Determination of the cellulase activity distribution in *Clostridium thermocellum* and *Caldicellulosiruptor obsidiansis* cultures using fluorescent substrate

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ABSTRACT

This study took advantage of resorufin cellobioside as a fluorescent substrate to determine the distribution of cellulase activity in cellulosic biomass fermentation systems. Cellulolytic biofilms were found withholding nearly four orders greater cellulase activity than their suspended counterpart in cellulose-fed cultures of both *Clostridium thermocellum* and *Caldicellulosiruptor obsidiansis*, which can be primarily attributed to the high cell concentration as well as the surface attachment-boosted cell-specific cellulase activity. This distribution pattern offered the shortest migration distance for cellulases to travel from cell to cellulose, which could be a strategy cellulolytic bacteria employed to achieve the best energy gain in the course of insoluble substrate utilization. For the same reason, cellulase should be closely associated with the surfaces of suspended cell in soluble substrate-fed culture, which has been verified with cellobiose-fed cultures of *C. thermocellum* and *C. obsidiansis*. This study addressed the importance of cellulase activity distribution in cellulosic biomass fermentation, and provided theoretical foundation for the leading role of biofilm in cellulose degradation. System optimization and reactor design taking into consideration of the superiority of biofilms in cellulosic biomass hydrolysis may promise an improved cellulosic biofuel process.

Keywords

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Introduction

In a continued effort to promote cellulosic biofuels for securing domestic energy, protecting the environment, and expanding agricultural markets, improvements are needed to further reduce the processing costs to enable large-scale production of cellulosic biofuels (Dale, 2011; Huang et al., 2011). As a major factor affecting the rate of feedstock-to-biofuels conversion, cellulose hydrolysis has been the focus of extensive research and various approaches have been developed in attempt to expedite this bottleneck in an inexpensive way (Lynd et al., 2008; Huang et al., 2011). Microbial cellulose hydrolysis is one cost savings strategy that researchers are currently pursuing (Lynd et al., 2008). Cellulolytic microbes such as *Caldicellulosiruptor obsidiansis* secrete a wide variety of catalytic enzymes, broadly termed cellulases, to solubilize the complex and rigid structure of cellulose into monomers or oligomers small enough for cell assimilation (Lochner et al., 2011). In some other microbes like *Clostridium thermocellum*, several types of cellulases are organized into an elaborate multifunctional supramolecular complex, known as the cellulosome, facilitating not only hydrolysis but also microbial attachment on the cellulosic biomass (Miron et al., 2001). Our earlier study exhibited that, when fed with cellulose, these two thermophiles colonized cellulose surfaces independent of cellulosome production, and cellulose degradation was highly correlated to microbial surface attachment (Wang et al., 2011b). These data suggested that cellulases were not uniformly distributed in the cellulosic fermentation system. Following secretion, cellulases may be released into bulk solution, physically associated with suspended cells, or retained within the biofilms on the cellulose surface (**Fig. 1**). To date, the distribution pattern of cellulase activity in a microbial cellulosic biomass fermentation system has not been determined, although the distribution of extracellular enzymes has been broadly studied for mixed culture systems of wastewater treatment or natural aquatic environment (Hollibaugh and Azam, 1983; Hoppe, 1983; Somville and Billen, 1983; Logan et al., 1987; Chróst, 1989; Mayer, 1989; Frølund et al., 1995; Confer and Logan, 1997). The purpose of this study was to investigate the distribution of cellulases in a model microbial fermentation system and to shed light on the relative contributions of differentially localized cellulases to cellulose degradation. To this end, resorufin cellobioside, a model substrate with two glucose molecules bound to a fluorescent molecule was used to detect cellulase activity by measuring fluorescence intensity. Both *C. thermocellum*, a microbe that produces cellulosomes, and *C. obsidiansis*, a microbe that does not produce cellulosomes, were used as model organisms to examine if the production of cellulosomes affects the distribution of cellulase activity. It is anticipated that results of this study in conjunction with results from previous studies of microbial cellulose utilization will help

comprehend the importance of cellulase distribution in cellulosic biomass fermentation, and aid in elucidating the mechanism behind the predominant role of biofilms in cellulose hydrolysis for cellulosic biofuel production (Wang et al., 2011a; Wang et al., 2011b).

1 Materials and Methods

1.1 Microbial growth and microscopic analysis

C. thermocellum (ATCC 1313) and *C. obsidiansis* (ATCC BAA2073) were used as model microorganisms in this study. Medium composition and cultivation conditions were described earlier (Wang et al., 2011b). Briefly, anaerobic tubes, each containing 10 ml medium and nitrogen gas headspace, were inoculated with 10^{10} L⁻¹ cells and incubated under anaerobic conditions at 60°C for *C. thermocellum* and 75°C for *C. obsidiansis* with moderate shaking (60 r/min). In cellulose-fed cultures, identical chads (diameter of 6.00 ± 0.04 mm) were stamped from regenerated cellulose membranes (0.2 µm pore size, Whatman RC58, UK), and added to the culture as the sole carbon source for microbial growth. In cellobiose-fed cultures, 0.4% cellobiose was added as the sole carbon source. Replicate cultures were incubated for 24 hours before analysis. Samples were prepared for microscopy as described earlier (Wang et al., 2011b). Briefly, samples were stained with Syto9 (Invitrogen, Carlsbad, CA) to visualize the distribution of bacterial cells using a confocal laser scanning microscope (Zeiss LSM 710, Jena, Germany). The suspended cell concentration was determined using a Thoma cell counting chamber (Blaubrand, Germany) and an Axioskop2 Plus microscope (Zeiss, USA) with phase contrast illumination. ImageJ software (Version 1.42q, NIH, USA) was used for image analysis and cell quantification (Wang et al., 2011b).

1.2 Cellulase activity analysis

A fluorescent cellulase assay kit (M1245, MarkerGene, US) was employed for cellulase activity analysis. This kit contains resorufin cellobioside, a substrate that releases a fluorescent compound, namely resorufin, upon cleavage. Activity measurements were obtained by determining fluorescence intensity at room temperature in a Plate Reader (SynergyMx, Bio-Tek Instruments, US) at Ex/Em = 571/585. Sample and reagent composition for each analysis in 96-well plates is described in **Table 1**. Briefly, 100 µl 0.5 mM resorufin cellobioside substrate was mixed with 100 µl samples or fresh medium, plus one piece of cellulose chad in the case of biofilm analysis. Cell-free cellulase samples were prepared by filtering the culture supernatant through a 0.22 µm syringe filter. Fluorescence

intensities were compared to a standard curve according to manufacturer's instructions (M1245, MarkerGene, USA).

2 Results and discussion

2.1 Cell distribution in cellulose- and cellobiose-fed cultures of *C. thermocellum* and *C. obsidiansis*

After 24 hours growth, both *C. thermocellum* and *C. obsidiansis* cells were found attaching to the cellulose chad surface (**Fig. 2**). During the same timeframe, the concentration of suspended cells (X) also increased nearly eight times from the inoculum concentration of 10^{10} cell/L to 8.80×10^{10} cell/L for *C. thermocellum* and to 8.00×10^{10} cell/L for *C. obsidiansis* (**Table 2**). Because the hydrolysate concentration in the supernatant is insufficient to support microbial growth (the cellulose chad is the sole carbon source), we concluded in our previous work that the rate of cell growth on and detachment from the cellulose likely explains the increased number of suspended cells (Wang et al., 2011a). Consistent with this, microscopic observation of the cellulose chad confirmed the majority of cells were associated with the cellulose surface with concentration on the scale of 10^{14} cell/L for both *C. thermocellum* and *C. obsidiansis* (**Table 2**). This is nearly four-orders of magnitude above the concentration of their suspended counterparts in the supernatant (**Table 2**). In contrast, the cultures of *C. thermocellum* and *C. obsidiansis* grown on a soluble substrate (cellobiose) reached a final cell concentration of only 10^{12} cell/L (**Table 2**).

2.2 Cellulase distribution in cellulose- and cellobiose-fed cultures of *C. thermocellum* and *C. obsidiansis*

Being excreted out of cellulolytic cells, cellulases need to drift and migrate all the way across the medium solution onto the surface of cellulosic biomass to bind with cellulose molecules for cellulolysis to occur (**Fig. 1**). Thus it can be seen, the migration distance, or in another words, the distribution of cellulase relative to the locations of their producers and targets plays a critical role in determination of the energy efficiency of cellulose degradation and microbial growth in a fermentation system. In light of the microbial distribution observed above, three possible locations can be hypothesized for cellulase activity to reside in a given cellulosic biomass fermentation system (**Fig. 1a**), i.e., (1) associated with the biofilms colonizing cellulosic biomass surfaces; (2) associated with cells suspended in supernatant; or (3) roaming in supernatant without any cell association. To test these hypotheses, cellulose chads were

sampled to quantify cellulase activity in association with biofilms. Culture supernatants were sampled to quantify the sum of the supernatant cellulase activity either with or without association with suspended cells. Supernatant filtrate through 0.22 μm pore-size syringe filters were used to quantify cell-free cellulase activity only. The extent of resorufin cellobioside hydrolysis measured with a plate-reader was normalized to the sample volume in **Figs. 3 and 4** to compare volumetric cellulase activity within each type of sample. In the case of biofilm, the sample volume was measured by multiplying chad surface area with biofilm thickness determined from confocal images, which was $2.21 \pm 0.42 \mu\text{m}$ for *C. thermocellum* and $2.02 \pm 0.23 \mu\text{m}$ for *C. obsidiansis* biofilms. The maximum volumetric resorufin cellobioside hydrolysis rates measured from profiles in **Figs. 3 and 4** were translated to the cellulase activity (V_m) in **Table 2**. Both resorufin cellobioside hydrolysis profiles (**Fig. 3**) and cellulase activity (**Table 2**) clearly show that the magnitudes of cellulase activity on per unit volume of biofilm basis were four-order above those of supernatants for both *C. thermocellum* and *C. obsidiansis* cultures, suggesting cellulases were overwhelmingly associated with biofilms, which may explain the leading role of biofilms in cellulose hydrolysis reported earlier (Wang et al., 2011b). In contrast, only marginal differences in cellulase activities were found between supernatants and filtrates, indicating most of the supernatant cellulases were actually not associated with suspended cells (**Fig. 3**). Comparing these two organisms, *C. thermocellum* appeared to have slightly greater cellulase activity over that of the *C. obsidiansis* in all three locations of the cellulose-fed cultures (**Fig. 3; Table 2**).

Without biofilm formation, cellulases should be either cell-free or associated with suspended cells in cellobiose-fed cultures (**Fig. 1b**). **Fig. 4** as well as **Table 2** exhibited that the supernatants of cellobiose-fed cultures of *C. thermocellum* and *C. obsidiansis* demonstrated five times faster cellulase activity than did their filtrates, indicating that the majority of cellulases was actually associated with the surfaces of suspended cell, with only minor activity in the cell-free supernatant. This is opposite to the cellulase distribution within the supernatant of cellulose-fed cultures (**Fig. 3**). The same was observed for both *C. thermocellum* and *C. obsidiansis* cultures (**Fig. 4**).

2.3 Cell-specific cellulase distribution in cellulose- and cellobiose-fed cultures of *C. thermocellum* and *C. obsidiansis*

The magnitude of cellulase activity is correlated with the quantity of cellulolytic cells contained in a sample, which might be a primary reason accounting for the exceptionally high cellulase activity measured with biofilms in both *C. thermocellum* and *C. obsidiansis* cultures (**Table 2**). To make a

comparison on an individual cell basis, cellulase activity (V_m) was normalized to the sample cell concentration (X) to calculate a cell-specific cellulase activity (V_m/X). Results show that individual biofilm cell still held an average of four-fold greater cellulase activity than did the suspended counterparts in cellulose-fed cultures of *C. thermocellum* and *C. obsidiansis* (**Table 2**). For both organisms, cellulose-fed suspended cells possessed eight times more cell-specific cellulase activity than did the cellobiose-fed suspended cells (**Tables 2 and 3**).

2.4 Mechanism of the cellulase activity distribution

Cellulases were synthesized and excreted by cellulolytic bacteria to hydrolyze the polymeric structure of cellulose for assimilation. To carry out hydrolysis activity, cellulases need to make contact and bind with cellulose molecules. This requires cellulases to travel to the cellulose surface upon excretion out of the cellulolytic cell surface. In turn, hydrolysate produced at the cellulase-cellulose binding site needs to migrate back to the surfaces of cellulolytic cell for ingestion. Basically, the length of this migration distance would significantly influence the growth efficiency of cellulolytic bacteria. A long transportation line would dilute the concentration of cellulases that can reach cellulose surface as well as the concentration of hydrolysate that cellulolytic cells can receive. Theoretically, the energy received from hydrolysate has to be greater than the energy spent on cellulase synthesis for cellulolytic bacteria to sustain. In this sense, the relative distance of cellulases to their producer and food source is so critical that it would determine whether the producer has the ability to persist and prosper. Undoubtedly, the closer this distance is, the better chance cellulolytic bacteria will have to prevail.

In cellulose-fed cultures, apparently, the shortest cellulase migration distance would be achieved when cellulolytic bacteria are attaching on cellulose surface in the form of biofilm, in which cellulase can be positioned right next to and in between the cell and cellulose (**Fig. 1a**). This strategic location will ensure the best cellulase exercise and hydrolysate capture for cellulolytic bacteria. Therefore, it should not be difficult to understand why both *C. thermocellum* and *C. obsidiansis* have concentrated their limited cellulase resources in biofilms on the surfaces of cellulose chad (**Table 2**). As for the minor amount of cell-free cellulases floating in the supernatant of cellulose-fed cultures, they were most likely originated from detached biofilms for two reasons. Firstly, suspended cells were positioned far from cellulose surface as compared to biofilm cells, so energetically it wouldn't be favorable for them to launch cellulases for cellulose utilization. Secondly, the growth of suspended cells should be minimum because of the extremely low hydrolysate level in the supernatant of a cellulose-fed culture (Wang et al.,

2011a). These provide good explanations for why cellulases in the supernatant of cellulose-fed *C. thermocellum* and *C. obsidiansis* were not cell-associated (**Table 2**). In contrast, due to the soluble nature of cellobiose, *C. thermocellum* and *C. obsidiansis* were completely immersed in their food broth in cellobiose-fed cultures (**Fig. 1b**). This means, a negligible cell-to-substrate gap can be presumed wherever cellulolytic cells wander in the medium solutions. Thus, cellulase distribution right at the cell-to-substrate interface, namely the surface of suspended cells, will give the shortest cellulase migration distance and thus the best energy gain (**Fig. 1b**). Probably for this reason, overwhelming cellulase activities were found associated with suspended cells in cellobiose-fed cultures of *C. thermocellum* and *C. obsidiansis* (**Table 2**). A general rule that can be found here is that: cellulases tend to find the narrowest interface between cellulolytic cells and cellulosic biomass to reside in. Interface like this is located within biofilms colonizing insoluble substrate surface or at the surfaces of cells suspended in a soluble substrate-fed culture (**Fig. 1**).

The advantage of biofilms in cellulosic biomass degradation has received widespread attention for decades (Weimer et al., 1993; Miron et al., 2001; Burrell et al., 2004; Song et al., 2005; Shinkai and Kobayashi, 2007; Wang et al., 2011b). This study provided fundamental mechanism underlining the efficacy of biofilms in retaining and exercising limited cellulase resources for focused cellulosis on cellulose surfaces (**Table 2**). Several intrinsic characteristics of biofilms may account for their superiority in cellulase withholding. First of all, biofilms usually pack thick cell crowd (**Fig. 2**) in slimy extracellular polysaccharides matrices (EPSs), which may directly hinder enzymes from being washed into convectional flow (Vetter et al., 1998; Flemming and Wingender, 2001; Wang and Chen, 2009). Excessive EPS production has been reported with numerous cellulolytic organisms (Desvaux and Petitdemange, 2001; Kenyon et al., 2005; Weimer et al., 2006) and thermophiles (Marshall et al., 2001; Nicolaus et al., 2004). Cellulases were also found with high adsorption affinity for EPSs (Lu et al., 2006). Cellulosomes are known to have ultrastructure in connection with the surface of *C. thermocellum* (Bayer and Lamed, 1986), however very little is known about how *C. obsidiansis* has retained massive cellulases on their surfaces without such a tethering structure. Finding from this study that both organisms didn't show much difference in cellulase activity distribution (**Figs. 3 and 4**) suggested some mechanism other than cellulosomes adhesion might exist and account for the excellent cellulase withholding ability of cellulolytic microbes. It is noteworthy that biofilms are actually renowned for their outstanding extracellular enzymes withholding ability. Wastewater treatment process has been utilizing biofilms as robust biocatalysts for decades. It has been estimated that over 90% of extracellular

enzymes remain cell-associated and immobilized within the biofilms structure (Chróst, 1989; Frølund et al., 1995; Confer and Logan, 1997). The same conclusion also extends to fungal biofilms when measuring the distribution of cellulase, lignocellulolytic enzymes, endoglucanase activity, or xylanase activity (Villena and Gutiérrez-Correa, 2006; Gamarra et al., 2010).

Compared on individual cell basis, biofilms formed by *C. thermocellum* and *C. obsidiansis* also demonstrated significantly higher cell-specific cellulase activity than their suspended counterparts (**Table 2**). This observation implied that *C. thermocellum* and *C. obsidiansis* might have shifted to a higher cellulase activity phenotype upon switching from suspended to attached lifestyles. Similar phenomena that biofilm formation boosted individual cell's cellulase yield and activity has drawn researchers' attention earlier (Lu et al., 2006; Gamarra et al., 2010). Evolution requires natural system to selectively reward microbes expressing superior phenotypes that best adapt to the culturing environment. For biofilms colonizing and utilizing an insoluble substrate such as cellulose, the system should selectively reward strains that exhibit increased cellulosis. A mass transport model by Fan et al. (2005) demonstrated that a hydrolysis-capable mutant with increased enzyme production will have a better chance to outcompete others when the enzyme is tethered with cell surfaces in both insoluble and soluble substrate-fed cultures. Besides, the swarming cellular environment of biofilms actually provides an ideal condition for cell-to-cell gene or signal horizontal transfer and thus mutant creation (Sorensen et al., 2005; Nadell et al., 2008). It has been demonstrated that proteins involved in virulence, adhesion, and resistance were more abundant under biofilm growth conditions (Allegrucci et al., 2006). Nevertheless, very little is currently known about how the expression of cellulase phenotype has been affected by biofilm attachment on cellulose surface. Our ongoing proteomic study holds promise to shed light on this unknown.

3 Conclusions

Cellulolytic biofilms accumulated nearly four orders greater cellulase activity than their suspended counterpart in cellulose-fed cultures of both *C. thermocellum* and *C. obsidiansis*. This pattern of biofilm-concentrated cellulase distribution offers the shortest migration distance for cellulases to migrate from cell to cellulose, which could be a strategy cellulolytic bacteria employed to achieve the best energy gain in the course of insoluble substrate utilization. For the same reason, cellulases were closely associated with the surfaces of suspended cells in soluble substrate-fed culture, which has been verified with cellobiose-fed cultures of *C. thermocellum* and *C. obsidiansis*.

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Fig. 1 Illustrative distributions of cell, cellulase, hydrolysate and cellobiose in (a) cellulose- and (b) cellobiose-fed cultures.

Fig. 2 Confocal images of *C. thermocellum* and *C. obsidiansis* biofilms attaching on cellulose chad.

Fig. 3 Resorufin cellobioside (RC) hydrolysis by biofilms, supernatant, and filtrate sampled from *C. thermocellum* and *C. obsidiansis* cultures feeding on cellulose chad; portraits the error range.

Fig. 4 Resorufin cellobioside (RC) hydrolysis by supernatant and filtrate sampled from *C. thermocellum* and *C. obsidiansis* cultures feeding on cellobiose; portraits the error range.

Fig. 1

Fig. 3

Fig. 4

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Table 1. Sample and reagent composition in 96-well plate for cellulase activity analysis.

	Cellulose-fed culture				Cellobiose-fed culture	
Sample	Control	Biofilm	Supernatant	Filtrate	Control	Supernatant
Composition	1 fresh-chad	1 biofilm-chad	1 fresh-chad	1 fresh-chad	1 fresh-chad	1 fresh-chad
	100 µl medium	100 µl medium	100 µl supernatant	100 µl filtrate	100 µl medium	100 µl supernatant
	100 µl substrate	100 µl substrate	100 µl substrate	100 µl substrate	100 µl substrate	100 µl substrate

Fresh-chad denotes blank cellulose chad; biofilm-chad denotes biofilm-colonized cellulose chad; medium denotes sterilized growth medium without carbon source; substrate denotes resorufin cellobioside; supernatant denotes bulk solution with suspended cells; filtrate denotes bulk solution without suspended cells.

Table 2. *C. thermocellum* or *C. obsidiansis* cellulase activity (V_m), cell concentration (X), and cell-specific cellulase activity (V_m/X)

Parameter	Cellulose-fed culture			Cellobiose-fed culture	
	Biofilm	Supernatant	Filtrate	Supernatant	Filtrate
<i>C. thermocellum</i>					
V_m (µmol/(L·min))	5212.50±324.50	0.50±0.01	0.39±0.01	0.93±0.01	0.18±0.11
X (cell/L)	$3.22±0.27×10^{14}$	$8.80±0.91×10^{10}$	-	$1.20±0.12×10^{12}$	-
V_m/X (µmol/(cell·min))	$1.62×10^{-11}$	$5.68×10^{-12}$	-	$7.75×10^{-13}$	-
<i>C. obsidiansis</i>					
V_m (µmol/(L·min))	4759.40±96.40	0.47±0.03	0.40±0.12	0.73±0.01	0.15±0.02
X (cell/L)	$1.80±0.11×10^{14}$	$8.00±0.88×10^{10}$	-	$1.00±0.23×10^{12}$	-
V_m/X (µmol/(cell·min))	$2.64×10^{-11}$	$5.88×10^{-12}$	-	$7.30×10^{-13}$	-

Graphical abstract

Cellulolytic biofilms were found withholding nearly four orders greater cellulase activity than their suspended counterpart in cellulose-fed cultures of both *Clostridium thermocellum* and *Caldicellulosiruptor obsidiansis*, which can be primarily attributed to the high cell concentration as well as the surface attachment-boosted cell-specific cellulase activity.