Bioresource Technology 101 (2010) 9560–9569

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Factors influencing cellulosome activity in Consolidated Bioprocessing of cellulosic ethanol

Chenggang Xu [a,1], Yong Qin [a,b,1], Yudong Li [a,c], Yuetong Ji [a], Jianzhong Huang [b], Houhui Song [a,*], Jian Xu [a,**]

[a] Qingdao Institute of BioEnergy and BioProcess Technology, Chinese Academy of Sciences, 189 Songling Rd., Qingdao, Shandong 266101, PR China
[b] Engineering Research Center of Industrial Microbiology, Fujian Normal University, Fuzhou, Fujian 350108, PR China
[c] Huaqiao University, Quanzhou, Fujian 362021, PR China

ARTICLE INFO

Article history:
Received 1 March 2010
Received in revised form 6 July 2010
Accepted 15 July 2010
Available online 10 August 2010

Keywords:
Cellulosome
Fungal cellulase
Consolidated Bioprocessing
Pretreatment
Clostridium thermocellum

ABSTRACT

The cellulosome, a multi-subunit protein complex catalyzing cellulose degradation in cellulolytic *Clostridium thermocellum*, plays a crucial role in Consolidated Bioprocessing (CBP) of lignocellulose into ethanol. Here, activity of cellulosome was tested under varying concentrations of chemical compounds derived from lignocellulose pretreatment and fermentation. We found that, firstly, the cellulolytic activity of cellulosome was actually promoted by formate, acetate and lactate; secondly, cellulosome was tolerant up to 5 mM furfural, 50 mM *p*-hydroxybenzoic acid and 1 mM catechol. Furthermore, the cellulosome exhibited higher ethanol tolerance and thermostability than commercialized fungal (*Trichoderma reesei*) cellulase. To probe the implication of these unique enzyme-features, *C. thermocellum* JYT01 was cultured under conditions optimal for cellulosome activity. This CBP system yielded 491 mM ethanol, the highest level reported thus far for *C. thermocellum* monocultures. These findings demonstrate the potential advantages of bacterial cellulosome, and provide a novel strategy for design, selection and optimization of the cellulosome–ethanologen partnership.

1. Introduction

Global warming, energy crisis and health concerns have demanded novel sustainable and renewable substitutes for petroleum-based liquid fuels (Hill et al., 2009). Cellulosic ethanol, one kind of renewable biofuels, has been regarded as a potential solution due to the reduced emission of greenhouse gas, lower toxicity, higher octane rating, greater combustion efficiency and wide availability of inexpensive substrates (Demain et al., 2005). Production of cellulosic biofuels generally starts with pretreatment of lignocellulose, followed by cellulose/hemicellulose hydrolysis and then fermentation of the liberated "sugar" (including oligo-, di- and monosaccharides). Consolidated Bioprocessing (CBP), which simultaneously combines efficient lignocellulosic biomass hydrolysis, unbiased utilization of the full-spectrum of the liberated sugars, and robust ethanolic fermentation in one bioreactor, has proven, in theory, to be feasible in energy conversion and be crucial in reducing biological processing costs (Lynd et al., 2008). However, no "CBP-organisms", that singularly combine all these features, have been identified in nature. Among the potential CBP-organisms, thermophilic bacteria as a group have attracted increasing attention since they are cellulolytic and ethanologenic under thermophilic conditions (Farrell et al., 2006; Georgieva et al., 2008; Shaw et al., 2008).

For instance, *Clostridium thermocellum*, a gram-positive, anaerobic and thermophilic bacterium, has been regarded as one potential CBP-organism due to its robust growth on crystalline cellulose (Lynd et al., 2002). However, intrinsic characteristics of *C. thermocellum* limit its immediate and direct applications in industrial ethanologenesis from cellulose. Growth of *C. thermocellum* is slowed down by ethanol at concentrations greater than 2% (v/v) (Herrero and Gomez, 1980), although laboratory-evolved strains viable in up to 8% (v/v) ethanol were reported (Rani and Seenayya, 1999). Furthermore, studies showed that ethanol and hydrogen production using *C. thermocellum* consistently suffered from low carbon-loading (less than 2% w/v) (Lynd and Grethlein, 1987; Lynd et al., 1989; Zhang and Lynd, 2005).

The "cellulose degradation and sugar release" is typically the rate-limiting step in cellulosic ethanol production. "Cellulosome", found in many thermophilic and mesophilic cellulose-degraders in nature including *C. thermocellum* (Demain et al., 2005), is the cellulase complex underlying cellulose degradation and sugar release in these organisms. Thus, system efficiency could potentially be improved by maximizing cellulosome activity and/or creating the synergy between cellulosome-based cellulolysis and the

* Corresponding author. Tel.: +86 532 8066 2653; fax: +86 532 8066 2654.
** Corresponding author. Tel.: +86 532 8066 2653; fax: +86 532 8066 2654.
E-mail addresses: songhh@qibebt.ac.cn (H. Song), xujian@qibebt.ac.cn (J. Xu).
[1] Both authors contributed equally to this work.

0960-8524/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2010.07.065

consequential sugar fermentation. However, it is unclear what the optimal conditions for cellulosome activity are under a CBP scheme. Optimal conditions for host cell growth do not necessarily match those for the enzymes produced. For instance, the cellulase of the mesophilic *Trichoderma reesei* is secreted at 30 °C, while exhibits its maximal enzymatic activity at 50 °C (Xia and Shen, 2004). Furthermore, during pretreatment of lignocellulose, a significant amount of compounds from side-reactions are released (Galbe and Zacchi, 2007; Liu et al., 2004) and are inhibitory to growth of fermenting cells (Klinke et al., 2003, 2004, 2001). These "pretreatment-derived inhibitors" are major barriers to an efficient CBP scheme due to the additional, generally prohibitive costs associated with their removal. Therefore, prospecting and construction of a CBP-organism in general, and engineering of promising contenders such as *C. thermocellum*, both demand a rigorous assessment of cellulosome activity in the CBP environment.

This study identified optimal enzymatic conditions for isolated cellulosomes and investigated the resistance of cellulosome to the pretreatment-derived and fermentation-produced compounds found in a CBP scheme. Furthermore, the experimentally established conditions were applied to *C. thermocellum* cultures to test whether conditions conducive for cellulosome activity were compatible with those for robust cellulose degradation and ethanol fermentation. Surprisingly, our results yielded the highest ethanol titer ever reported in *C. thermocellum*.

2. Methods

2.1. Material

All chemicals were purchased from Merck, Roche, USP, Ameresco or Sigma at the highest purity available. *C. thermocellum* JYT01, tolerating up to 3% (w/v) ethanol, is a derivative of *C. thermocellum* LQRI, kindly provided by Prof. Jizhong Zhou, University of Okalahoma, USA. *C. thermocellum* JYT01 was grown anaerobically in GS-2 medium (Johnson et al., 1981). The commercial cellulase (LS002610, Worthington Biochemical Corp., USA, hereafter referred to as "the cellulase") derived from the fungus *T. reesei* was used as a reference in all experiments. Microcrystalline cellulose was Avicel PH 101 (particle size ~50 μm) from Sigma.

2.2. Cellulosome preparation and enzyme activity measurement

Cellulosome was extracted from *C. thermocellum* according to the method described (Morag et al., 1992). Protein concentration was measured by the Bradford assay kit (Bio-rad) to maintain the batch-to-batch consistency of cellulosome.

To identify the optimal pH enabling maximal sugar release, 0.04 mg cellulosomes were added to 10 mg/ml Avicel crystalline cellulose in 1 ml GS-2 buffer (11 mM KH_2PO_4, 16.7 mM K_2HPO_4, 50 mM MOPS, 10 mM sodium citrate) with pH ranging from 4 to 10 (Macarron et al., 1993). Cellulose degradation reactions were incubated at 50 °C for 8 h (Boer and Koivula, 2003; Johnson et al., 1982). The enzyme activity in the first 8 h, in which the initial reaction rate could be modeled by Michaelis–Menten equation (Lee and Fan, 1983), was evaluated throughout this study (unless indicated otherwise).

To identify the optimal temperature enabling maximal sugar release, 0.04 mg cellulosomes were added to 10 mg/ml Avicel crystalline cellulose in 1 ml GS-2 buffer. The pH of the solution was 6.5, which was determined in pH experiments enabling maximal sugar release. The cellulase was incubated in GS-2 buffer in the same manner with the exception that the solution was buffered to pH 5.0, which was experimentally determined to induce maximal sugar release for cellulase. The reactions were incubated at temperatures ranging from 4 to 80 °C for 8 h.

The definition of one international unit (IU) of cellulosome enzyme activity is the amount of cellulosomes required to release 1 μmol "reducing sugar" in 1 h from Avicel microcrystalline cellulose at 65 °C/pH 6.5 (Han et al., 2005). One IU of enzyme activity for the cellulase is defined as the amount of cellulase required to release 1 μmol reducing sugar in 1 h from the Avicel microcrystalline cellulose at 55 °C/pH 5.0 (Liu et al., 2010). Thus, in the following experiments, a constant 2.78 IU enzyme was used per 10 mg Avicel microcrystalline cellulose, and the enzyme activities were measured via the release of reducing sugar.

The released sugar concentration was estimated by dinitrosalicylic acid (DNS) method using glucose as standard as described (Ghose, 1987), at time points of 0, 5, 15, 30, 60, 120, 240, and 480 min. The absorbance was measured at 490 nm rather than 540 nm as indicated by (Ghose, 1987), which was determined by full wavelength scanning at a GE GeneQuant1300 spectrophotometer (single beam, 190–1110 nm) (Fig. S1).

2.3. Effect of pretreatment-derived inhibitors (organic acids, furfural, phenolic compounds) and fermentation products (ethanol, organic acids) on cellulosome activity

Approximately 2.78 IU cellulosome or commercial cellulase were mixed with 10 mg Avicel microcrystalline cellulose in 1 ml GS-2 buffer containing organic acids and corresponding organic salts, ethanol, and phenols as indicated below. Concentrations of organic acid anions (sodium salts of formate, acetate and lactate) ranged from 0 to 1 M. Concentrations of organic acids (formic acid, acetic acid and lactic acid) ranged from 0 to 0.8 M. Concentrations of ethanol ranged from 0 to 3.2 M. Concentration of furfural, *p*-hydroxybenzoic acid and catechol ranged from 0 to 100 mM. Reactions were incubated at 65 °C/pH 6.5 for cellulosomes, or at 55 °C/pH 5.0 for cellulase in all experiments except those with organic acids.

Initial buffer pH for enzymatic assays containing organic acids was 7.4, which was consistent with the pH of GS-2 medium for the growth of *C. thermocellum*. Changes in the pH were monitored after addition of varying concentrations of formic acid, acetic acid or lactic acid.

The reactions were incubated for 8 h in a water bath. To measure sugar concentration in the reaction, 20 μl samples were removed from each tube at time points of 0, 1, 2, 4 and 8 h. The reducing sugar concentrations were measured by the colorimetric DNS method as described above for all experiments except assays containing furfural due to its reaction with DNS. Thus, D-glucose in furfural-containing samples was measured by the D-glucose kit (Megazyme, Ireland) following manufacturer's instructions. All experiments were performed in triplicate.

2.4. Thermostability of cellulosomes

Cellulosome and commercial cellulase (described above) were incubated in GS-2 buffer at temperatures ranging from 4 to 70 °C with durations ranging from 6 h to 6 days, followed by re-measurement of the enzyme activity. Reactions were incubated for 1 h at 65 °C/pH 6.5 for samples containing cellulosome or at 55 °C/pH 5.0 for cellulase. Concentration of reducing sugars was measured by colorimetric DNS method. Fresh cellulosome and cellulase were used as controls and their corresponding activities were designated as 100%. In parallel, 2.78 IU of cellulosome and fungi cellulase were incubated with 10 mg Avicel cellulose at 50 °C and at their corresponding optimized pH described above for 7 days (168 h). Samples were taken at intervals to measure reduced sugar concentrations.

2.5. Cellulose degradation of C. thermocellum JYT01 in the presence of exogenous formate, acetate and lactate in Hungate tubes

C. thermocellum JYT01 was cultured for 7 days at 60 °C without pH control in a sealed Hungate tube containing 100 ml GS-2 medium and 10 g/L cellulose. Exogenous formate, acetate and lactate were added at final concentrations of 50 mM, 25 mM and 25 mM, respectively; these concentrations enable maximal sugar release, as determined in Section 2.4. Cellulose concentration was measured using the H_2SO_4/phenol method as described (Haigler and Weimer, 1991). On day 7, the residual sugar and ethanol produced in the medium were measured.

Formate, acetate and lactate were measured using an ion chromatography system (Dionex ICS-3000) equipped with an IonPac® AG11-HC column (4 × 250 mm). Ethanol concentrations were quantified using an enzymatic K-ethanol kit (Megazyme, Ireland) according to manufacturer's protocol.

2.6. Ethanolic fermentation of C. thermocellum JYT01 monocultures in a 5 L bioreactor

Seed culture of *C. thermocellum* JYT01 was prepared in GS-2 medium supplemented with 0.5% cellulose, and transferred into 2 L of N_2-flushed GS-2 medium containing 0.5% Avicel microcrystalline cellulose in a 5 L bioreactor (Sartorius Stedim Biostat B-plus) with 1/100 inoculation ratio. Fermentation was performed at 60 °C with low speed stirring (40 rpm) and starting pH 7.4. Starting cellulose concentration was 5 g/L. After the first 24 h, whenever cellulose concentration was below 1 g/L, 20 g cellulose in GS-2 medium was loaded into the bioreactor. The pH was started and maintained as pH 7.4 from 0 to 36 h, changed to pH 6.5 gradually from 36 to 60 h, and kept constant at pH 6.5 from 60 h until the end of experiment (18 days). Concentrations of cellulose, sugar, ethanol, formate, acetate and lactate were measured every 12 h.

3. Results and discussion

3.1. Effect of pH and temperature on the activities of cellulosome and cellulase

To examine the pH and temperature ranges for cellulosome-mediated cellulose degradation, the cellulolytic activity was evaluated in the presence of cellulose substrate. Given that optimal temperature might change when varying pH, the optimal pH for cellulolytic activity was determined first at 50 °C, followed by determination of optimal temperature under the optimal pH. Maximum sugar release by cellulosome activity was observed at pH 6.5 (Fig. 1A) and at 70 °C (Fig. 1C). Cellulose was degraded by cellulosome with sugar release rates of 7.2×10^{-3}, 7.5×10^{-3}, 10.3×10^{-3}, 11.2×10^{-3} and 13.1×10^{-3} mg/min when examined at 50, 55, 60, 65, and 70 °C, respectively, at the optimal pH 6.5 (Fig. 1C). In contrast, maximal sugar release from cellulose by *T. reesei*-derived cellulase occurred at pH 5.0 (Fig. 1B) and temperature 50 °C (Fig. 1D). Cellulose was degraded by cellulase with sugar release rate of 14.8×10^{-3}, 14.4×10^{-3}, 14.1×10^{-3}, 13.8×10^{-3}, 11.1×10^{-3} mg/min when examined at 50, 55, 60, 65, and 70 °C, respectively, at the optimal pH 5.0 (Fig. 1D). This was consistent with previous findings that endoglucanase and cellobiohydrolase, the major components of cellulase from *T. reesei*, exhibited maximal enzymatic activity at pH 4–5 (Macarron et al., 1993) and 60 °C (Boer and Koivula, 2003). For cellulosome, our finding was consistent with previous observations that crude cellulosome exhibited maximal activity at 70 °C/pH 6.1 (Johnson et al., 1982). Hence, it was concluded that the maximal cellulosome-mediated cellulose degradation could be achieved at 70 °C/pH 6.5, while maximal cellulase-mediated cellulose degradation occurred at 50 °C/pH 5.0. Of great interest is that optimal conditions for growth of *C. thermocellum* (60 °C/pH 7.4) and for cellulosome activity (70 °C/pH 6.5) are in such proximity, which is beneficial to CBP system where *C. thermocellum* could be inoculated into bioreactor directly. Given that the activity of cellulosome was drastically reduced by 59% at 70 °C after 6 h, as compared to fresh cellulosome (Fig. 4C), the optimal temperature for cellulosome enabling maximal sugar release was designated as 65 °C.

3.2. Effect of organic acid anions (formate, acetate and lactate) on the activities of cellulosome and cellulase

Organic acids (e.g. acetic acid, lactic acid) are always concurrently generated during ethanol production by *C. thermocellum*. Organic acid itself causes both pH changes and anion group accumulation in aqueous solution. The pH changes drastically altered enzymes capability for cellulose degradation (Fig. 1A and B). To determine whether organic acid anions affect cellulosome-mediated cellulose degradation, enzymatic activity was quantified in the presence of sodium salts of formate, acetate and lactate (Fig. 2). Maximum cellulosome–cellulolytic activity was observed at 25 mM formate, while its activity was partially inhibited when formate concentration exceeded 0.5 M (Fig. 2A). During the first 8 h, within 1 mM to 0.5 M range, formate promoted cellulosome activity by 9.2% and 7.5%, while 1 M formate inhibited the activity by 16%, as compared to 0 mM formate (Fig. 2A). Similarly, for acetate, at the tested concentrations below 200 mM, cellulosome activity increases ranging from 17% (for 200 mM acetate) to 41% (for 25 mM acetate) were observed (Fig. 2C). Nevertheless, the cellulosome activities were also increased in the presence of lactate below 50 mM (Fig. 2E), as compared to 0 mM lactate, albeit only 5% and 17% increases were observed in the presence of 50 and 25 mM lactate, respectively. Therefore interestingly, these anions, which were found inhibiting cell growth, in fact promote cellulosome activity even at relatively higher concentrations, and negatively affect cellulosome activity only beyond a particular concentration. The concentrations of organic acids tested in this study are reasonable at industrial production of cellulosic ethanol. For example, formate and acetate were released by pretreatment to high concentrations (217 and 186 mM, respectively) in pretreated wheat straw and hardwood (Klinke et al., 2004) (Table 1). They were also produced by *C. thermocellum* cells and reached 60 and 230 mM (Fig. 6D). Taken together, formate and acetate could reach 277 and 416 mM, respectively, in a CBP system where both lignocellulose degradation and fermentation are combined in one bioreactor. Therefore, the formate and acetate at such concentrations, which are considered inhibitory to growth of cell, are still under the ranges of cellulosome tolerance.

However, these positive effects of organic anions on cellulosome were not found in the *T. reesei*-derived cellulase (Fig. 2B, D and F). It was recently observed that acetate at 150 mM improves ethanol yield of *Thermoanaerobacter ethanolicus* 39E and *C. thermocellum* for unknown reasons, whereas lactate exhibited no significantly effects on ethanol production (He et al., 2009). Therefore, further studies into why low concentrations of organic acid anions promote cellulosome activity are required. In particular, cellulosome activity significantly increased (by 41%) when adding less acetate, and reached maximum at 25 mM acetate. One possible explanation may be that active sites of certain cellulosome components harbor a moiety for acetate-binding, enabling substrate recognition in the presence of cellulose-acetate compounds (Correia et al., 2008).

Furthermore, at high concentrations of acetate and lactate, cellulosome exhibited significantly higher tolerance, as compared to *T. reesei*-derived cellulase. For instance, at 1 M acetate and

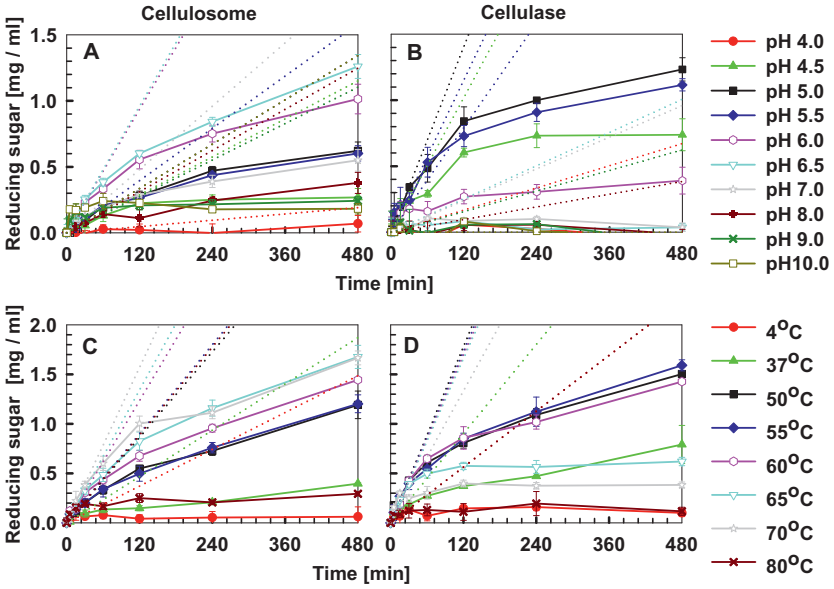

Fig. 1. Effects of pH and temperature on cellulolytic activities of cellulosome and cellulase. Enzymatic activity was measured via concentration of reducing sugar, which was released from 10 mg/ml Avicel microcrystalline cellulose in the presence of 0.04 mg cellulosome (A and C) and 0.02 mg cellulase (B and D). The reactions were incubated at 50 °C and pH ranging from pH 4.0 to 10 for 8 h (A and B). (C) Cellulosome activity was determined at pH 6.5 and temperatures ranging from 4 to 80 °C. (D) Cellulase activity was determined at pH 5.0 and temperatures ranging from 4 to 80 °C. All experiments were performed in triplicate and are shown with standard deviations. Degradation rates of cellulose were approximated by fitting the curves within the first 30 min, which are shown as regression lines (dotted lines).

lactate, the cellulosome still maintained 71% (Fig. 2C) and 43.7% (Fig. 2E) cellulolytic activity after 8 h at 65 °C, as compared to 0 mM organic anions, whereas cellulase activities were completely inhibited in the presence of 1 M acetate (Fig. 2D) and lactate (Fig. 2F). However, the cellulase activity was not affected by acetate at a concentration below 100 mM, which is consistent with previous observation that acetate below 50 mM failed to alter *T. reesei* cellulase activity (Szengyel and Zacchi, 2000). In contrast to *T. reesei, C. thermocellum* is an acetic acid producing thermophilic bacterium, indicating that proteins of *C. thermocellum* are evolutionarily acetate-adapted, potentially explaining the higher acetate tolerance of cellulosome than fungus cellulase.

Taken together, it was concluded that formate, acetate and lactate increased cellulolytic activities of cellulosome at a concentration below 100, 200 and 50 mM, respectively, whereas formate above 0.5 M, acetate above 1 M and lactate above 0.5 M were inhibitory to cellulosomal activity. Therefore, Clostridial cellulosome exhibits CBP-friendly features in comparison to the fungal cellulase, and might be a competitive alternative in enzymatic lignocellulose hydrolysis.

3.3. Effect of organic acids (formic acid, acetic acid and lactic acid) on the activities of cellulosome and cellulase

Those experiments at Sections 3.1 and 3.2 had been conducted either at different pH (same organic anions) or at different organic anion concentrations (same pH). However, in a CBP scheme, organic acids secreted by the metabolizing cells would gradually acidify the reaction. To evaluate how cellulosome and cellulase activities were affected in a gradually acidifying system, cellulose degradation assays were performed in the presence of formic acid (Fig. 3A and B), acetic acid (Fig. 3C and D) and lactic acid (Fig. 3E and F) ranging from 0 to 0.8 M in GS-2 buffer. The pH of the reaction system was measured after addition of the organic acids. These results demonstrated that over 80% of the cellulosome activity were lost when formic acid, acetic acid and lactic acid reached 0.1 M (Fig. 3A), 0.2 M (Fig. 3C) and 0.1 M (Fig. 3D), respectively. This was caused by pH dropping below 5.0, which was far beyond the optimal pH 6.5 of cellulosome. The *T. reesei*-derived cellulase was more resistant to acids than cellulosome (Fig. 3B, D and F), probably because the optimal pH of cellulase for cellulose degradation was 5.0.

Taken together, formic acid above 0.1 M, acetic acid above 0.2 M and lactic acid above 0.1 M can drastically acidify the GS-2 buffer system, resulting in inhibition of cellulosome activity. Therefore, pH control is essential to maintain the activity of cellulosome.

3.4. Effect of ethanol, furfural and phenols on the activities of cellulosome and cellulase

In a general CBP scheme, ethanol concentration could reach 1.5 M (7%, w/v) in industrialized cellulosic ethanol production (Lynd et al., 2008). To test whether cellulosome was sensitive to ethanol, the cellulosome activity was evaluated in the presence of up to 3.2 M ethanol. This experiment demonstrated that cellulosome activity decreased 17%, 21%, 36% and 55% after 8 h in the presence of 0.4, 0.8, 1.6 and 3.2 M ethanol, respectively, as compared to 0 mM ethanol (Fig. 4A). In contrast, activity of the *T. reesei*-derived cellulase had decreased 20%, 40%, 70% and 92% at ethanol concentrations of 0.4, 0.8, 1.6 and 3.2 M, respectively (Fig. 4B). Therefore, cellulosome exhibited higher ethanol tolerance than cellulase. We speculate that the ethanol tolerance of cellulosome could be traced to the "adapted evolution" inside the ethanolic microenvironment of the ethanologenic *C. thermocellum.*

Therefore it was concluded that cellulosome exhibited potential advantage over commercial cellulase regarding ethanol tolerance, the crucial features demanded by CBP, in which cellulose hydrolysis and fermentation take place in a single bioreactor. Due to the same reason, improving ethanol tolerance of cellulolytic enzymes is of great potential to industrial CBP from cellulose.

Furfural and phenols, released during pretreatment of lignocellulosic materials, are unfavorable side-products due to their inhibitory effects on cell growth (Klinke et al., 2004). High concentrations of phenolic compounds, primarily *p*-hydroxybenzoic acid and catechol, are found in pretreated lignocellulosic biomass (Klinke et al., 2004). To examine whether these degradation

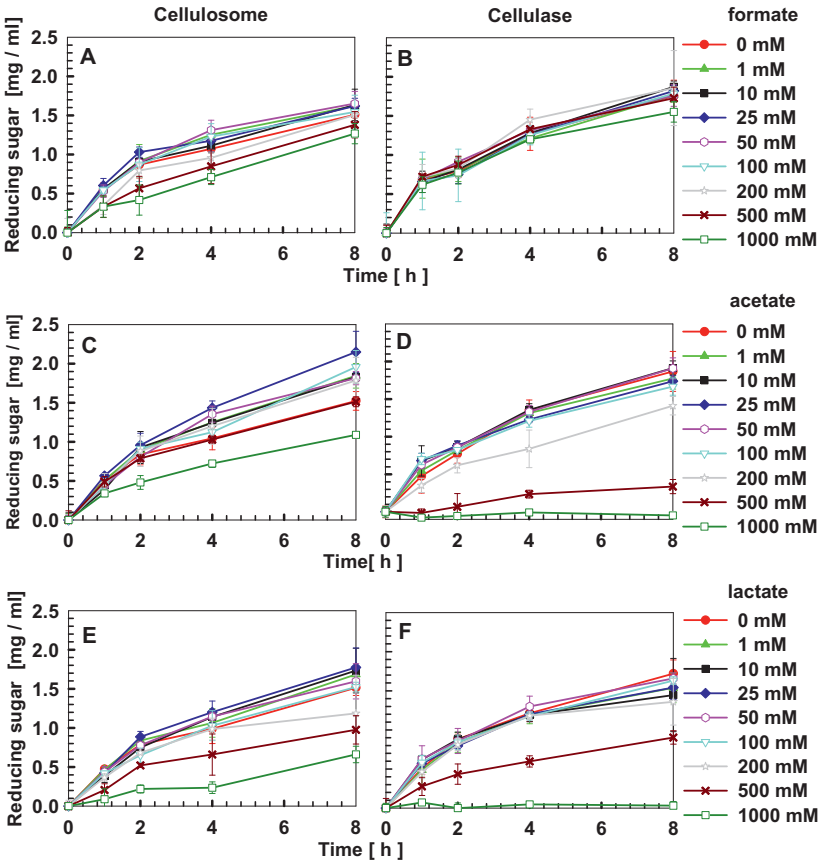

Fig. 2. Effects of organic acid anions on cellulolytic activities of cellulosome and cellulase. Cellulosome and cellulase activities were measured via concentrations of the reducing sugar, which was released from 10 mg/ml Avicel microcrystalline cellulose in the presence of 2.78 IU cellulosome or 2.78 IU cellulase. Reactions were incubated at pH 6.5 for cellulosome (A, C and E) and pH 5.0 for cellulase (B, D and F) in GS-2 buffer in the presence of formate (A and B), acetate (C and D) or lactate (E and F) sodium salts with final concentrations ranging from 0 to 1000 mM as indicated. Reactions were incubated at 65 and 55 °C water bath for cellulosome and cellulase, respectively. All experiments were performed in triplicate and are shown with their standard deviations.

products had any effects on cellulosome activity, cellulose degradation experiments were performed in the presence of furfural (Fig. 4C and D), *p*-hydroxybenzoic acid (Fig. 4E and F) and catechol (Fig. 4G and H) ranging from 0 to 100 mM. These results demonstrated that cellulosome activity was reduced 15% and 62% in the presence of 10 and 100 mM furfural, respectively, after 8 h, as compared to 0 mM furfural (Fig. 4C), whereas cellulase activity was unchanged under the same conditions (Fig. 4D). In addition, cellulosome activity was reduced 5% and 34% after 8 h in the presence of 50 and 100 mM *p*-hydroxybenzoic acid, respectively (Fig. 4E), whereas cellulase activity was drastically reduced 27% and 82% when examined in the presence of 50 and 100 mM *p*-hydroxybenzoic acid, respectively (Fig. 4F). In contrast, the cellulosome activity was reduced 42% and 56% after 8 h in the presence of 25 and 50 mM catechol, respectively (Fig. 4G), whereas cellulase activity was reduced 40% and 48% when examined in the presence of 25 and 50 mM catechol, respectively (Fig. 4H), as compared with 0 mM catechol. Therefore, improvement of tolerance to the pretreatment-derived products is important to both cellulosome and cellulase in simultaneous saccharification and co-fermentation.

Table 1
The minimal inhibitory concentrations (MIC) of the degradation products to *C. thermocellum*-derived cellulosome and *T. reesei*-derived cellulase.

	Highest level reported so far in pretreated hydrolysates (mM)	MIC[g] to cellulosome (mM)	MIC[g] to cellulase (mM)
Formate	217[a]	>1000	>1000
Acetate	186[b]	1000	500
Lactate	132[c]	500	500
Furfural	40[b]	25	>100
Ethanol	1520[d]	1600	800
p-Hydroxybenzoic acid	56[e]	>100	100
Catechol	9[f]	25	25

[a] Larsson et al. (1999), concentration up to 100 mM actually promotes cellulosome activity.
[b] Taherzadeh et al. (1997), concentration up to 200 mM acetate actually promotes cellulosome activity.
[c] Product of fermentation; 132 mM produced in this study. Concentration up to 50 mM actually promotes cellulosome activity.
[d] Assuming 7% (w/v) ethanol produced from fermentation in a CBP system (Lynd et al., 2008).
[e] Lee et al. (1999).
[f] Larsson et al. (2000).
[g] MIC is the minimal inhibitory concentration that results in loss of approximately 50% enzymatic sugar release activity after 8 h.

The enzymatic activities of cellulosome and cellulase were evaluated by the concentration of reducing sugars following the DNS method as described (Ghose, 1987). Both cellobiose (major end product of cellulosome, Lamed et al. (1991)) and glucose (major end product of cellulase, Chirico and Brown (1987)) could be quantitatively estimated by this colorimetric method. However, in the presence of furfural, the DNS method could result in a background absorption error due to absorbance of furfural at the wavelength around 500 nm, as well as a reaction between furfural and DNS (data not shown). Therefore, the D-glucose kit (Megazyme), which was based on the glucose oxidase/peroxidase enzymatic

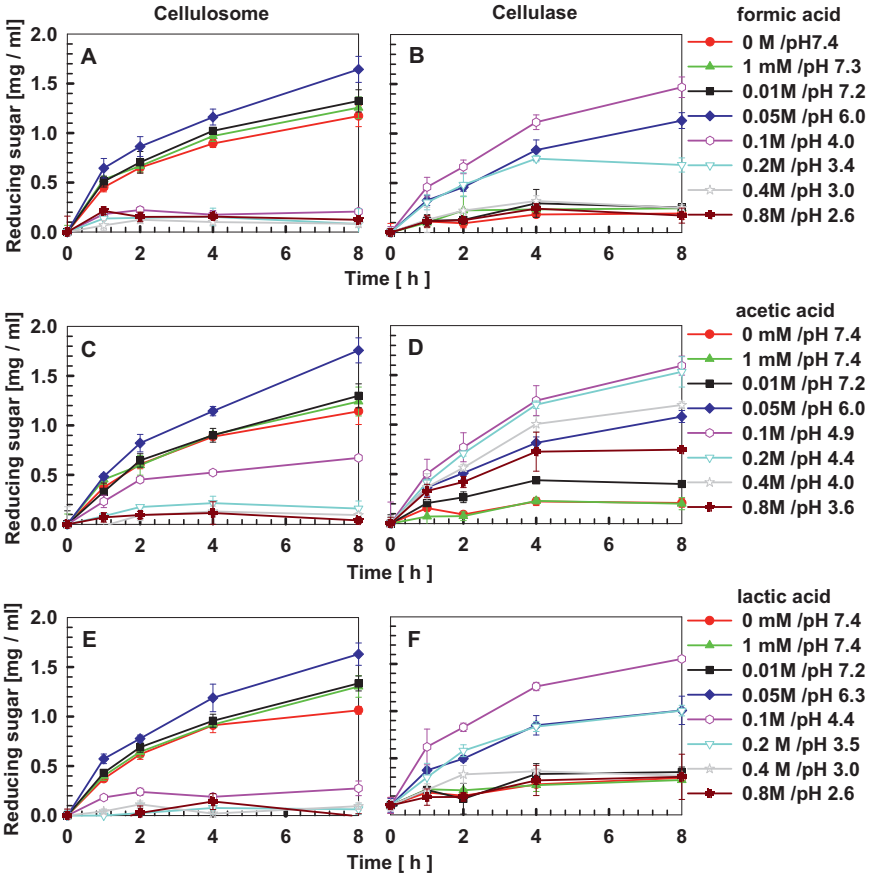

Fig. 3. Effects of organic acids on cellulolytic activities of cellulosome and cellulase. Cellulosome (A, C and E) and cellulase (B, D and F) activities were determined in the presence of formic acid (A and B), acetic acid (C and D) and lactic acid (E and F) ranging from 0 to 0.8 M as indicated. Reactions containing 10 mg/ml Avicel microcrystalline cellulose and 2.78 IU cellulosome or 2.78 IU cellulase were performed in GS-2 buffer and incubated at 65 and 55 °C water bath for cellulosome and cellulase, respectively. The initial pH of GS-2 buffer was 7.4. The pH of the GS-2 buffer system after adding the organic acids is shown on the right side of each organic acid concentration. All experiments were performed in triplicate and are shown with standard deviations.

procedures, was chosen to measure the reducing D-glucose in reactions (Fig. 4C and D). This result further suggested that glucose accounted for 17% of total reducing sugars in the end products of cellulosome (by comparing Fig. 4A and C), whereas 40% soluble glucose was present in the end products of cellulase (by comparing Fig. 4B and D)

Taken together, it was concluded that cellulosome was tolerant to 5 mM furfural, 50 mM *p*-hydroxybenzoic acid and 1 mM catechol without significant changes of its activity, whereas cellulase was tolerance to 100 mM furfural, 10 mM *p*-hydroxybenzoic acid and 1 mM catechol.

All chemical compounds released during pretreatment of lignocellulose might be inhibitory to cell growth at specific concentrations (Klinke et al., 2003, 2004, 2001). This could be reflected from the enzymes activities of specific cells. Therefore, the minimal inhibitory concentrations (MIC) of pretreatment-derived inhibitors and fermentation products to cellulosome and fungal cellulase activities were summarized in Table 1, in which cellulosome was found more tolerant to certain chemical inhibitors as described than cellulase, and thus could be more competitive in industrial CBP.

3.5. Thermostability of cellulosome and cellulase

In general, the cellulosic ethanol production under CBP mode may last for days or weeks at high temperature (Georgieva et al., 2008). To evaluate the thermostability of cellulosome and cellulase, these enzymes were incubated from 4 to 70 °C for 6 days. Then, the activities were reevaluated by sugar release. After 6 days incubation at 50, 65 and 70 °C, the activities of cellulosome were reduced 35%, 58% and 79%, respectively, as compared with fresh cellulosomes (Fig. 5A). In contrast, the cellulase activities were almost completely lost at temperatures higher than 65 °C (Fig. 5B), albeit 63% activity remains at 50 °C after 6 days. Thermostable enzymes offer great potential in the hydrolysis of lignocellulosic substrates in a prolonged fermentation process. Up to date, the majority of industrial cellulases used for cellulosic ethanol have been isolated from mesophilic organisms, mainly fungi. Thermophilic bacteria have, however, drawn greater interests as sources of such highly active and thermostable enzymes (Taylor et al., 2009; Viikari et al., 2007). Meanwhile, their thermostability was confirmed by monitoring released sugar under the same assay conditions. After 7-day incubation at 50 °C, the cellulose conversion rate by cellulosome was up to 76%, while fungi cellulase was 50% (Fig. 5C). Cellulase lost nearly all activities after 3 days (Fig. 5B and C). These findings suggested that cellulosomes could be more competitive through potentially achieving significant saving in the amount of externally added enzymes.

3.6. Cellulose degradation by cultured C. thermocellum JYT01 in sealed Hungate tubes without pH control

We demonstrated that activity of isolated cellulosome was promoted by low concentrations of formate, acetate and lactate

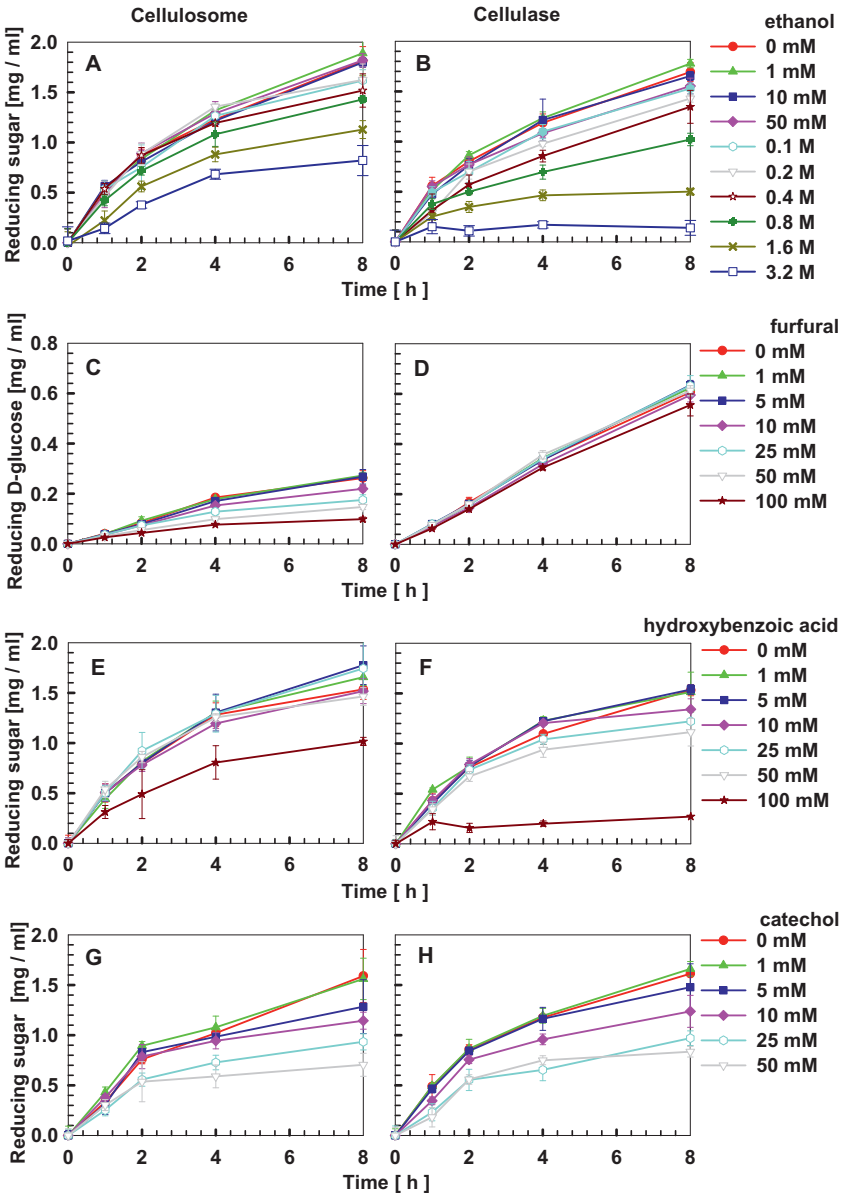

Fig. 4. Effects of ethanol, furfural and phenols on cellulolytic activities of cellulosome and cellulase. Cellulosome (A, C, E and G) and cellulase (B, D, F and H) activities were measured via concentrations of the reducing sugar. Reactions were incubated for 8 h at 65 °C/pH 6.5 for cellulosome or 55 °C/pH 5.0 for cellulase in GS-2 buffer in the presence of 0 mM to 3.2 M ethanol (A and B), 0 to 100 mM furfural (C and D), *p*-hydroxybenzoic acid (E and F) or catechol (G and H). D-glucose concentration was measured by D-glucose kit. All experiments were performed in triplicate and are shown with standard deviations.

in vitro (Fig. 2). To test whether exogenous organic acid anions could promote cellulose hydrolysis in a living-cell culture, the ethanol adapted *C. thermocellum* JYT01 was grown in GS-2 medium supplemented with or without formate, acetate or lactate. The promoting effects as measured by sugar release of formate, acetate and lactate on cellulose degradation of the isolated cellulosome were not observed in cultures of *C. thermocellum* (Fig. 6A). However, exogenous acetate and lactate increased ethanol yield by 16% and 10% (Fig. 6B) as compared to 0 mM exogenous organic salts, while formate caused more sugar accumulation (Fig. 6C). This sugar accumulation might account for gas (H_2 and CO_2) accumulation in the sealed bottle (Bothun et al., 2004). These results also showed that cellulose was completely degraded after 7 days in all cultures (Fig. 6A), suggesting that cellulosomes released by the cells were functional in the presence of exogenous organic acids. However, the conversion rate from cellulose to ethanol was merely 10.4–15.3%, indicating that this culture system was not optimal due to its inability to control pH and gas pressure in the sealed tubes.

It was concluded that 50 mM formate, 25 mM acetate or 25 mM lactate did not significantly alter cellulose degradation by *C. thermocellum*, albeit acetate slightly increased the ethanol yield.

3.7. Cellulosic ethanolic fermentation of *C. thermocellum* JYT01 in a bioreactor with pH control

To further probe the implication of these unique cellulosome-features to cellulosic ethanol production, we examined the performance of *C. thermocellum* under the pH and temperature conditions optimized for cellulosome activity. *C. thermocellum* JYT01 was fermented in a 5 L bioreactor with 2 L working volume. The bioreactor was loaded with cellulose with initial concentrations of 5 g/L, and was fed again with 20 g cellulose whenever its concentration fell below 1 g/L. Exogenous organic acid salts were

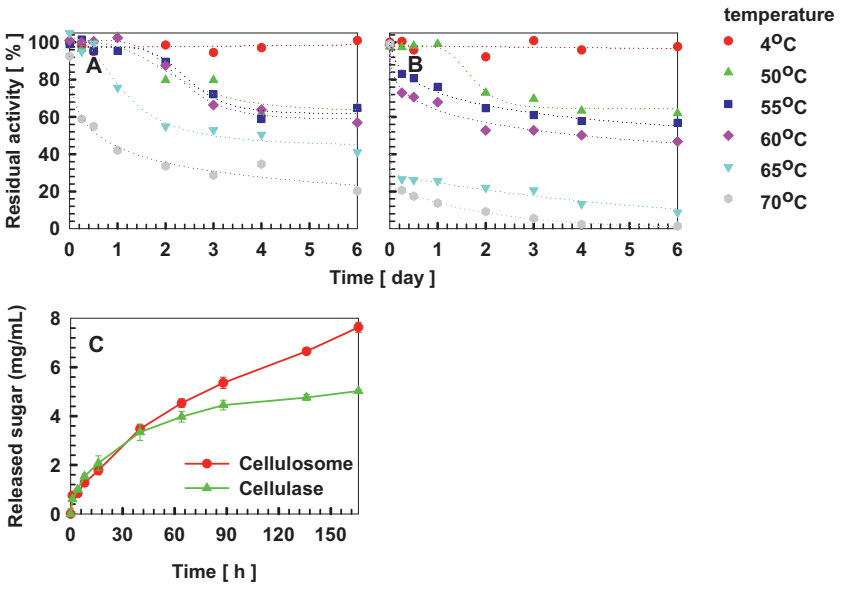

Fig. 5. Thermostability of cellulosome and cellulase. Cellulosome (A) and cellulase (B) activities were measured via concentrations of the reducing sugar. Reactions were incubated at pH 6.5 for cellulosome and pH 5.0 for cellulase in GS-2 buffer at temperature ranging from 4 to 70 °C (A and B). The curves were fitted with dotted lines as shown. Cellulose was incubated with cellulosome (pH 6.5) and cellulase (pH 5.0) at 50 °C for 168 h (C). The experiments performed in triplicate are shown with their standard deviations.

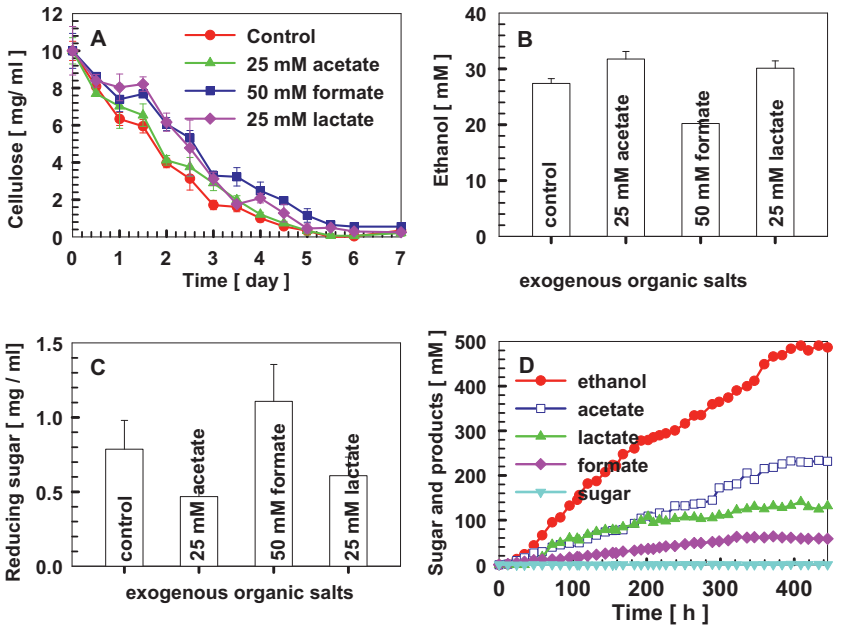

Fig. 6. Cellulose degradation and fermentation by *C. thermocellum* JYT01 in Hungate tubes and bioreactors. Four sets of *C. thermocellum* JYT01 cultures were grown in parallel in GS-2 medium supplemented with or without (control) 25 mM acetate, 50 mM formate or 25 mM lactate (A, B and C). Cellulose concentration was measured every 12 h (A). Concentrations of ethanol (B) and residual sugar (C) were measured at the end of experiments (on day 7). *C. thermocellum* JYT01 was cultured in a 5 L bioreactor with 2 L working volume (D). Concentrations of end products were measured every 12 h.

not added into the bioreactor due to their inhibitory effects on cell growth. After 18 days fermentation, a total of 137 g/L cellulose was completely degraded and no sugar accumulation was detected in the whole process (Fig. 6D), indicating that all reducing sugars released from cellulose were completely utilized by *C. thermocellum*. Furthermore, final ethanol concentration reached 491 mM (Fig. 6D), which was the highest yield reported so far using monoculture of *C. thermocellum* and has almost reached the cell's maximal ethanol tolerance (3%, w/v). This finding demonstrates the value and potential of defining and strategically tuning cellulosome activity in a CBP scheme.

However, 64 mM formate, 234 mM acetate and 132 mM lactate were also generated (Fig. 6D), suggesting that these branched pathways at least partially reduced ethanol yield. The average cellulose-to-ethanol conversion rate was only 17% after 154 h fermentation in contrast to the theoretically rate of 50%, indicating that *C. thermocellum* JYT01 needs to be engineered or further evolved to improve its ethanologenic capability. One possible strategy might be knocking out the branched organic acids pathways. However, even in the metabolically engineered thermophilic bacterium lacking organic-acid-producing pathways, the conversion rate from a mixture of monosaccharides to ethanol was only 38% (Shaw et al., 2008), albeit

37 g/L ethanol was produced ultimately. This monosaccharide-to-ethanol conversion rate was substantially lower than *Saccharomyces cerevisiae* (Lau and Dale, 2009), which produced 40 g/L ethanol with a conversion rate of 47%. To address this, a second strategy could be introducing other ethanologenic microbes in CBP systems in addition to *C. thermocellum*. However, we observed that no sugar accumulations were detected in bioreactor during the whole process of *C. thermocellum* culture (Fig. 6D); this might severely limit the performance of the introduced ethanologenic microbes. A third strategy that circumvents this challenge could use crude cellulosome as cellulase substitute in cellulosic biomass hydrolysis, while an ethanologenic thermophilic bacterium or thermotolerant yeast could be used to produce ethanol. Towards a CBP framework, these findings demonstrated the significance of defining and tuning cellulosome activity, suggested rational strategies to maximize cellulosome-ethanologen synergy and unleash the full potential of cellulosome, and should help guiding the herculean yet urgent endeavors in developing and engineering CBP systems.

4. Conclusions

In this study, the effects of organic acids, organic acid anions, ethanol, furfural and phenols on cellulosome activities were investigated and the minimal inhibitory concentrations (MIC) of these compounds to cellulosome and cellulose activities were summarized. In particular, formate, acetate and lactate with concentrations below 100, 200 and 50 mM, respectively, could increase cellulosome activities for cellulose degradation. Moreover, cellulosome exhibited higher ethanol tolerance and thermostability than cellulase, and was tolerant up to 5 mM furfural, 50 mM *p*-hydroxybenzoic acid and 1 mM catechol. Ultimately, 491 mM ethanol was generated from cellulose using the ethanol adapted *C. thermocellum* JYT01.

Acknowledgements

We thank Jason Huff, University of Alabama at Birmingham for critical reading of the manuscript. This work was funded by grants KSCX1-YW-11C2 from the Chinese Academy of Sciences, 30870572 from the National Natural Science Foundation of China, China-Israel Cooperation Project "Designer Cellulosome Systems" from the Ministry of Science and Technology of China, JQ200822 from the Young Investigator Award of Shandong Province to J.X. and by Shandong Provincial Key Laboratory of Energy-Microbe Genetics and CAS Key Laboratory of Biofuels.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2010.07.065.

References

Boer, H., Koivula, A., 2003. The relationship between thermal stability and pH optimum studied with wild-type and mutant *Trichoderma reesei* cellobiohydrolase Cel7A. Eur. J. Biochem. 270, 841–848.

Bothun, G.D., Knutson, B.L., Berberich, J.A., Strobel, H.J., Nokes, S.E., 2004. Metabolic selectivity and growth of *Clostridium thermocellum* in continuous culture under elevated hydrostatic pressure. Appl. Microbiol. Biotechnol. 65, 149–157.

Chirico, W.J., Brown Jr., R.D., 1987. Purification and characterization of a beta-glucosidase from *Trichoderma reesei*. Eur. J. Biochem. 165, 333–341.

Correia, M.A., Prates, J.A., Bras, J., Fontes, C.M., Newman, J.A., Lewis, R.J., Gilbert, H.J., Flint, J.E., 2008. Crystal structure of a cellulosomal family 3 carbohydrate esterase from *Clostridium thermocellum* provides insights into the mechanism of substrate recognition. J. Mol. Biol. 379, 64–72.

Demain, A.L., Newcomb, M., Wu, J.H., 2005. Cellulase, clostridia, and ethanol. Microbiol. Mol. Biol. Rev. 69, 124–154.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M., Kammen, D.M., 2006. Ethanol can contribute to energy and environmental goals. Science 311, 506–508.

Galbe, M., Zacchi, G., 2007. Pretreatment of lignocellulosic materials for efficient bioethanol production. Adv. Biochem. Eng. Biotechnol. 108, 41–65.

Georgieva, T.I., Mikkelsen, M.J., Ahring, B.K., 2008. Ethanol production from wet-exploded wheat straw hydrolysate by thermophilic anaerobic bacterium *Thermoanaerobacter* BG1L1 in a continuous immobilized reactor. Appl. Biochem. Biotechnol. 145, 99–110.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268.

Haigler, C.H., Weimer, P.J., 1991. Biosynthesis and Biodegradation of Cellulose. Marcel Dekker, Inc., New York.

Han, S.O., Yukawa, H., Inui, M., Doi, R.H., 2005. Effect of carbon source on the cellulosomal subpopulations of *Clostridium cellulovorans*. Microbiology 151, 1491–1497.

He, Q., Lokken, P.M., Chen, S., Zhou, J., 2009. Characterization of the impact of acetate and lactate on ethanolic fermentation by *Thermoanaerobacter ethanolicus*. Bioresour. Technol. 100, 5955–5965.

Herrero, A.A., Gomez, R.F., 1980. Development of ethanol tolerance in *Clostridium thermocellum*: effect of growth temperature. Appl. Environ. Microbiol. 40, 571–577.

Hill, J., Polasky, S., Nelson, E., Tilman, D., Huo, H., Ludwig, L., Neumann, J., Zheng, H., Bonta, D., 2009. Climate change and health costs of air emissions from biofuels and gasoline. Proc. Natl. Acad. Sci. USA 106, 2077–2082.

Johnson, E.A., Madia, A., Demain, A.L., 1981. Chemically defined minimal medium for growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum*. Appl. Environ. Microbiol. 41, 1060–1062.

Johnson, E.A., Sakajoh, M., Halliwell, G., Madia, A., Demain, A.L., 1982. Saccharification of complex cellulosic substrates by the cellulase system from *Clostridium thermocellum*. Appl. Environ. Microbiol. 43, 1125–1132.

Klinke, H.B., Olsson, L., Thomsen, A.B., Ahring, B.K., 2003. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of *Saccharomyces cerevisiae*: wet oxidation and fermentation by yeast. Biotechnol. Bioeng. 81, 738–747.

Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Appl. Microbiol. Biotechnol. 66, 10–26.

Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2001. Potential inhibitors from wet oxidation of wheat straw and their effect on growth and ethanol production by *Thermoanaerobacter mathranii*. Appl. Microbiol. Biotechnol. 57, 631–638.

Lamed, R., Kenig, R., Morgenstern, E., Calzada, J., De Micheo, F., Bayer, E., 1991. Efficient cellulose solubilization by a combined cellulosome-β-glucosidase system. Appl. Biochem. Biotechnol. 27, 173–183.

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N.-O., 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enz. Microb. Technol. 24, 151–159.

Larsson, S., Quintana-Sáinz, A., Reimann, A., Nilvebrant, N.-O., Jönsson, L., 2000. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by *Saccharomyces cerevisiae*. Appl. Biochem. Biotechnol. 84–86, 617–632.

Lau, M.W., Dale, B.E., 2009. Cellulosic ethanol production from AFEX-treated corn stover using *Saccharomyces cerevisiae* 424A(LNH-ST). Proc. Natl. Acad. Sci. USA 106, 1368–1373.

Lee, W., Lee, J., Shin, C., Park, S., Chang, H., Chang, Y., 1999. Ethanol production using concentrated oak wood hydrolysates and methods to detoxify. Appl. Biochem. Biotechnol. 78, 547–559.

Lee, Y.H., Fan, L.T., 1983. Kinetic studies of enzymatic hydrolysis of insoluble cellulose: II. Analysis of extended hydrolysis times. Biotechnol. Bioeng. 25, 939–966.

Liu, K., Lin, X., Yue, J., Li, X., Fang, X., Zhu, M., Lin, J., Qu, Y., Xiao, L., 2010. High concentration ethanol production from corncob residues by fed-batch strategy. Bioresour. Technol. 101, 4952–4958.

Liu, Z.L., Slininger, P.J., Dien, B.S., Berhow, M.A., Kurtzman, C.P., Gorsich, S.W., 2004. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J. Ind. Microbiol. Biotechnol. 31, 345–352.

Lynd, L.R., Grethlein, H.E., 1987. Hydrolysis of dilute acid pretreated mixed hardwood and purified microcrystalline cellulose by cell-free broth from *Clostridium thermocellum*. Biotechnol. Bioeng. 29, 92–100.

Lynd, L.R., Grethlein, H.E., Wolkin, R.H., 1989. Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum*. Appl. Environ. Microbiol. 55, 3131–3139.

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How biotech can transform biofuels. Nat. Biotechnol. 26, 169–172.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. 66, 506–577.

Macarron, R., Acebal, C., Castillon, M.P., Dominguez, J.M., de la Mata, I., Pettersson, G., Tomme, P., Claeyssens, M., 1993. Mode of action of endoglucanase III from *Trichoderma reesei*. Biochem. J. 289 (Pt 3), 867–873.

Morag, E., Bayer, E.A., Lamed, R., 1992. Affinity digestion for the near-total recovery of purified cellulosome from *Clostridium thermocellum*. Enz. Microb. Technol. 14, 289–292.

Rani, K.S., Seenayya, G., 1999. High ethanol tolerance of new isolates of *Clostridium thermocellum* strains SS21 and SS22. World J. Microbiol. Biotechnol. 15, 173–178.

Shaw, A.J., Podkaminer, K.K., Desai, S.G., Bardsley, J.S., Rogers, S.R., Thorne, P.G., Hogsett, D.A., Lynd, L.R., 2008. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. Proc. Natl. Acad. Sci. USA. 105, 13769–13774.

Szengyel, Z., Zacchi, G., 2000. Effect of acetic acid and furfural on cellulase production of *Trichoderma reesei* RUT C30. Appl. Biochem. Biotechnol. 89, 31–42.

Taherzadeh, M.J., Eklund, R., Gustafsson, L., Niklasson, C., Lidén, G., 1997. Characterization and fermentation of dilute-acid hydrolyzates from wood. Ind. Eng. Chem. Res. 36, 4659–4665.

Taylor, M.P., Eley, K.L., Martin, S., Tuffin, M.I., Burton, S.G., Cowan, D.A., 2009. Thermophilic ethanologenesis: future prospects for second-generation bioethanol production. Trends Biotechnol. 27, 398–405.

Viikari, L., Alapuranen, M., Puranen, T., Vehmaanpera, J., Siika-Aho, M., 2007. Thermostable enzymes in lignocellulose hydrolysis. Adv. Biochem. Eng. Biotechnol. 108, 121–145.

Xia, L., Shen, X., 2004. High-yield cellulase production by *Trichoderma reesei* ZU-02 on corn cob residue. Bioresour. Technol. 91, 259–262.

Zhang, Y.H., Lynd, L.R., 2005. Cellulose utilization by *Clostridium thermocellum*: bioenergetics and hydrolysis product assimilation. Proc. Natl. Acad. Sci. USA 102, 7321–7325.