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Factors influencing cellulosome activity in Consolidated Bioprocessing of cellulosic ethanol

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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

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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.