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Determination of the cellulase activity distribution in *Clostridium thermocellum* and *Caldicellulosiruptor obsidiansis* cultures using a 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 to express nearly four orders greater cellulase activity compared to planktonic cultures of *Clostridium thermocellum* and *Caldicellulosiruptor obsidiansis*, which can be primarily attributed to the high cell concentration and surface attachment. The formation of biofilms results in cellulases being secreted close to their substrates, which appears to be an energetically favorable stategy for insoluble substrate utilization. For the same reason, cellulases 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 designs that promote biofilm formation in cellulosic biomass hydrolysis may promise an improved cellulosic biofuel process.

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-biofuel conversion, cellulose hydrolysis has been the focus of extensive research and various approaches have been developed in an attempt to overcome this bottleneck in an inexpensive way (Lynd et al., 2008; Huang et al., 2011). Microbial cellulose hydrolysis is one economical 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).

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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 environments (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 determine 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 hr before analysis. Samples were prepared for microscopy as described earlier (Wang et al., 2011b). Briefly, samples were stained with Syto9 (Invitrogen, Carlsbad, California, USA) 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, Wertheim, Baden-Württemberg, Germany) and an Axioskop2 Plus microscope (Zeiss, Oberkochen, Baden-Württemberg, Germany) with phase contrast illumination. ImageJ software (Version 1.42q, NIH, Bethesda, Maryland, 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, Eugene, Oregon, USA) 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,Winooski, Vermont, USA) 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, Eugene, Oregon, USA).

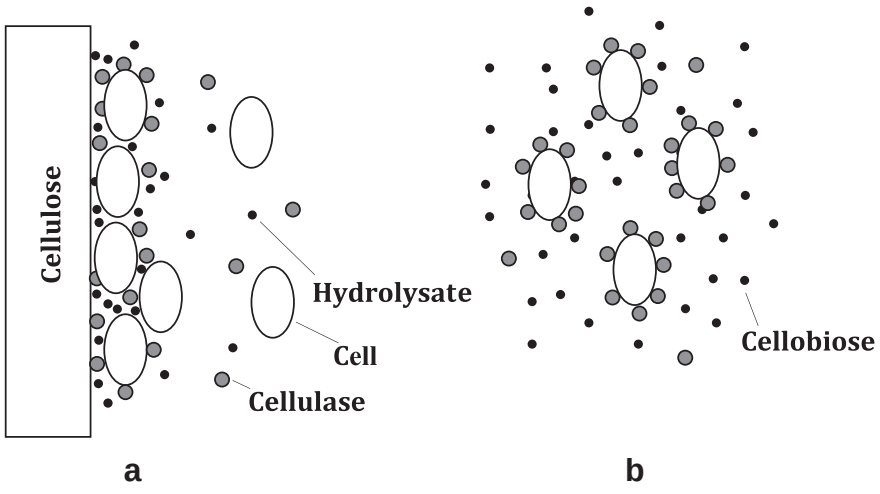

Fig. 1 – Illustrative distributions of cell, cellulase, hydrolysate and cellobiose in a) cellulose- and b) cellobiose-fed cultures.

2. Results and discussion

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

After 24 hour 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^{-1} to 8.80×10^{10} cell L^{-1} for *C. thermocellum* and to 8.00×10^{10} cell L^{-1} 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 that the majority of cells were associated with the cellulose surface with a concentration on the scale of 10^{14} cell L^{-1} for both *C. thermocellum* and *C. obsidiansis* (Tables 2 and 3). This is nearly four-orders of magnitude above the concentration of suspended cells (Tables 2 and 3). 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^{-1} (Tables 2 and 3).

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

Once secreted, cellulases must bind to cellulose molecules for cellulose degradation. (Fig. 1). Thus the distance between the cellulase-producing cell and the cellulose substrate 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, cellulases may be (Fig. 1a), *i.e.*, i) associated with the surface attached cells, or iii) present in the 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 was 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 surface attached biofilms, the sample volume was measured by multiplying chad surface area with biofilm thickness determined from confocal images, which was 2.21 ± 0.42 μm for *C. thermocellum* and 2.02 ± 0.23 μm 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 (Tables 2 and 3) clearly show that cellulase activity per unit volume of biofilm were four-orders of magnitude greater than those of supernatants for both *C. thermocellum* and *C. obsidiansis* cultures, suggesting that cellulases were overwhelmingly associated with biofilms, which may explain the prominent 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 that 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; Tables 2 and 3).

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 Tables 2 and 3 show that the supernatants of cellobiose-fed cultures of *C. thermocellum* and *C. obsidiansis* demonstrated five times higher cellulase activity than did their filtrates, indicating that the majority of cellulases were actually associated with the surfaces of suspended cells, 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 (Tables 2 and 3). 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 cells in a biofilm still held an average of

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

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

Note: 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.

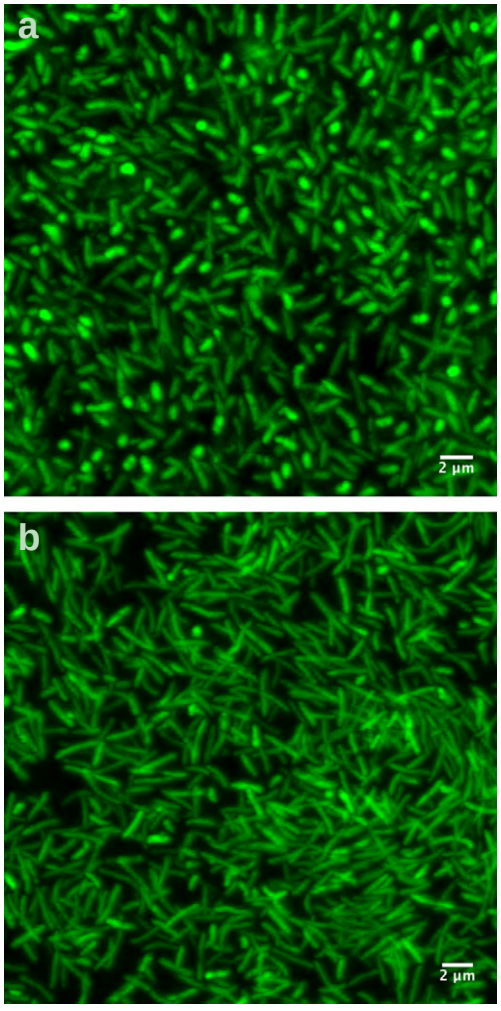

Fig. 2 – Confocal images of a) *C. thermocellum* and b) *C. obsidiansis* biofilms attaching on cellulose chad.

four-fold greater cellulase activity than did the suspended cells in cellulose-fed cultures of *C. thermocellum* and *C. obsidiansis* (Tables 2 and 3). 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 secreted 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 secretion 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 cells for ingestion. In short, the distance between the cell and the site of cellulose degradation would significantly influence the growth efficiency of cellulolytic bacteria. A long distance between the cell and cellulose substrate would have a negative impact on growth due to diffusion of both the cellulases and hydrolysate. Theoretically, the energy received from hydrolysate has to be greater than the energy spent on cellulase synthesis for cellulolytic bacteria to grow. 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, the shortest distance between the cellulase-producing cell and the cellulose substrate 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 should result in high cellulase activity 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 (Tables 2 and 3). Similarly, growth of *C. thermocellum* and *C. obsidiansis* in soluble cellobiose resulted in cellulase activity associated with the cell surface. Thus, cellulase distribution right at the cell-to-substrate interface, namely the surface of suspended cells, was the most energetically favorable (Fig. 1b). Interestingly, the cellulase activity associated with cells growing in a biofilm was higher than that of cells growing in suspension even after cell numbers were normalized. This suggests that the formation of a biofilm is advantageous for cellulolytic bacteria (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 suggests a fundamental mechanism underlining the efficacy of biofilms in retaining limited cellulase resources for focused cellulosis on cellulose surfaces (Tables 2 and 3). First of all, biofilms are characterized by high numbers of cells encapsulated in extracellular polysaccharide matrices (EPS) which may directly limit diffusion of cellulase enzymes (Vetter et al., 1998; Flemming and Wingender, 2001; Wang

Table 2 – *C. thermocellum* 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
V_m (μmol L^{-1} min^{-1})	5212.50 ± 324.50	0.50 ± 0.01	0.39 ± 0.01	0.93 ± 0.01	0.18 ± 0.11
X (cell L^{-1})	3.22 ± 0.27 × 10^{14}	8.80 ± 0.91 × 10^{10}	–	1.20 ± 0.12 × 10^{12}	–
V_m/X (μmol $cell^{-1}$ min^{-1})	1.62 × 10^{-11}	5.68 × 10^{-12}	–	7.75 × 10^{-13}	–

Table 3 – *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
V_m (μmol L^{-1} min^{-1})	4759.40 ± 96.40	0.47 ± 0.03	0.40 ± 0.12	0.73 ± 0.01	0.15 ± 0.02
X (cell L^{-1})	1.80 ± 0.11 × 10^{14}	8.00 ± 0.88 × 10^{10}	–	1.00 ± 0.23 × 10^{12}	–
V_m/X (μmol $cell^{-1}$ min^{-1})	2.64 × 10^{-11}	5.88 × 10^{-12}	–	7.30 × 10^{-13}	–

and Chen, 2009). Indeed, 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). Moreover, some cellulases show a high adsorption affinity for EPS (Lu et al., 2006). While *C. thermocellum* cellulases are docked to the cell surface via cellulosomes, (Bayer and Lamed, 1986), little is known about how *C. obsidiansis* cellulases are associated with the cell surface. Despite the presence or absence of a cellulosome, there was little difference in the cellulase activity between *C. thermocellum* and *C. obsidiansis* (Figs. 3 and 4). It is noteworthy that biofilms are known to limit diffusion of extracellular enzymes. Indeed, biofilms have been used as robust biocatalysts in wastewater processing for decades. It has been estimated that over 90% of extracellular enzymes remain cell-associated and immobilized within the biofilm 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 the individual cell basis, biofilms formed by *C. thermocellum* and *C. obsidiansis* demonstrated significantly higher cell-specific cellulase activity than their suspended cells also suggests that cellulase production may be triggered by surface attachment and biofilm growth, consistent with previous studies (Lu et al., 2006; Gamarra et al., 2010). 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. It has been demonstrated that proteins involved in virulence, adhesion, and resistance were more abundant under biofilm growth conditions (Allegrucci et al., 2006). Future studies are needed to determine whether cellulase production or activity is affected by surface attachment or biofilm growth.

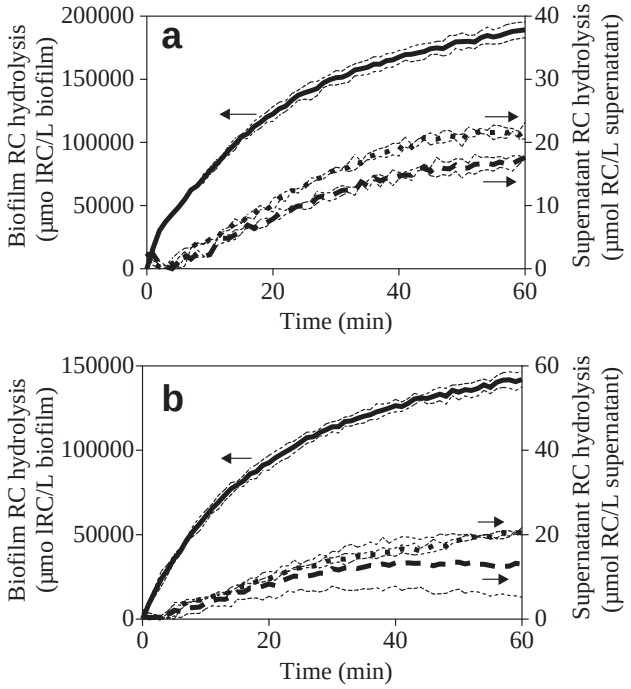

Fig. 3 – Resorufin cellobioside (RC) hydrolysis by biofilms (——), supernatant (- - -), and filtrate (– –) sampled from (a) *C. thermocellum* and (b) *C. obsidiansis* cultures feeding on cellulose chad; ····· indicates the error range.

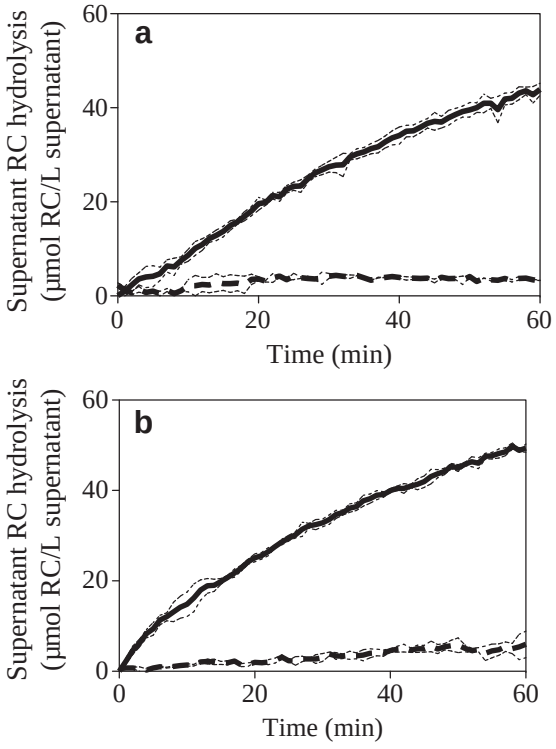

Fig. 4 – Resorufin cellobioside (RC) hydrolysis by supernatant (——) and filtrate (– –) sampled from a) *C. thermocellum* and b) *C. obsidiansis* cultures feeding on cellobiose; ····· indicates the error range.

3. Conclusions

Cellulolytic biofilms accumulated nearly four orders of magnitude greater cellulase activity than suspended cells in cellulose-fed cultures of both *C. thermocellum* and *C. obsidiansis*. Biofilms are likely to retain high cellulase activity and promote growth due to limited diffusion of cellulase enzymes and hydrolysate. The proximity of cellulase-producing cells with the substrate offers an energetically favorable strategy for 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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