RESEARCH

Pleiotropic regulation of a glucose-specific PTS in *Clostridium acetobutylicum* for high-efficient butanol production from corn stover without detoxification

Youduo Wu[1], Yidi Bai[1], Daojing Zhang[2], Chi Cheng[1], Lijie Chen[1], Fengwu Bai[3] and Chuang Xue[1*]

Abstract

Background: Corn stover (CS) is evaluated as the most favorable candidate feedstock for butanol production via microbial acetone–butanol–ethanol (ABE) fermentation by *Clostridium acetobutylicum*. By independent acid pretreatment and enzymatic hydrolysis, fermentable sugars (mainly glucose and xylose) were released, of which glucose was naturally utilized as the most preferred carbon source by *C. acetobutylicum*. However, the ABE fermentation using corn stover hydrolysate (CSH) without detoxification is typically limited to poor sugars utilization, butanol production and productivity. In the presence of pretreatment-derived inhibitors, the intracellular ATP and NADH, as important factors involved in cell growth, solventogenesis initiation and stress response, are exceedingly challenged owing to disrupted glucose phosphotransferase system (PTS). Therefore, there is a necessity to develop effective engineering approaches to overcome these limitations for high-efficient butanol production from CSH without detoxification.

Results: PTS-engineered *C. acetobutylicum* strains were constructed via overexpression and knockout of gene *glcG* encoding glucose-specific PTS IICBA, which pleiotropically regulated glucose utilization, cell growth, solventogenesis and inhibitors tolerance. The PTS^{GlcG}-overexpressing strain exhibited high fermentation efficiency, wherein butanol production and productivity was 11.1 g/L and 0.31 g/L/h, compared to those of 11.0 g/L and 0.15 g/L/h with the PTS^{GlcG}-deficient strain. During CSH culture without detoxification, the PTS^{GlcG}-overexpressing strain exhibited desirable inhibitors tolerance and solventogenesis with butanol production of 10.0 g/L, increased by 300% and 400% compared to those of 2.5 and 2.0 g/L with the control and PTS^{GlcG}-deficient strains, respectively. As a result of extra glucose and 10 g/L $CaCO_3$ addition into CSH, butanol production and productivity were further maximized to 12.5 g/L and 0.39 g/L/h. These validated improvements on the PTS^{GlcG}-overexpressing strain were ascribed to not only efficient glucose transport but also its cascading effects on intracellular ATP and NADH generation, solventogenesis initiation and inhibitors tolerance at the exponential growth phase.

Conclusion: The PTS^{GluG} regulation could be an effective engineering approach for high-efficient ABE fermentation from lignocellulosic hydrolysates without detoxification or wastewater generation, providing fundamental information for economically sustainable butanol production with high productivity.

Keywords: *Clostridium acetobutylicum*, Corn stover, PTS regulation, Inhibitor tolerance, Butanol productivity

*Correspondence: xue.1@dlut.edu.cn
[1] School of Bioengineering, Dalian University of Technology, No 2 Linggong Road, Dalian 116024, China
Full list of author information is available at the end of the article

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Background

Considering the worldwide depletion of fossil fuel resources, fluctuating crude oil prices as well as growing environmental concerns, sustainable butanol production via clostridial acetone–butanol–ethanol (ABE) fermentation is arousing intensive attention due to the unique physicochemical properties of butanol as a promising substitute for fossil-based energy [1–3]. Corn stover (CS) is well accepted as the most favorable candidate feedstock for sustainable ABE fermentation due to its widespread availability and high residue yield [4–6]. Followed by indispensable acid pretreatment and enzymatic hydrolysis, fermentable sugars (mainly glucose and xylose) are released from CS [4, 7], of which glucose is utilized as the most preferred carbon source. Although H_2SO_4-pretreatment is cost-effective and widely used for preparing corn stover hydrolysate (CSH) at industrial scale, the major drawback of this pretreatment process is the formation of degradation products such as weak acids, furan derivatives and phenolic compounds, which exhibit combined toxicities on clostridia [4, 7]. As a result, the fermentative process is severely inhibited without apparent sugar utilization, cell growth or solventogenesis, thus making butanol production from lignocellulosic feedstocks less sustainable and cost-competitive.

In recent years, numerous efforts have been made on development of pretreatment and detoxification methods, butanol recovery techniques, as well as strain reinforcements via mutagenesis, genetic manipulation and metabolic perturbations [8–17]. Particularly, significant improvements have been achieved with engineered solventogenic strains by either eliminating carbon catabolite repression or enhancing xylose metabolism. However, these genetically engineered strains were tested in synthetic media rather than detoxified lignocellulosic hydrolysates [11, 14–17]. Until now, of the best available strains for CS-based ABE fermentation, the highest ABE production and productivity of 26.3 g/L and 0.31 g/L/h were achieved with *C. beijerinckii* P260 using over-limed H_2SO_4-pretreated CSH, while no fermentation was observed using non-detoxified hydrolysate [18]. Despite physical, chemical or biological detoxification methods developed for eliminating the toxicities of inhibitors, the resulting wastewater emission, energy cost, sugar loss and low productivity remain hurdles for the economic viability and environmental sustainability at industrial scale [4, 7, 8, 19]. Therefore, there is a necessity to develop inhibitor-tolerating strains applicable to CSH culture without detoxification or wastewater generation.

When exposed to high levels of inhibitors like HMF and furfural, *C. acetobutylicum* cells are affected by uncoupling cellular functions associated with nutrient transport, cell replication, enzyme catalysis and stress response, resulting in inner accumulation of toxic metabolites and shortage of carbon, energy and reducing power [7]. In addition, ATP and NADH are drained during stress response such as heat shock proteins biosynthesis, efflux pumps and inhibitors transformation, thus shifting metabolism towards acid production to compensate for the decreased ATP supply [20]. Taken together, the significant issues for CHS culture lie in the disruption of glucose-specific PTS as well as ATP- and NADH-draining effects of microbial inhibitors [7]. More recently, micronutrient zinc as medium addictive affected ABE fermentation at metabolic and transcriptomic levels of *C. acetobutylicum* [21–23]. The upregulated expression of gene *glcG* encoding glucose-specific PTS IICBA led to efficient glucose transport, which evoked cascading effects on cofactors generation, solventogenesis initiation and stress tolerance [22]. Based on these findings, the objective of this work is to develop PTS^{GlcG}-engineered *C. acetobutylicum* applicable to CSH culture without detoxification. Finally, PTS^{GlcG} overexpression exerted pleiotropic roles facilitating CSH culture, and thus could be an effective engineering approach for high-efficient butanol production from lignocellulosic feedstocks.

Results and discussions

Pleiotropic regulation of PTS^{GluG} on ABE fermentation

The mixture of glucose and xylose was first selected as carbon sources by the wild-type strain L7, control strain L7(pPthl), PTS^{GlcG}-overexpressing strain L7(GlcG) and PTS^{GlcG}-deficient strain L7(ΔGlcG). Comparative profiles and results were illustrated in Fig. 1 and Table 1, respectively. Similar to strain L7, 45.6 g/L glucose and 8.6 g/L xylose were slowly utilized by strain L7(pPthl) with butanol production and productivity of 11.0 g/L and 0.18 g/L/h. The acetate production achieved the highest level of 4.7 g/L at 12 h and then decreased to 0.8 g/L until the end of fermentation. As for strain L7(ΔGlcG), 46.8 g/L glucose and 13.7 g/L xylose were simultaneously consumed, by which xylose utilization was promoted while little difference was observed on glucose utilization, reaching butanol production and productivity of 11.0 g/L and 0.15 g/L/h. In addition, less than 2.5 g/L acetate was detected during the whole fermentation. These observations were identical to the results as recently reported [11], wherein disrupting PTS^{GlcG} facilitated simultaneous utilization of glucose, xylose and arabinose by *C. acetobutylicum*. During batch culture with strain L7(GlcG), 46.6 g/L glucose was rapidly depleted within 24 h, achieving an average consumption rate of 1.94 g/L/h, compared to those of 0.84 and 0.89 g/L/h from strains L7(pPthl) and L7(ΔGlcG), respectively. However, only 5.3 g/L xylose was utilized due to rapid butanol production up to 11.1 g/L and its severe toxicity on cells. Finally, butanol
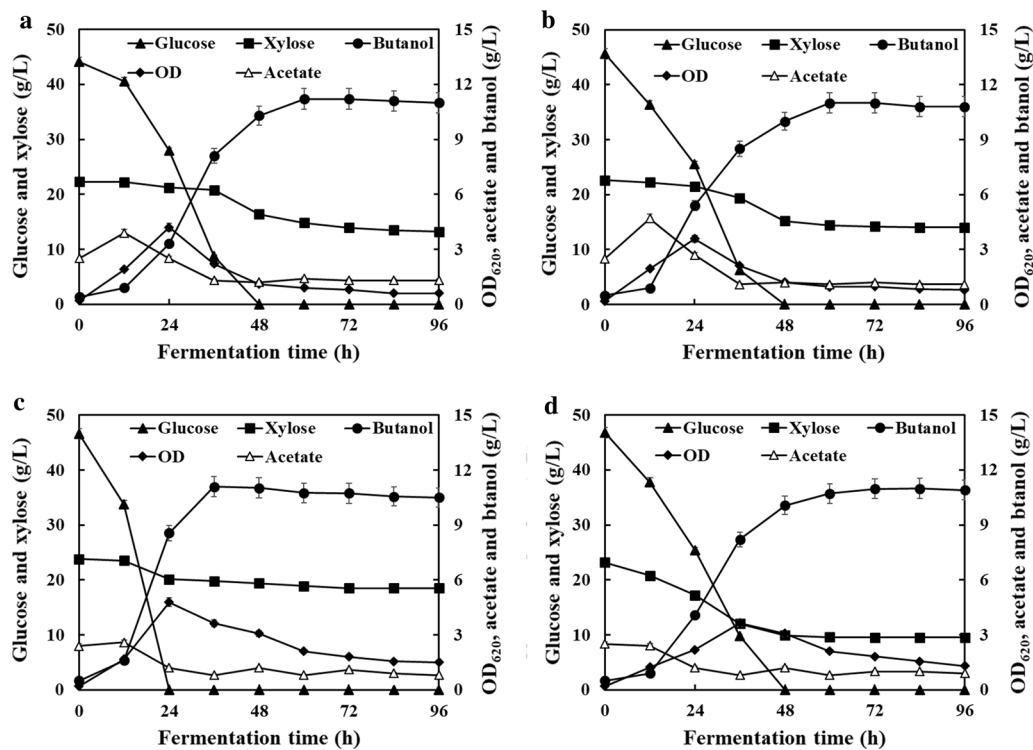
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Fig. 1 PTS^{GlcG}-mediated regulation on ABE fermentation using glucose/xylose mixture as carbon sources by strains of **a** *C. acetobutylicum* L7; **b** *C. acetobutylicum* L7(pPthl); **c** *C. acetobutylicum* L7(GlcG); **d** *C. acetobutylicum* L7(ΔGlcG)

productivity of 0.31 g/L/h was greatly increased by 72.2% and 106.7% compared to those of 0.18 and 0.15 g/L/h from strains L7(pPthl) and L7(ΔGlcG), respectively. In particular, strain L7(GlcG) produced only 2.6 g/L acetate at 12 h and grew fast with a peak OD$_{620}$ of 4.8 at 24 h, compared to that of 3.6 at 36 h for strain L7(ΔGlcG), indicating PTS^{GlcG}-mediated pleiotropic roles in glucose/xylose utilization, exponential growth and solventogenesis initiation.

Especially for xylose consumption within the first 36 h of fermentation, only 2.5 and 3.2 g/L xylose were utilized by strains L7 and L7(pPthl), respectively, indicating that severe carbon catabolite repression on xylose consumption occurred in the presence of glucose. Finally, 6.6 and 5.4 g/L xylose were consumed by strains L7 and L7(pPthl) from 36 h to the end of fermentation. Similarly, as low as 3.7 g/L xylose was consumed within the first 24 h of fermentation by strain L7(GlcG). However, in the absence of glucose, only 1.6 g/L xylose was further consumed from 24 h to the end of fermentation due to the severe inhibition of high butanol concentration (> 9 g/L). Despite more xylose consumed rapidly by PTS^{GlcG}-deficient strain within the first 48 h in the presence of glucose, the fermentative process remained inefficient due to low butanol yield and prolonged metabolism. Until recently,

efficient glucose/xylose culture has been achieved by adding calcium carbonate and zinc sulfate, wherein butanol production and productivity were maximized to 13.9 g/L and 0.35 g/L/h [23]. Global transcriptomic and proteomic results showed that CaCO$_3$ and ZnSO$_4$ could act upon overall cellular functions associated with sugar utilization, central carbon metabolism and stress response at global levels [22, 24–26]. Particularly, followed by zinc addition, the expression of gene *glcG* was 3.62-fold upregulated at the exponential growth phase of *C. acetobutylicum*. Additionally, intracellular metabolites analysis showed that central carbon flux was earlier redistributed towards solventogenesis owing to increased supplies of ATP and NADH as driving forces [22]. Hence, fermentation efficiency depends on not only strains used but a rapid shift towards solventogenesis, which was mainly attributed to the coordination of metabolic cues and their cascading effects on *C. acetobutylicum*.

Enhanced inhibitors tolerance during CSH culture without detoxification

The basic chemical compositions of unpretreated CS solids were 40.8% cellulose, 15.2% hemicellulose, 32.1% acid insoluble lignin and ash. Followed by acid pretreatment, the pretreated CS solids were mainly composed

Table 1 Comparative results of batch ABE fermentation by *C. acetobutylicum* strains

Strain	Sugar utilized (g/L)	Products (g/L)					$Y_{B/ABE}$ (g/g)	$P_{B/ABE}$ (g/L/h)
		Acetate	Butyrate	Acetone	Butanol	Ethanol		
7% glucose/xylose (2:1) mixture as carbon sources								
L7	44.1±1.1/9.1±0.9	1.3±0.2	1.0±0.2	6.5±0.4	11.2±0.5	1.2±0.1	0.21/0.36	0.19/0.32
L7(pPthl)	45.6±1.5/8.6±0.8	1.1±0.1	0.9±0.1	6.0±0.3	11.0±0.5	1.8±0.1	0.20/0.35	0.18/0.31
L7(GlcG)	46.6±1.3/5.3±0.8	0.8±0.1	0.5±0.1	6.6±0.5	11.1±0.4	1.9±0.2	0.21/0.38	0.31/0.54
L7(ΔGlcG)	46.8±1.5/13.7±1.1	0.9±0.1	0.4±0.1	6.1±0.3	11.0±0.5	1.2±0.1	0.18/0.30	0.15/0.25
Non-detoxified H_2SO_4-pretreated CSH as carbon sources[a]								
L7	15.6±1.3	2.4±0.2	2.1±0.3	1.8±0.1	2.5±0.2	0.5±0.1	0.16/0.31	0.07/0.13
L7(pPthl)	15.2±1.1	2.8±0.2	2.3±0.2	1.7±0.1	2.5±0.2	0.5±0.1	0.16/0.31	0.07/0.13
L7(GlcG)	45.4±1.6	1.3±0.1	1.1±0.1	6.0±0.3	10.0±0.4	1.3±0.1	0.22/0.38	0.28/0.48
L7(ΔGlcG)	12.3±1.4	1.7±0.2	1.4±0.2	1.2±0.1	2.0±0.1	0.4±0.1	0.16/0.29	0.04/0.08
Non-detoxified enzymatically H_2SO_4-pretreated CSH as carbon sources								
L7	16.5±1.3[b]	2.6±0.2	2.2±0.3	1.4±0.1	3.0±0.2	0.4±0.1	0.18/0.29	0.08/0.13
L7(pPthl)	15.6±1.0[b]	2.9±0.3	2.3±0.2	1.5±0.1	2.7±0.1	0.5±0.1	0.17/0.30	0.08/0.13
L7(GlcG)	34.5±1.9[b]	1.8±0.2	1.5±0.2	3.9±0.2	7.2±0.3	0.9±0.1	0.21/0.35	0.20/0.33
	45.0±1.5[c]	2.7±0.3	2.1±0.2	5.6±0.3	10.0±0.3	1.0±0.1	0.22/0.37	0.28/0.46
	52.5±2.0[d]	1.5±0.1	1.2±0.2	6.6±0.4	11.0±0.4	1.6±0.1	0.21/0.37	0.34/0.60
	60.0±1.8[e]	2.1±0.2	1.7±0.1	6.7±0.4	12.5±0.4	1.8±0.2	0.21/0.35	0.39/0.66

[a] With glucose supplemented to 60 g/L total sugars

[b] CSH with 45 g/L total sugars and no nutrients added

[c] CSH with 45 g/L total sugars and 10 g/L $CaCO_3$ added

[d] CSH with glucose supplemented to 60 g/L total sugars and no nutrients added

[e] CSH with glucose supplemented to 60 g/L total sugars and 10 g/L $CaCO_3$ added

of 56.5% cellulose, 4.5% hemicellulose, 34.7% acid insoluble lignin and ash. The recovery rates of solid, cellulose and hemicellulose were 57.8%, 79.5% and 17.1%, respectively. In the H_2SO_4-pretreated CSH, the initial concentration of total sugars was 22.0 g/L (3.0 g/L glucose, 15.5 g/L xylose and 3.5 g/L arabinose), and the initial concentrations of acetate, furfural and HMF as the major inhibitory compounds were 4.0, 1.5, and 0.9 g/L, respectively. After 10% inoculation, the concentration of total sugars was adjusted to ~60 g/L by adding glucose. As shown in Fig. 2 and Table 1, the regulatory impacts of PTS^{GlcG} on inhibitors tolerance were further investigated using the non-detoxified H_2SO_4-pretreated CSH. Similar to strain L7, strain L7(pPthl) exhibited poor cell growth and solventogenesis, wherein 2.5 g/L butanol was produced from 15.2 g/L total sugars (mostly glucose) with an average sugar consumption rate of 0.42 g/L/h. The residual acetate decreased to 2.8 g/L. As for strain L7(ΔGlcG), only 12.3 g/L total sugars (8.8 g/L glucose and 3.5 g/L xylose) were utilized with an average sugar consumption rate of 0.34 g/L/h, resulting in slightly decreased butanol production of 2.0 g/L. The residual acetate decreased to 1.7 g/L. It should be noted that 1.3 g/L furfural and 0.8 g/L HMF remained in the fermentation broth, implying poor

metabolic transformation of furfural and HMF by strains L7(pPthl) and L7(ΔGlcG).

Particularly for strain L7(GlcG), as high as 45.4 g/L total sugars (42.7 g/L glucose and 2.7 g/L xylose) was rapidly utilized within 36 h, reaching a peak OD_{620} of 3.0 and an average sugar consumption rate of 1.26 g/L/h. Finally, butanol production of 10.0 g/L was achieved together with yield and productivity of 0.22 g/g and 0.28 g/L/h, respectively, which increased by 300%, 37.5% and 300% compared to those of strain L7(pPthl). More strikingly, the residual acetate, furfural and HMF significantly decreased to 1.3, 0.3 and 0.1 g/L, respectively, demonstrating that these pretreatment-derived inhibitors could be largely transformed into less toxic compounds by strain L7(GlcG), thus making the non-detoxified CSH culture sustainable. For example, the oxidation–reduction potential (ORP) was maintained at the range of −550 to −520 mV, which was consistent with the active cellular metabolisms by PTS^{GlcG} overexpression. Correspondingly, the ORP increased rapidly after 24 h culture with both strains L7(pPthl) and L7(ΔGlcG), wherein cell death and premature termination occurred owing to their insufficient supports on carbon, energy and reducing power. Until now, the highest butanol production of 14.5 g/L was achieved by *C. beijerinckii* P260 from
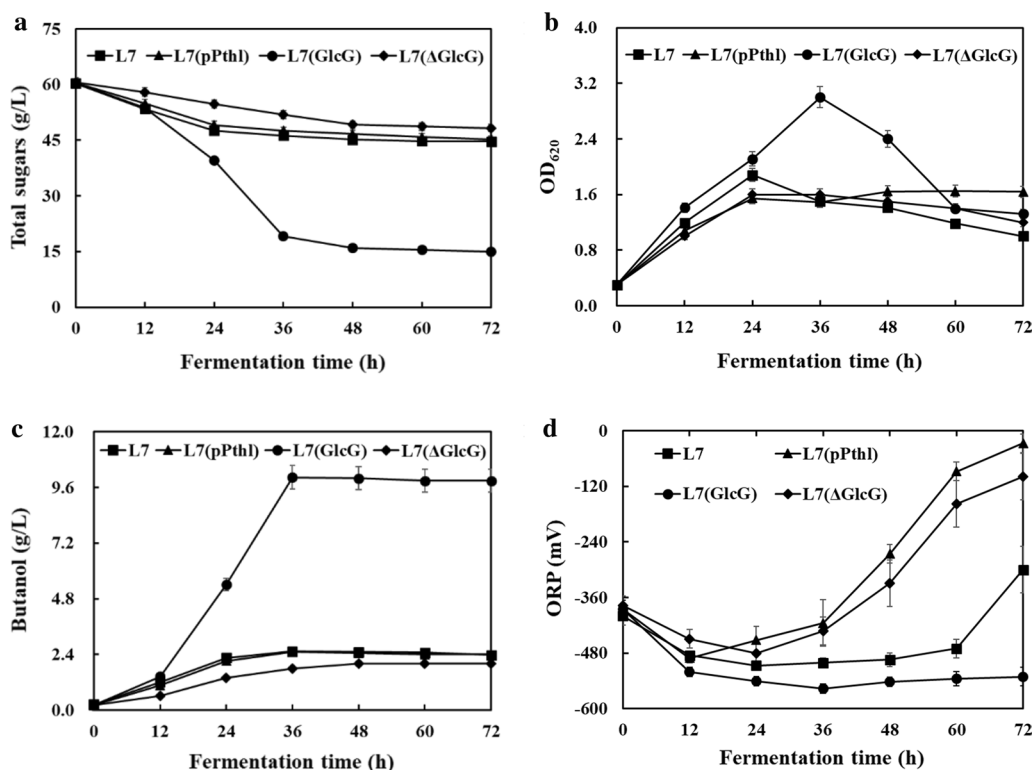
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Fig. 2 PTS^{GlcG}-mediated impact on microbial inhibitors tolerance of *C. acetobutylicum* using non-detoxified H$_2$SO$_4$-pretreated CSH. Comparative fermentation profiles of **a** total sugars; **b** OD$_{620}$; **c** butanol; **d** ORP

detoxified H$_2$SO$_4$-pretreated CSH (60.3 g/L total sugars by adding glucose) [18], however, butanol productivity of 0.17 was much lower than that of 0.28 g/L/h in non-detoxified CSH culture by strain L7(GlcG).

Microbial inhibitors such as furfural and 5-hydroxymethyl furfural (HMF) could be naturally transformed into less inhibitory products via the NADH-dependent metabolic detoxification process in *Clostridium* strains [7], which in turn hinders a metabolic shift towards solventogenesis due to excess NADH consumption in *C. acetobutylicum* [27]. Furthermore, the decreased ATP supply impairs cell growth and stress response, including membrane modification, heat shock proteins biosynthesis and efflux pumps. Therefore, boosting intracellular ATP and NADH supplies has been validated as a rational approach for combating these negative effects of inhibitors [28–31]. For instance, glycerol as a co-substrate with glucose (2:1, mol/mol) could generate additional ATP and NADH and exert positive effects on cell growth, alcohol/aldehyde dehydrogenases activities and furfural/HMF transformation [12]. When subjected to 5 g/L furfural during ABE fermentation, glycerol generated a 1.8-fold increase in NADH level, which accounted for 2.3-fold increase in furfural detoxification rate, glucose utilization and

butanol production compared to the control without glycerol addition [12]. However, the naturally poor glycerol utilization remains a bottleneck for most solventogenic clostridia strains, leading to unsustainable ATP/NADH support as well as unnecessary waste [32, 33]. Therefore, the demonstrated PTS^{GlcG} overexpression possibly boosted intracellular ATP/NADH supplies and evoked cascading effects on inhibitors tolerance.

Increased intracellular ATP and NADH supplies by overexpressing PTS^{GlcG}

For the sake of better understanding the intracellular energy and reducing power changes in *C. acetobutylicum* after PTS^{GlcG} overexpression, glucose-based batch culture was performed to analyze ATP and NADH levels of strains L7(pPthl) and L7(GlcG), respectively. As shown in Fig. 3, strain L7(GlcG) exhibited efficient glucose utilization and earlier solventogenesis, wherein 12.6 g/L butanol was achieved from 59.4 g/L glucose within only 28 h. Therefore, as high as 2.12 and 0.45 g/L/h of glucose consumption rate and butanol productivity were also achieved, respectively, which increased by 130.4% and 125% compared to those of strain L7(pPthl). Furthermore, the exponential growth of strain L7(GlcG) was
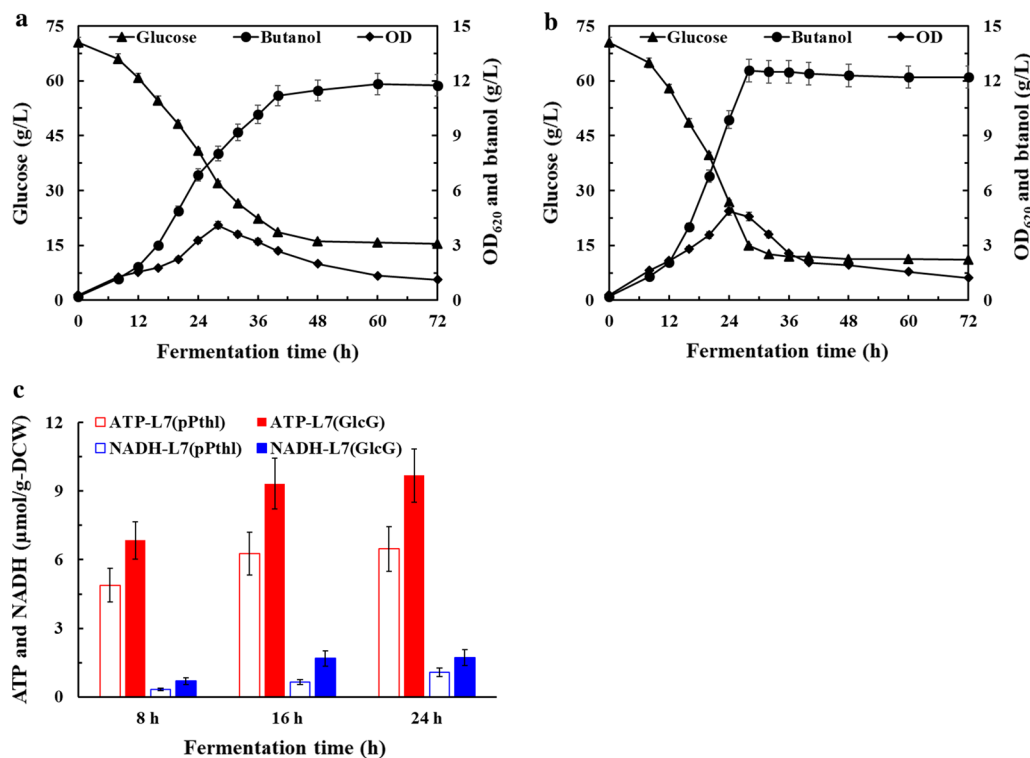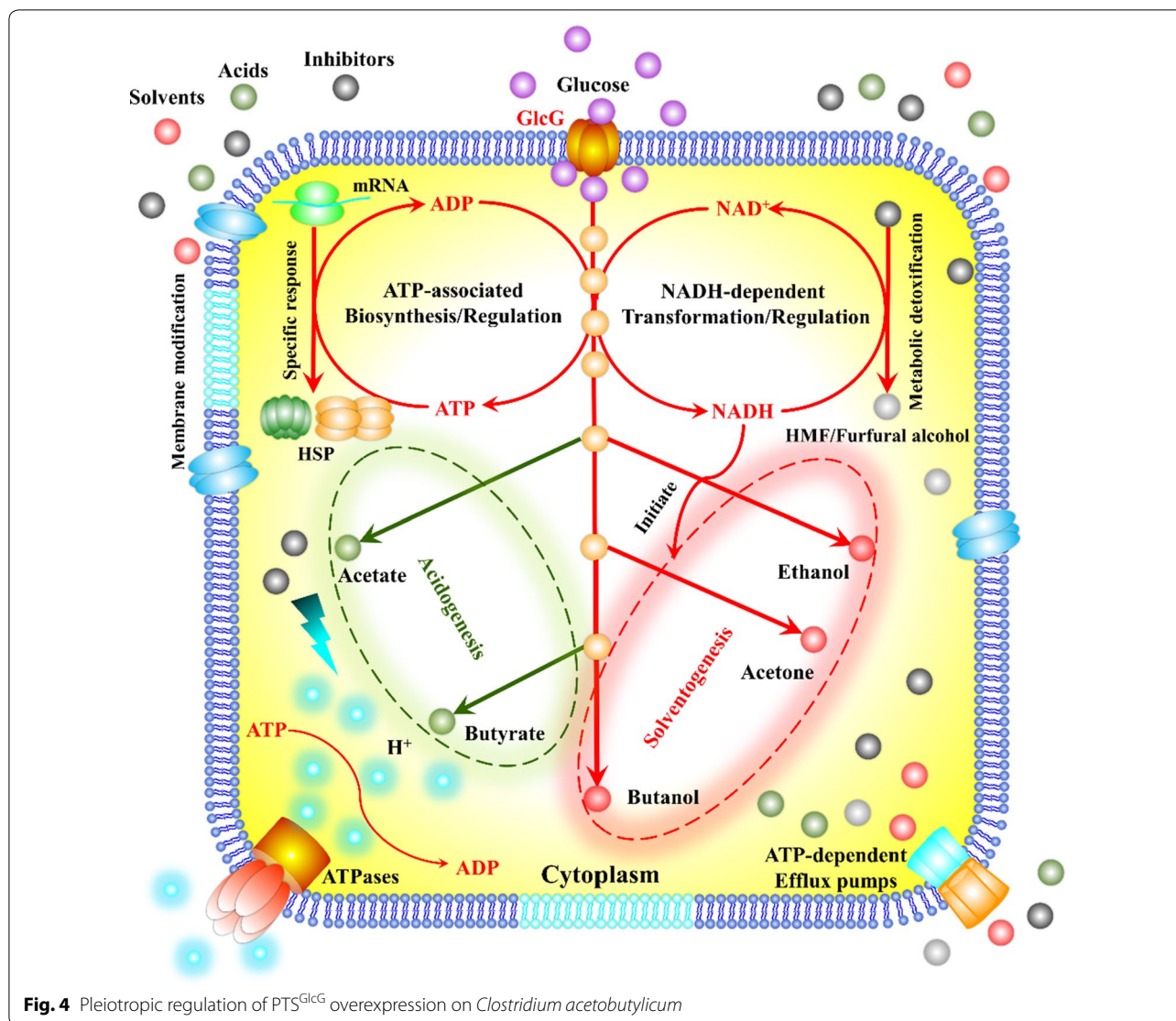
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Fig. 3 PTS^{GlcG}-mediated effects on ABE fermentation using glucose as sole carbon source. Comparative profiles of **a** glucose, OD$_{620}$ and butanol of strain L7(pPthl); **b** glucose, OD$_{620}$ and butanol of strain L7(GlcG); **c** ATP and NADH of strains L7(pPthl) and L7(GlcG)

significantly enhanced with a peak OD$_{620}$ of 4.9 obtained at 24 h, compared to that of 4.1 obtained at 28 h for strain L7(pPthl). Since strain L7(GlcG) exhibited higher fermentation efficiency than strain L7(pPthl), the sampling times were 8, 16 and 24 h of the exponential growth phase for both strains. At the three sampling times of 8, 16 and 24 h, the ATP levels of strain L7(GlcG) were 6.85, 9.33 and 9.69 μmol/g-DCW, respectively, which increased by 40.4%, 49.0% and 49.8% compared to those of strain L7(pPthl), thus leading to 23.1%, 55.6% and 48.5% increase on the OD$_{620}$. More importantly, the NADH levels of strain L7(GlcG) were 0.69, 1.68 and 1.73 μmol/g-DCW, respectively, which increased by 52.2%, 61.9% and 37.6% compared to those of strain L7(pPthl), which contributed to earlier solventogenesis initiation and NADH-driven inhibitors transformation. Therefore, as illustrated in Fig. 4, the experimental results demonstrated that overexpressing PTS^{GlcG} played pleiotropic roles regulating glucose transport and its cascading effects on cofactors generation, solventogenesis initiation and inhibitors tolerance of *C. acetobutylicum*.

High-efficient ABE fermentation from non-detoxified CSH
To achieve cost-effective lignocellulosic butanol production, the enzymatically H$_2$SO$_4$-pretreated CSH was directly utilized for batch culture without detoxification (Fig. 5). Similarly, strains L7 and L7(pPthl) both exhibited poor fermentation performance and produced less than 3 g/L butanol at 36 h. As for strain L7(GlcG), 7.2 g/L butanol was produced from 34.5 g/L total sugars within 36 h. However, as high as 10.5 g/L residual sugars (mainly xylose) remained in the fermentation broth. As a result of 10 g/L CaCO$_3$ addition, all sugars were rapidly depleted by strain L7(GlcG) within 36 h, wherein butanol production and productivity were further maximized to 10.0 g/L and 0.28 g/L/h. It should be noted that, under the same culture condition, butanol production was still limited to ~6.0 g/L with strains L7 and L7(pPthl), respectively, implying potential redistribution of central carbon flux towards butanol biosynthesis by overexpressing PTS^{GlcG}. Actually, 100 g dry corn stover yielded ~45 g fermentable sugars (27.8 g glucose, ~14 g xylose and ~3.2 g arabinose) in this study. Given the fact that as high as ~66 g fermentable sugars could be extracted from 100 g dry CS [4], more fermentable sugars (especially glucose) could be released from pretreated corn stover if possible. Therefore, the high concentration of total sugars was further adjusted to 60 g/L by adding glucose after 10% inoculation. As expected, 60 g/L total sugars were all depleted by strain L7(GlcG) in the presence of only 10 g/L CaCO$_3$.

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Fig. 4 Pleiotropic regulation of PTS^{GlcG} overexpression on *Clostridium acetobutylicum*

Butanol production and productivity of 12.5 g/L and 0.39 g/L/h could be achieved, which increased by 316.7% and 387.5% compared to those of strain L7(pPthl), making the detoxification process, wastewater generation and medium optimization unnecessary for non-detoxified CSH culture.

As summarized in Table 2, despite considerable efforts on improving CS-based ABE fermentation [10, 18, 34–36], high-efficient CSH culture remains a significant issue in synthetical consideration of sugar utilization, butanol production and productivity. For instance, Qureshi et al. reported that the highest butanol production of 14.5 g/L was achieved with productivity of 0.17 g/L/h by *C. beijerinckii* P260 using detoxified H$_2$SO$_4$-pretreated CSH [18]. Zhang et al. reported that 7.1 g/L butanol was produced with much lower productivity of 0.10 g/L/h from detoxified CSH by *C. acetobutylicum* ATCC 824 [36]. Until recently, butanol productivity was improved to 0.19 g/L/h using detoxified enzymatically NaOH-pretreated CSH by *C. beijerinckii* CC101 [37]. As reported by Gao et al., the butanol-tolerant *C. acetobutylicum* strain 206 was screened by NTG mutagenesis and proven to be robust for lignocellulosic ABE fermentation, wherein butanol productivity up to 0.14 g/L/h could be achieved using non-detoxified enzymatically NaOH-pretreated CSH [38]. However, no solventogenic clostridia strains to date are available for high-efficient CSH culture. Therefore, the demonstrated PTS^{GlcG} overexpression in this study was validated as a feasible engineering approach for high-efficient butanol production from lignocellulosic feedstocks without detoxification or wastewater generation.
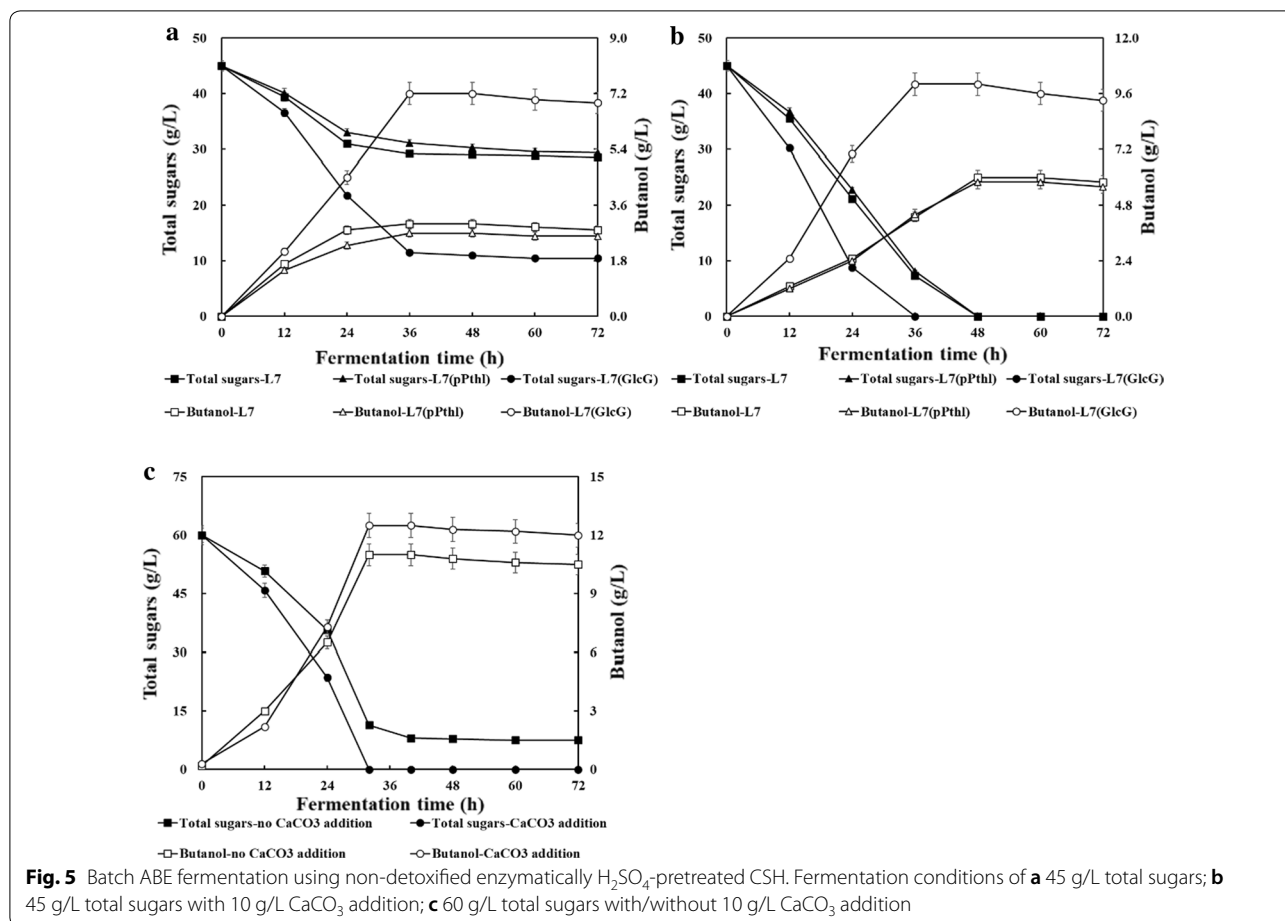
Fig. 5 Batch ABE fermentation using non-detoxified enzymatically H_2SO_4-pretreated CSH. Fermentation conditions of **a** 45 g/L total sugars; **b** 45 g/L total sugars with 10 g/L $CaCO_3$ addition; **c** 60 g/L total sugars with/without 10 g/L $CaCO_3$ addition

Table 2 Comparisons of batch ABE fermentation using detoxified or non-detoxified CSH

Strain	Pretreatment method	Detoxification method	Products			References
			Butanol/ABE (g/L)	Yield (g/g)	Productivity (g/L/h)	
C. acetobutylicum ATCC 824	Alkaline twin-screw extrusion	Washing	7.1/11.2	0.18/0.29	0.10/0.16	Zhang et al. [36]
C. acetobutylicum ATCC 824	Steam explosion	Activated charcoal	NA/12.4	NA/0.30	NA/0.17	Wang and Chen [35]
C. beijerinckii P260	1% (v/v) H_2SO_4	Overliming	14.5/26.3	0.24/0.44	0.17/0.31	Qureshi et al. [18]
C. beijerinckii P260	1% (v/v) H_2SO_4	Overliming	NA/24.4	NA/0.44	NA/0.30	Qureshi et al. [34]
C. beijerinckii CC101	2% (v/v) NaOH	Washing	11.2/19.8	0.28/0.49	0.19/0.33	Xue et al. [37]
C. acetobutylicum ATCC 824	Steam explosion	ND	2.2/3.7	NA/NA	0.03/0.05	Wang and Chen [35]
C. acetobutylicum strain 206	2% (v/v) NaOH	ND	9.8/15.4	0.26/0.41	0.14/0.21	Gao et al. [38]
C. beijerinckii P260	1% (v/v) H_2SO_4	ND	No fermentation			Qureshi et al. [18]
C. acetobutylicum L7	1% (v/v) H_2SO_4	ND	3.0/4.8	0.18/0.29	0.08/0.13	This study
C. acetobutylicum L7(GlcG)	1% (v/v) H_2SO_4	ND	12.5/21.0	0.21/0.35	0.39/0.66	This study

NA not available, *ND* not detoxified

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Conclusions

In this study, PTS^{GlcG}-engineered *C. acetobutylicum* strains were constructed via gene *glcG* overexpression and knockout to address the major problems during non-detoxified CSH culture. The PTS^{GlcG}-overexpressing strain exhibited remarkable improvements in glucose utilization, exponential growth and inhibitors tolerance, which was ascribed to efficient glucose transport but also its cascading effects on intracellular ATP and NADH generation, solventogenesis initiation and inhibitors tolerance. Finally, 12.5 g/L butanol was achieved within 32 h of non-detoxified CSH culture, resulting in significantly improved butanol productivity up to 0.39 g/L/h, and thus making butanol production from lignocellulosic feedstocks more economically sustainable and environmentally friendly.

Materials and methods

Bacterial strains, primers and plasmids

All bacterial strains and plasmids used in this study are listed in Table 3. Generally, strain *C. acetobutylicum* L7, adapted from the wild-type strain *C. acetobutylicum* ATCC 824 as previously reported [21], was

Table 3 Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristics	Source/references
Bacterial strains		
E. coli		
DH5α	Host cells for gene cloning and plasmids amplification	Invitrogen
DH10B	Strain used to methylate the vector	Invitrogen
C. acetobutylicum		
ATCC 824	Wild-type strain	ATCC
L7	Adapted from *C. acetobutylicum* ATCC 824	[21]
L7(pPthl)	Control strain, L7 containing the control plasmid pIMP1-Pthl	This study
L7(GlcG)	L7 containing the plasmid pIMP1-Pthl-glcG	This study
L7(ΔGlcG)	PTS^{GlcG}-deficient strain via intron-mediated knockout	This study
Plasmids		
pAN1	Φ3TI, p15A origin; *Spe^r*	[39]
PSY6	Group II intron, ltrA	[41]
pIMP1	*Amp^r*; *MLS^r*; repL, ColE1 origin, shuttle vector	[11]
pIMP1-Pthl	Control vector carrying *thl* promoter, derived from pIMP1	[11]
pIMP1-Pthl-glcG	Derived from pIMP1-Pthl with *glcG* overexpression in L7	This study
PSY-GlcG	Vector for intron insertion at 269/270 bp of *glcG* in L7	This study

used for constructing the control and PTS-engineered strains. Strains *E. coli* DH5α and DH10B cultured with LB medium or agar containing 10 μg/mL ampicillin and 50 μg/mL spectinomycin were used for plasmids amplification and in vivo methylation. Based on the *C. acetobutylicum* ATCC 824 genome, gene *glcG* synthesized by Sangon (Shanghai, China) was digested with the *SalI/KpnI* restriction sites and then ligated into the control plasmid pIMP1-P$_{thl}$, yielding the target plasmid pIMP1-P$_{thl}$-GlcG. Plasmids pIMP1-P$_{thl}$ and pIMP1-P$_{thl}$-GlcG were first methylated in *E. coli* DH10B and then electroporated into strain L7, respectively, according to the standard protocols [39]. The resulting cells were then cultured on RCM (Thermo Fisher, Oxoid Ltd.) agar containing 50 μg/mL erythromycin for single colony selection, yielding the control strain L7(pPthl) and PTS^{GlcG}-overexpressing strain L7(GlcG). According to the method described by Xiao et al., the PTS^{GlcG}-deficient strain L7(ΔGlcG) was constructed via intron-mediated knockout system with an intron inserted at 269/270 bp of gene *glcG* [11], and all the related primers were synthesized by Sangon (see Additional file 1: Table S1). All the commercial enzymes were purchased from New England Biolabs (Beverly, MA).

Acid pretreatment of corn stover

The CS (Pioneer variety) used in this study was obtained from a local farmer in Shandong and milled into 1–2 mm particles using a hammer mill. As documented by Malmierca et al., one hundred grams of milled CS was soaked with 1 L 1% (v/v) dilute H_2SO_4 solution in a stirred tank at 121 °C for 90 min, followed by cooling to room temperature and adjusting pH to 5.0 using 10 M NaOH [40]. The initial concentration of total sugars in the H_2SO_4-pretreated CSH was 22 g/L, which was further added to 60 g/L with extra glucose when necessary.

Enzymatic hydrolysis of acid-pretreated corn stover

As optimized by Xue et al., the resulting H_2SO_4-pretreated CS mixture containing 30 mM citrate was hydrolyzed using cellulase (Tianjin Novozymes Biotechnology, China) with 20 FPU/g-CS at 50 °C, pH 4.8 and 200 rpm for 72 h [37]. The enzymatically H_2SO_4-pretreated CSH was obtained by centrifugation at 8000×*g* for 5 min to remove sediments, and then stored at 4 °C for subsequent batch culture without detoxification process or medium optimization. Followed by 10% inoculation during CSH culture, the initial concentration of total sugars was ~45 g/L, which was added to 60 g/L with extra glucose when necessary.

Media and culture conditions

The media for pre-culture and seed culture were as previously described [21, 23]. The standard fermentation

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medium is composed of (g/L): glucose or glucose/xylose mixture (2:1, w/w) 70.0, yeast extract 2.0, K_2HPO_4 0.50, KH_2PO_4 0.50, $MgSO_4 \cdot 7H_2O$ 0.20, $MnSO_4 \cdot H_2O$ 0.10, $FeSO_4 \cdot 7H_2O$ 0.01, CH_3COONH_4 3.22, para-amino-benzoic acid 0.01 and biotin 0.01. The CSHs mentioned above were directly utilized for batch ABE fermentation, respectively. All CSHs and media were sterilized at 121 °C for 15 min, followed by cooling to room temperature and added with 10 μg/mL erythromycin when necessary. The initial pH for batch culture was adjusted to 5.5 using 3 M H_2SO_4 or 3 M NaOH after 10% inoculation. All chemical reagents used in this study were of analytical grade or equivalent and purchased from Sangon.

Batch ABE fermentation

Batch ABE fermentation was carried out in a stirred tank containing CSH or standard medium under anaerobic conditions as previously described [21]. After 10% inoculation, the initial fermentation pH was adjusted to 5.5 using 3 M H_2SO_4 or NaOH. All the experiments were triplicated and samples were taken for analyzing cell growth, residual sugar(s), acids and ABE production. Particularly, intracellular ATP and NADH were quantified during batch culture using non-detoxified H_2SO_4-pretreated CSH.

Analysis of intracellular ATP and NADH

During batch culture using glucose as the sole carbon source, *C. acetobutylicum* cells were collected at 8, 16 and 24 h by centrifugation at $10,000 \times g$ for 3 min at -10 °C. The resulting cell pellets were quenched immediately with 500 μL solution mixture of methanol, acetonitrile and water (40:40:20, v/v, -40 °C), and then frozen in liquid nitrogen for preparing crude extracts. According to our previous study [22], LC–MS/MS analysis was conducted for ATP quantification with an ACCELA HPLC system (Thermo Scientific, CA) equipped with an XBridge BEH Amide column (100 mm × 2.1 mm I.D., 2.5 μm, Waters, Ireland). Mass monitoring was achieved using a TSQ Quantum Ultra triple quadrupole mass analyzer (Thermo Scientific, CA) equipped with a heated electrospray ionization source (HESI). NADH assay was performed using a commercial kit (Sigma, MO). Cell pellets were first lysed using a Qiagen Tissue Lyser LT (Qiagen, Germany) at 50 oscillations/s for 3 min in the NADH extraction buffers (Sigma, MO), the resulting lysate was then used for NADH quantification at 450 nm with an iMark™ microplate reader (Bio-Rad, CA).

Analytical methods

As previously described, cell growth was measured at 620 nm using a spectrophotometer (Thermo Spectronic, USA) [22]. The chemical compositions of unpretreated and pretreated CS solids were analyzed according to Laboratory Analytical Procedures [19].

The total sugars in CSH were determined by 3,5-dinitrosalicylic acid (DNS) method at 540 nm using the spectrophotometer. ABE were determined by a gas chromatography (Agilgent 6890A GC). Sugars (glucose, xylose and arabinose), acids (acetate and butyrate) and major inhibitors (furfural and HMF) were analyzed by a high-performance liquid chromatography (Waters 1525 HPLC). All the standard chemicals of sugars, acids and ABE were of quality HPLC gradient grade and purchased from Sigma-Aldrich (Saint-Louis, Missouri, USA). The butanol or ABE yield ($Y_{B/ABE}$) was calculated as total butanol or ABE produced divided by the total sugars used and is expressed in g/g. The butanol or ABE productivity ($P_{B/ABE}$) was calculated as total butanol or ABE produced divided by the fermentation time used and is expressed in g/L/h.

Supplementary information

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Author details
[1] School of Bioengineering, Dalian University of Technology, No 2 Linggong Road, Dalian 116024, China. [2] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China. [3] School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China.

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