.0–18.1 g/L) along with similar yields (0.22 ~ 0.26 g/g) of butanol were obtained as compared to the SHF process (Fig. 4**B–E** and Table 1).

3.4. Comparison of results from this study with that from previous studies

Butanol production with lignocellulosic biomass as feedstock has been investigated extensively by many research groups. We compared the results from this study to those from the previous literatures. Guan et al. (2016) reported that 9.7 g/L butanol (with a yield of 0.13 g/g and a productivity of 0.10 g/L/h) could be produced with *C. acetobutylicum* using PMS as the feedstock. In another study, 16.1 g/L butanol was produced with the engineered *C. tyrobutyricum* Δ*ack-adhE2* strain from cassava bagasse (Huang et al., 2019). The same strain could produce 15.8 g/L butanol from cotton stalk hydrolysates (Li et al., 2019). In a report using switchgrass as the feedstock, 14.5 g/L butanol was produced in *C. beijerinckii* P260 (Qureshi et al., 2010). Based on such comparison, to the best of our knowledge, our strain in this study could produce the highest level of butanol (16.8 g/L) as well as the highest butanol productivity (0.45 g/L/h) in a batch fermentation among all the published studies. Our results demonstrated that the proposed bioprocess in this study holds a great promise for efficient and economical production of biobutanol from low-value lignocellulosic carbon sources.

4. Conclusions

PMS and CSL were used for biobutanol production with *C. tyrobutyricum* Δ*cat1::adhE2*. CSL, rich in nutrients, can not only be used to replace expensive nitrogen sources for fermentation but also provide LA to enhance butanol production. PMS can be readily hydrolyzed into mono-sugars with a yield of over 80%. During SHF using PMS hydrolysates as substrate supplemented with 5% CSL, 16.5 g/L butanol was obtained. Compared to SHF, SRHF, by recycling the cellulase, was demonstrated to be more effective for PMS enzymatic hydrolysis along with comparable butanol production. Our results demonstrated an efficient bioprocess for biobutanol production from industrial wastes.

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