Mathematical modeling of hydrolysate diffusion and utilization in cellulolytic biofilms of the extreme thermophile *Caldicellulosiruptor obsidiansis*

Zhi-Wu Wang, Scott D. Hamilton-Brehm, Adriane Lochner, James G. Elkins *, Jennifer L. Morrell-Falvey *

**BioEnergy Science Center, Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA**

**Abstract**

In this study, a hydrolysate diffusion and utilization model was developed to examine factors influencing cellulolytic biofilm morphology. Model simulations using *Caldicellulosiruptor obsidiansis* revealed that the cellulolytic biofilm needs to generate more hydrolysate than it consumes to establish a higher than bulk solution intra-biofilm substrate concentration to support its growth. This produces a hydrolysate surplus that diffuses through the thin biofilm structure into the bulk solution, which gives rise to a uniform growth rate and hence the homogeneous morphology of the cellulolytic biofilm. Model predictions were tested against experimental data from a cellulose-fermenting bioreactor and the results were consistent with the model prediction and indicated that only a small fraction (10–12%) of the soluble hydrolysis products are utilized by the biofilm. The factors determining the rate-limiting step of cellulolytic biofilm growth are also analyzed and discussed.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

The simultaneous solubilization and fermentation of lignocellulosic biomass into alcohols through a consolidated bioprocess would improve the economics of renewable fuel production (Lynd et al., 2008). While much has been described regarding the enzymatic hydrolysis of biomass (Bayer et al., 2006; Himmel et al., 2010), other surface-related phenomena remain poorly characterized. Some microorganisms that efficiently degrade biomass and convert the hydrolysate into fermentation end-products form biofilms (Jensen et al., 2009; O’Sullivan et al., 2009; Wang and Chen, 2009). Microbial biofilms demonstrate diverse morphological and structural characteristics under different cultivation conditions (van Loosdrecht et al., 2002). In general, the processes of internal mass diffusion and utilization play an important role in shaping the morphology of biofilms, such that the biofilm structure will tend to become heterogeneous and porous when substrate diffusion is rate-limiting, but homogeneous and compact when substrate utilization becomes the rate-limiting step (van Loosdrecht et al., 2002). This holds true for both pure and mixed cultures (Park et al., 1998; Trulear and Characklis, 1982).

Many studies of cellulose-degrading microbes have described the formation of biofilms with thin and uniform morphologies (Miron et al., 2001; Weimer et al., 1993). The underlying mechanisms that may constrain the morphology of cellulolytic biofilms, however, remain unknown. Unlike well-characterized biofilms that feed on soluble substrates, cellulolytic microbes must first hydrolyze the cellulose substrate and then utilize the hydrolysate for growth. In environments where cellulose is the primary carbon source, growth and survival of non-cellulolytic bacteria alongside cellulolytic microorganisms is thought to be supported by hydrolysate cross-feeding (Scheifinger and Wolin, 1973; Wells et al., 1995). This explanation requires that the rate of substrate hydrolysis and diffusion in a cellulolytic biofilm is faster than the rate of hydrolysate utilization by the biofilm, though this prediction has not been fully tested. Previous studies regarding cellulose fermentation by microbes, however, suggest that substrate hydrolysis is the rate-limiting step in a system which includes both attached and non-attached cellulolytic bacteria (Lynd et al., 2002). To provide additional insight into cellulolytic biofilm formation and growth, a mathematical model for substrate utilization and diffusion kinetics is described based on calculated and experimentally-derived parameters using *Caldicellulosiruptor obsidiansis* (ATCC BAA-2073) as a representative microorganism. *C. obsidiansis* is a recently described, extremely thermophilic and cellulolytic microbe isolated from Obsidian Pool in Yellowstone National Park. This microorganism has the capacity to utilize both cellulose and xylan while fermenting hexose and pentose sugars to lactate, acetate, ethanol, hydrogen and carbon dioxide (Hamilton-Brehm et al., 2010). Members of the genus *Caldicellulosiruptor* are known to express heat-stable multifunctional/multidomain cellulases and hemicellulases that act in concert to hydrolyze plant biomass...
(Bayer et al., 2006). Model simulations in this study suggest that hydrolysate utilization by the cellulolytic biofilm is the rate-limiting step for *C. obsidiansis* biofilm growth.

### 2. Methods

#### 2.1. Experimental design

*C. obsidiansis* was isolated and cultured in serum bottles as described previously (Hamilton-Brehm et al., 2010). Regenerated amorphous cellulose membranes with 0.2 μm pore size (Whatman RC58, Maidstone, Kent, UK) were used as a model substrate in this study. Identical chads with a mean diameter of 7.37 ± 0.03 mm and weight equal to 1.60 ± 0.14 mg were stamped from the membrane and used as the sole carbon source for *C. obsidiansis* growth. One cellulose chad was added to 50 ml of nutrient medium without yeast extract in an anaerobic serum bottle, inoculated with 2 × 10⁵ cells ml⁻¹, and incubated at 75 °C on a 100 rpm shaker. This gives an equivalent to 0.03 g L⁻¹ of initial cellulose concentration. This low initial cellulose concentration was intentionally designed to avoid accumulation of bulk solution hydrolysate to the extent necessary to support planktonic cell growth. This is confirmed by undetectable levels of reducing sugar and lack of dividing cells in the bulk solution during the experiment (data not shown). Replicate serum bottles were prepared and 3 serum bottles were randomly sampled and sacrificed for analysis every 4 h and the results from each time point were averaged. For the Avicel fermentation, the fermentor setup and growth conditions were as reported previously (Hamilton-Brehm et al., 2010), except that 30 g L⁻¹ rather than 15 g L⁻¹ of Avicel was used in this study.

#### 2.2. Intrinsic kinetic parameters determination

Serum bottles containing nutrient medium and varying cellulose concentrations (0–4 g L⁻¹) were inoculated with approx. 1.0 × 10⁷ cells ml⁻¹. The serum bottles were incubated at 75 °C and sampled every 2 h for 24 h to determine mid-log phase specific growth rates for each respective cellulose concentration. The maximum specific growth rate (**μ**max) and half saturation constant (Ki) were regressed from the Monod equation from the cellulose concentration-dependent specific growth rate plot. The observed growth yield (Yobs) was calculated by measuring the log-phase cellulose consumption and *C. obsidiansis* cell growth. All experiments were performed in triplicate.

#### 2.3. Microscopic analysis

Cellulose chads were collected from serum bottles and stained with Syto 9, a fluorescent nucleic acid dye (Invitrogen, Carlsbad, CA), to visualize the distribution of *C. obsidiansis* cells using a confocal laser scanning microscope (Leica TCS SP2, Mannheim, Germany). The mean thickness of each cellulose chad was determined by measuring the change in the Z dimension based on focusing the confocal microscope on the top and bottom of the chad at 10 randomly chosen positions. Biofilm thickness was measured from the cross-sectional image. The planktonic cell concentration was determined using a Thoma cell counting chamber (Blaubrand, Wertheim, Germany) and an Axioskop2 Plus microscope (Zeiss, Thornwood, NY, USA) with phase contrast illumination. ImageJ software (Version 1.42q, NIH, Bethesda, MD) was used for image analysis. The ImageJ 3D viewer and 3D object counter plug-ins were utilized to reconstruct the biofilm 3D structure and determine the biofilm cell density.

#### 2.4. Analytical methods

Samples collected from the fermentation were immediately stored at 4 °C and analyzed within 24 h. The concentration of reducing sugars in the growth medium was determined using dinitrosalicylic acid (DNS) reagent according to (Miller, 1959). Briefly, each *C. obsidiansis* fermentation sample was centrifuged at 16,060 × g for 5 min and the supernatant was then passed through a 0.22 μm nylon filter (PALL, Port Washington, NY) to remove residual cells. DNS reagent (100 μl) was added to 50 μl of filtered supernatant in a 96 well, 0.2 ml thin wall plate, which was sealed with an aluminium microplate seal and incubated in a thermal cycler at 99 °C for 5 min. Fifty microlitres of the reaction mixture was then transferred to a 96 well optical bottom plate (NUNC, Thermo Scientific, Rochester, NY) and diluted with 100 μl of deionized water. The absorbance was read at 540 nm and the amount of reducing sugars calculated as glucose equivalents, based on a glucose standard curve. The densities of the cellulose chads were measured according to standard methods (APHA, 1998). The residual Avicel was measured gravimetrically as previously described (Hamilton-Brehm et al., 2010). All measurements were done in triplicate.

#### 2.5. Modeling parameters

##### 2.5.1. Model description and assumptions

In general, a cellulolytic biofilm consists of four compartments: (i) cellulose substratum, (ii) microbial biofilm, (iii) diffusional boundary layer and (iv) bulk solution (Fig. 1). The primary objective of this model was to describe the hydrolysate transport and conversion within the biofilm and the boundary layer compartments. The hydrolysate is assumed to be produced at the cellulose-biofilm interface and utilized for microbial growth as it diffuses through the biofilm at a given influx of J_in (M L⁻² T⁻¹). Surplus hydrolysate, if any, diffuses through the biofilm into the boundary layer at an efflux of J_out. If the hydrolysate is not completely consumed in the boundary layer by cells that detached from the biofilm surface, a residual boundary layer substrate flux, namely J_b-out, would leak into the bulk solution. Given these hydrolysate flux definitions, the fraction of substrate utilization by the cellulolytic biofilm and boundary layer, can be reflected by the expressions (J_in - J_out)/J_in and (J_out - J_b-out)/J_in, respectively. In addition, J_b-out/J_in reflects the percent of hydrolysate leakage into the bulk solution. Cellulose, a dimer of two glucose molecules, was chosen as the model substrate in this study because it is a...
common growth substrate for cellulolytic bacteria. Also, the
growth rate does not appear to differ greatly when using celllobiose
versus other celldextrins (Russell, 1985). The following assump-
tions were made in the development of the cellulolytic biofilm
model: (1) Only cells in the biofilm are responsible for substrate
hydrolysis; (2) the biofilm structure is isotropic in physical, chemi-
cal and biological properties; (3) the bulk solution is homoge-
neously mixed; and (4) cellulose (and not other nutrients) is the
limiting factor for biofilm growth.

2.5.2. Modeling substrate diffusion and utilization in biofilm and
boundary layer

Because the hydrolysate will be at least partially consumed as it
diffuses through the biofilm, a diffusion flux reduction should oc-
cur which can be described according to Fick’s second law and ex-
pressed as,

\[ (-D_e) \frac{dS}{dx} = v \]  

in which \( v \) is the hydrolysate flux reduction rate \( (M \ L^{-3} \ T^{-1}) \), \( S \) is the
hydrolysate concentration \( (M \ L^{-3}) \), \( D_e \) is the effective substrate
diffusion coefficient inside the biofilm \( (L^2 \ T^{-1}) \) and \( L \) is the perpendicu-
lar distance from cellulose surface. The substrate utilization
kinetics in a biofilm are defined in the same manner as with
planktonic cells but the internal soluble substrate concentration is
adjusted to reflect the environment near the attached cells
(Rittmann and McCarty, 2001). Thus, the substrate utilization
rate in a cellulolytic biofilm can be expressed with the Monod
equation,

\[ v = \frac{\rho_x \mu_{\text{max}} S}{Y_{\text{obs}} K_s + S} \]  

where \( \rho_x \) is the biomass density \( (M \ L^{-3}) \), \( \mu_{\text{max}} \) and \( K_s \) are the
maximum specific growth rate \( (T^{-1}) \) and half-saturation constant
\( (M \ L^{-3}) \), and \( Y_{\text{obs}} \) is the observed growth yield of \( C. \ obsidiansis \) cells
(M M). Substituting Eqs. (2) into (1) gives the following expres-
sion that describes substrate diffusion and utilization in a cellulo-
ytic biofilm:

\[ (-D_e) \frac{dS}{dx} = \frac{\mu_{\text{max}} S X_b}{K_s + S} Y_{\text{obs}} \]  

Substrate utilization in the boundary layer is rarely considered
in models for biofilms growing on soluble substrates, which is
probably because the boundary layer is very thin compared to
the thickness of the biofilm layer (Rittmann and McCarty, 2001).
Because of the thin structure of the cellulolytic biofilm, however,
the significance of this parameter was investigated further. Similar
to the equations above, the substrate diffusion and utilization in
the boundary layer can be described by,

\[ (-D_e) \frac{dS}{dx} = \frac{\mu_{\text{max}} S X_b}{K_s + S} Y_{\text{obs}} \]  

where \( D \) is the hydrolysate diffusion coefficient in water \( (L^2 \ T^{-1}) \)
and \( X_b \) is the cell concentration within the boundary layer.

2.5.3. Modeling cell diffusion in the boundary layer

A fraction of the attached cells should detach from the cellulo-
ytic biofilm surface and migrate across the boundary layer into
the bulk liquid. Different from substrate diffusion, the flux of cells into
the bulk solution might increase due to cell growth and division
during detachment and diffusion. Therefore, the diffusion and
growth of those detached cells in the boundary layer should also
follow Fick’s second law and Monod equation in the same manner
as in Eq. (4), i.e.,

\[ D_e \frac{d^2X}{dx^2} = \frac{\mu_{\text{max}} S X_b}{K_s + S} \]  

where \( D_e \) is the cell diffusion coefficient \( (L^2 \ T^{-1}) \).

2.5.4. Boundary conditions

To solve Eqs. (3)–(5) requires the definition of several boundary
conditions. For Eq. (3), the substrate flux into the biofilm at its
interface with the cellulose surface can be described according to Fick’s first law,

\[ (-D_e) \frac{dS}{dx} = J_{in} \]

The substrate efflux from the biofilm, if any, should be continu-
ous at the interface between the biofilm and the boundary layer,
and so is the substrate concentration. Accordingly,

\[ (-D_e) \frac{dS}{dx} = (-D_e) \frac{dS}{dx} \bigg|_{x=-L_t} \]

\[ S_{L_t-\delta} = S_{L_t-\delta} \]

in which \( L_t \) is the biofilm thickness, \( L_t^- \) and \( L_t^+ \) are the perpen-
dicular distance measured from the inner and outer side of the biofilm-
boundary layer interface to the cellulose surface, respectively. Eq.
(4) shares the boundary conditions derived from Eqs. (7) and (8)
with Eq. (3). The same concept applies to the boundary layer-bulk
solution interface, where

\[ (-D_e) \frac{dS}{dx} |_{x=L_t+\delta} = J_{b-out} \]

\[ S_{L_t+\delta} = S_{L_t+\delta} \]

in which \( S_s \) is the supernatant hydrolysate concentration \( (M \ L^{-3}) \),
and \( L_b \) refers to the boundary layer thickness.

In Eq. (5), the planktonic cell concentration and the cell
detachment rate measured in the bulk solution can be used to define the
boundary conditions at the interface between the boundary layer
and bulk liquid,

\[ D_e \frac{dX}{dx} |_{x=L_t+\delta} = \frac{R_{det} V}{A} \]

\[ X_{L_t+\delta} = X_p \]

in which \( R_{det} \), \( V \), \( A \), and \( X_p \) stand for the bacterial detachment rate
\( (M \ L^{-3} \ T^{-1}) \), bulk solution volume \( (L^3) \) and the surface area \( (L^2) \) of
cellulose that is covered by the biofilm and the planktonic cell
concentration \( (M \ L^{-3}) \), respectively.

To solve differential Eqs. (3)–(5), at least two of the bound-
ary conditions defined in Eqs. 6, 9, and 10 must be known in view
of the function continuity in Eqs. (7) and (8). In addition, the value
\( X_b \) in differential Eq. (4) relies on the solution from differential
Eq. (5) which can be solved only when both Eqs. (11) and (12)
are known. More importantly, since differential Eqs. (3)–(5) share
common variables such as \( S \) and \( X_b \) that vary at different
locations, these three differential equations must be solved simultane-
ously. Due to this complexity, Matlab R2009a was used to determine nume-
rical solutions to these equations.

2.5.5. Estimation of other modeling parameters

The crowded cellular environment in a cellulolytic biofilm cre-
ates resistance for hydrolysate diffusion. The biofilm cell density
dependent mass diffusion coefficient has been recognized and
quantified with an empirical model developed by Fan et al.
(1990), and expressed as,
in which \( m \) denotes the single cell dry mass (Table 1). \( D_e/D \) is estimated to be 0.18 in this study (Table 1). This ratio is within the range reported for biofilms at comparable cell density (Stewart, 1998). The temperature dependent \( D \) value can be converted through the Stokes–Einstein equation. \( D_e \) is chosen from published reports for cells with similar morphology to \( C. \) \( \text{obsidiansis} \) without mobility (Kim, 1996). To date, no reliable method for estimating the boundary layer thickness \( (L_b) \) on a suspended particle in a bulk solution has been reported. In order to determine an appropriate value for \( L_b \), a wide range of values were chosen as input for the model system and the simulation results were compared to experimental data to determine an appropriate value. All other parameters used in this model are listed in Table 1.

3. Results and discussion

3.1. Morphology of the cellulolytic biofilm grown on cellulose chad surfaces

One cellulose chad was sampled and stained with Syto9 to determine the morphology of the \( C. \) \( \text{obsidiansis} \) biofilm after 68 h growth using confocal microscopy. Optical sections were collected and used to reconstruct a 3-dimensional image of the biofilm growing on the cellulose substrate (data not shown). The thickness of this biofilm was randomly measured at various positions and found to be in the range of 9–11 \( \mu \)m. After the first 20 h of growth, the maximum thickness of the biofilm remained constant until the end of the experiment (72 h) and cross-sections showed that \( C. \) \( \text{obsidiansis} \) cells were evenly distributed throughout the biofilm (data not shown). Moreover, the biofilm did not display any obvious porosity or mushroom-like structures commonly seen in biofilms grown on soluble substrates (van Loosdrecht et al., 2002). This uniform morphology suggests that the substrate concentration is in a state of homogeneous distribution throughout the biofilm. To test this hypothesis, a model was developed to describe the hydrolysate diffusion and utilization in cellulolytic biofilm.

3.2. Modeling hydrolysate diffusion and utilization in a cellulolytic biofilm

During biofilm formation, thinning of the cellulose chad was observed, which is consistent with the chad being used as a carbon source for microbial growth. The reduction in chad thickness due to microbial degradation was determined at multiple time points during the 72 h experiment (Fig. 2A). Thinning of the chad was first observed after 28 h of growth (Fig. 2A). From this point, the cellulose chad thickness decreased at a relatively steady rate (Fig. 2A). This rate of thinning was independent of the planktonic cell concentration measured in the bulk liquid (Fig. 2B), suggesting that the thinning was due to a constant rate of substrate hydrolysis by attached cells but not planktonic cells, which is consistent with published reports (Jensen et al., 2009). From these data, the hydrolysate flux per chad surface area into the biofilm in Eq. (6) can be approximated as,

\[
J_{in} = \rho_c R_{bsu}
\]

in which \( \rho_c \) is the cellulose chad density, and \( R_{bsu} \) is the chad thinning rate estimated from Fig. 2A. Because the initial experimental conditions were designed to prevent planktonic cell growth, the cell detachment rate per surface area \( (R_{det}) \) can be estimated from Fig. 2B at the stage when the cellulose chad surface was fully covered by the cellulolytic biofilm, namely after 40 h incubation as observed by microscopy, and substituted into Eq. (11). Similarly, the substrate concentration in the bulk liquid \( (S_b) \) in Eq. (10) is negligible based on the experimental design. Further, the value of \( K_p \) in Eq. (12) was also determined experimentally (Fig. 2B). Using these values, the hydrolysate diffusion and utilization model was numerically solved with these four boundary conditions at various scales of \( L_b \) (Table 2). The specific biofilm growth rate \( (\mu_f) \) in Table 2 is calculated from the model through a mass balance of biofilm growth, i.e.,

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Values</th>
<th>Units</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \rho_c )</td>
<td>Biofilm cell density</td>
<td>( 1.69 \times 10^{11} )</td>
<td>cells cm(^{-3})</td>
<td>This study</td>
</tr>
<tr>
<td>( \rho_p )</td>
<td>Cellulose chad density</td>
<td>( 289.07 \pm 3.22 )</td>
<td>kg m(^{-3})</td>
<td>This study</td>
</tr>
<tr>
<td>( D_e )</td>
<td>Effective diffusion coefficient</td>
<td>( 0.18 \times D )</td>
<td>cm(^2) s(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( D (75 , ^\circ C) )</td>
<td>Cellulose diffusion coefficient</td>
<td>( 1.38 \times 10^{-5} )</td>
<td>cm(^2) s(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( D (30 , ^\circ C) )</td>
<td>Cell diffusion coefficient</td>
<td>( 5.71 \times 10^{-4} )</td>
<td>cm(^2) s(^{-1})</td>
<td>(Kurath and Swanson, 1961)</td>
</tr>
<tr>
<td>( D_b (75 , ^\circ C) )</td>
<td>Biofilm thickness</td>
<td>( 9 \times 10^{-6} )</td>
<td>cm(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( \mu_b )</td>
<td>Biofilm specific growth rate</td>
<td></td>
<td>h(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( \mu_{max} )</td>
<td>Maximum specific growth rate</td>
<td>0.72</td>
<td>h(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( K_s )</td>
<td>Half-reaction coefficient</td>
<td>0.38</td>
<td>g L(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( X_{obs} )</td>
<td>Observed growth yield</td>
<td>( 3.40 \times 10^{12} )</td>
<td>cells g(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( L_b )</td>
<td>Cellulose chad surface area</td>
<td>85.28</td>
<td>mm(^2)</td>
<td>This study</td>
</tr>
<tr>
<td>( J_{bsu} )</td>
<td>Hydrolysate flux into biofilm</td>
<td>( 5.33 \times 10^{-5} )</td>
<td>g h(^{-1}) cm(^{-2})</td>
<td>This study</td>
</tr>
<tr>
<td>( J_{bs} )</td>
<td>Hydrolysate flux out of biofilm</td>
<td>( 5 \times 10^{-4} )</td>
<td>g h(^{-1}) cm(^{-2})</td>
<td>This study</td>
</tr>
<tr>
<td>( J_{bsu} )</td>
<td>Hydrolysate flux out of boundary layer</td>
<td></td>
<td>g h(^{-1}) cm(^{-2})</td>
<td>This study</td>
</tr>
<tr>
<td>( V )</td>
<td>Medium volume</td>
<td>50</td>
<td>ml</td>
<td>This study</td>
</tr>
<tr>
<td>( R_{det} )</td>
<td>Detachment rate</td>
<td>( 4.59 \times 10^{5} )</td>
<td>cells ml(^{-1}) h(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( R_{bsu} )</td>
<td>Chid thinning rate</td>
<td>3.69</td>
<td>( \mu )m h(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( m_{cell} )</td>
<td>Cellular unit dry mass</td>
<td>( 6.58 \pm 1.21 \times 10^{-10} )</td>
<td>mg cell(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( \mu (75 , ^\circ C) )</td>
<td>Water dynamic viscosity</td>
<td>( 3.69 \times 10^{-4} )</td>
<td>kg m(^{-1}) s(^{-1})</td>
<td>(CRC, 2003)</td>
</tr>
<tr>
<td>( \mu (30 , ^\circ C) )</td>
<td>Boundary layer thickness</td>
<td>( 7.77 \times 10^{-4} )</td>
<td>kg m(^{-1}) s(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( S_b )</td>
<td>Supernatant substrate concentration</td>
<td>0</td>
<td>g L(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( X_p )</td>
<td>planktonic cell concentration</td>
<td>( 1.10 \times 10^{12} )</td>
<td>cells ml(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( X_b )</td>
<td>Boundary layer cell concentration</td>
<td></td>
<td>cells ml(^{-1})</td>
<td>This study</td>
</tr>
<tr>
<td>( \eta )</td>
<td>Effectiveness factor</td>
<td></td>
<td></td>
<td>(Grady et al., 1999)</td>
</tr>
<tr>
<td>( \eta^* )</td>
<td>Modified effectiveness factor</td>
<td></td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>
Table 2

| $L_b$ (μm) | $J_{in}$ (g cm$^{-2}$ h$^{-1}$) | $J_{out}$ (g cm$^{-2}$ h$^{-1}$) | $J_{in}$ (g cm$^{-2}$ h$^{-1}$) | $\frac{|J_{in} - J_{out}|}{J_{in}}$ (%) | $\frac{|J_{in} - J_{out}|}{J_{out}}$ (%) | $\frac{J_{in}}{J_{out}}$ (%) | $\mu_f$ (h$^{-1}$) |
|------------|-------------------------------|-------------------------------|-------------------------------|---------------------------------|---------------------------------|----------------------------|-------------------|
| 8          | $5.33 \times 10^{-5}$         | $5.29 \times 10^{-5}$         | $5.29 \times 10^{-5}$         | 0.75                            | 0.00                            | 99.25                     | 0.01              |
| 80         | $5.33 \times 10^{-5}$         | $5.23 \times 10^{-5}$         | $5.23 \times 10^{-5}$         | 1.88                            | 0.00                            | 98.12                     | 0.02              |
| 800        | $5.33 \times 10^{-5}$         | $4.72 \times 10^{-5}$         | $4.71 \times 10^{-5}$         | 11.44                           | 0.19                            | 88.37                     | 0.13              |

3.3. Hydrolysate flux

The results of the hydrolysate diffusion and utilization simulations over the range of boundary layer thicknesses ($L_b$) show a positive relationship between $L_b$ and $\mu_f$, such that a thicker boundary layer helps the biofilm retain more hydrolysate in terms of $J_{in}$ in the course of diffusion, and thus gives rise to a faster $\mu_f$ (Table 2). This positive relationship differs from models based on biofilm growth on a soluble substrate in which $L_b$ plays a negative role and must be attenuated with intensive shear force (Grady et al., 1999). The role of the boundary layer in retaining hydrolysate within the biofilm may account for the differing impacts of $L_b$ on cellulolytic biofilms versus biofilms grown on soluble substrates (Fig. 1). Experimental data indicating that the cellulolytic biofilm reaches and maintains a constant thickness (around 10 μm) suggests a mass balance has been reached between the rate of cell growth and detachment (data not shown). Based on these data, the specific growth rate of the biofilm can be inferred from its specific detachment rate determined experimentally as $\mu_f = 0.13$ h$^{-1}$ according to Eq. (16),

$$\mu_f = \frac{R_{ave} V}{\rho_s \tau A}$$

Comparing this calculated $\mu_f$ value derived from experimental data to the $L_b$-dependent simulated $\mu_f$ values reported in Table 2, a boundary layer value of 800 μm appears to be an appropriate thickness for the C. obsidiansis biofilm growing on a cellulose chad. This value is consistent with typical $L_b$ values reported in bioreactors ranging from 100 to 1200 μm (Bishop et al., 1997). Although the estimated boundary layer thickness is within this range, it may be larger than expected in this study for the following reasons. First, in this experimental design, the cell culture was grown with moderate shaking (100 rpm) compared to the more vigorous mixing conditions in a mechanically mixed bioreactor. Second, the boundary layer is predicted to increase with the suspended particle size, so the size of the cellulose chad (7.37 ± 0.03 μm in diameter) may artificially result in a larger $L_b$. Third, according to the Stokes–Einstein equation, the mass diffusion coefficient is doubled and the water viscosity is halved at 75 °C (Table 1), which allows for greater diffusion distance in the boundary layer. Lastly, cellodextrin, the substrate used in this study, gives the greatest mass diffusion coefficient among all cellodextrins due to its small molecular size, which may also lead to an overestimation of the boundary layer thickness (Kurath and Swanson, 1961).

The simulations based on this experimental design (Table 2) predict that only 11% of the hydrolysate will be utilized by a cellulolytic biofilm grown to a thickness of 10 μm despite the high cell density. A small fraction of the hydrolysate would be consumed in the boundary layer and the remaining sugars (88%) would diffuse through the biofilm into the bulk solution (Table 2). The phenomenon of hydrolysate efflux through the biofilm indicated by an increased concentration of sugar in the bulk liquid has been observed in many other cellulose fermentations (Lo et al., 2009; Lu et al., 2006). This hydrolysate efflux suggests that the rate of substrate utilization by the surface adhered cells is slower than the rate of diffusion of soluble sugars out of the biofilm.

3.4. Hydrolysate concentration

A simulation of the hydrolysate profile across the biofilm and boundary layer is presented in Fig. 2C. This simulation shows a diffusion profile with the maximum hydrolysate concentration at the cellulose surface and the minimum concentration in bulk solution. The motive force maintaining this gradient is microbial cellulose degradation at the cellulose surface which produces hydrolysate...
at a rate of $J_{in} = 5.33 \times 10^{-5}$ g cm$^{-2}$ h$^{-1}$ that diffuses into the biofilm (Table 2). The extensive efflux ($J_{out}$ in Table 2) indicates that the thin cellulolytic biofilm is incapable of consuming all of the hydrolysate influx. As a consequence, the hydrolysate diffuses through the biofilm and gives rise to an efflux of $J_{out}$ into the boundary layer. Although up to $2 \times 10^7$ C. obsidiansis cells ml$^{-1}$ are distributed in the boundary layer, the amount of substrate utilized by growing cells in the boundary layer is minimal compared to the total influx. For this reason, 88% of the hydrolysate escapes into the bulk solution (Table 2). The hydrolysate concentration profile is quite uniform across the biofilm (Fig. 2C) implying a uniform growth rate would result with respect to film thickness. Moreover, this uniform hydrolysate profile suggests that the hydrolysate influx exceeds the hydrolysate utilization capacity of the biofilm despite its high cell density, i.e., the rate-limiting step for cellulolytic biofilm growth lies in its substrate utilization rather than hydrolysis (Fig. 2C). Consistent with previous reports (van Loosdrecht et al., 2002), the rate-limitation in substrate utilization may explain the homogenous thin biofilm morphology observed microscopically (data not shown).

3.5. Verification of the hydrolysate efflux from the cellulolytic biofilm

Due to the minimal initial substrate concentrations in the cellulose chad experiment, no measurable sugar concentration could be detected in the supernatant, even though it is predicted in the model simulation (data not shown). In order to test whether hydrolysate efflux was occurring during biofilm growth, C. obsidiansis was grown in the presence of a larger amount of initial feedstock (30 g L$^{-1}$ Avicel) and the fermentation was followed for 120 h (Fig. 3). Like previous experiments, a constant Avicel hydrolysis rate was observed, which was independent of planktonic cell concentration which increased from $10^7$ to $10^8$ cell ml$^{-1}$ over the course of the experiment (Fig. 3A). Because planktonic cells quickly consume any hydrolysate that diffuses through the biofilm into the bulk liquid, it is hypothesized that the presence of leaked hydrolysate in the bulk liquid may be detectable only in the early stages of batch fermentation when the planktonic cell concentration is low. To test this, the reducing sugar and planktonic cell concentrations were measured during early and mid-log-phase growth. As predicted, an accumulation of reducing sugar in the bulk liquid was observed during the first 5 h of the fermentation when the planktonic cell concentration was below $5 \times 10^7$ cells ml$^{-1}$ (Fig. 3B). As the planktonic cell concentration increased, reducing sugars in the bulk liquid were no longer detected during the log phase, suggesting that the soluble substrate was consumed by planktonic cells (Fig. 3B). Similar soluble carbohydrate profiles have been reported during Avicel fermentation by other cellulolytic microorganisms (Lu et al., 2006). It is important to note that at later stages of the fermentation process, substrate hydrolysis by the biofilm becomes the overall rate-limiting step when considering the biofilm and planktonic cells as a whole system, which is consistent with published reports (Lynd et al., 2002).

3.6. Why does a hydrolysate efflux occur in a cellulolytic biofilm?

Both model analysis and experimental results indicate that the cellulolytic biofilm consumes only a fraction of the hydrolysate generated by microbial hydrolysis and the rest of the hydrolysate diffuses into the bulk liquid. The hydrolysate “leakage” is often observed in cellulose fermentation (Lo et al., 2008; Lu et al., 2006) and was originally inferred from the cross-feeding phenomenon. As early as the 1950s, it was recognized that starch-digesting ruminal bacteria outnumbered cellulolytic bacteria in cattle rumen despite the fact that the cattle’s diet was primarily cellulose (Bryant and Burkey, 1953). Later, Scheiﬁnger and Wolin (1973) demonstrated successful co-cultivation of a cellulolytic and a non-cellulolytic bacterial species with cellulose as the sole carbon source, and the two microbes were found at roughly equal numbers. Russell (1985) subsequently showed that cellodextrins generated by cellulolytic bacteria were consumed by the non-cellulolytic microbial species. Interestingly, cross-feeding between pure cultures of attached and non-attached cells similar to the ﬁnding in this study was also observed previously (Wells et al., 1995). Results from this study indicate that a hydrolysate concentration gradient must exist between the cellulosic biofilm and the bulk solution to ensure a high enough intra-biofilm hydrolysate concentration for biofilm growth (Fig. 2C). This gradient is maintained by the continuous hydrolysate efflux from the biofilm and is particularly important when the carbohydrate concentration in the bulk solution is low (Fig. 2C). In a natural system, one would expect the hydrolysate concentration in the bulk solution to be low as non-cellulolytic microbes consume soluble compounds (Johnson et al., 1985). As a consequence, the main mechanism to produce a hydrolysate concentration gradient compatible with cellulolytic biofilm growth is to allow hydrolysate diffusion out of the biofilm into the bulk liquid.

One question that still remains is whether conditions exist in which hydrolysate efflux does not occur, i.e., hydrolysate diffusion but not utilization becomes the rate-limiting step for cellulolytic biofilm growth. The model system developed in this study may provide some insight into this question. Effectiveness factor ($\eta$) is a routine indicator used to evaluate whether a biofilm is subjected to substrate utilization or diffusion limitation (Grady et al., 1999). It is defined as the ratio of the bioreaction rate under diffusion limiting and non-limiting conditions. $\eta$ gives a value of approximately 1 when substrate utilization is the rate limiting determinant and a value less than 1 when diffusion is the limiting factor. However, this indicator does not apply to cellulolytic biofilms in which the mass diffusion limitation starts to play a significant role as mentioned above, i.e., diffusion resistance helps hold more hydrolysate.

![Fig. 3. Fermentation of Avicel by C. obsidiansis in a bioreactor.](image-url)
within the biofilm for higher substrate utilization rate. This positive
effect of diffusion limitation in a cellulolytic biofilm will lead
to $\eta > 1$, implying that $\eta$ has lost its original physical meaning
defined for a biofilm grown on a soluble substrate diffused from bulk
solution. For this reason, a modified effectiveness factor ($\eta^*$) is defined
to evaluate the relative contributions of substrate utilization and
diffusion rates in a cellulolytic biofilm,

$$\eta^* = \frac{\text{Hydrolysate utilization rate per surface area}}{\text{Hydrolysate influx rate per surface area}}$$

$$= \frac{L_f \frac{\text{max} \ \text{q}_{\text{obs}}}{L_m \ \text{in}}}{L_m}$$

(17)

In this equation, $\eta^*$ is a dimensionless parameter. When $\eta^* < 1$,
substrate utilization is the rate-limiting step for biofilm growth. When
celullose is the sole carbon source, $J_m$ should be the upper-
limit of the hydrolysate utilization rate in the biofilm, and thus
only $\eta^* \leq 1$ is possible. This means that a cellulolytic biofilm
cannot have a theoretical substrate utilization rate that exceeds
the substrate diffusion rate. However, conditions may exist in
which $\eta^*$ approaches 1 when the process is close to the diffusion
limitation. To determine which parameters impact the value of
$\eta^*$ most significantly, the effects of $J_m$, $S_i$, $L_r$ and $L_s$ on the value of $\eta^*$ were studied with reference to a baseline established with parameters adapted from Fig. 2C. Each profile is simulated with a single parameter varied at a time so that the effect of each parameter can be compared. These simulations indicate that all parameters except $J_m$ play a positive role on $\eta^*$, although with different
sensitivities (Fig. 4). $\eta^*$ appears to be more sensitive to changes in $L_r$ and $L_s$ but less to $L_s$ as $S_i$ increases. For example, $\eta^*$ quickly approaches a value of 1 when $L_r$ increases 5-fold or $J_m$ decreases 5-fold with respect to the baseline. In contrast, a 500-fold change in $L_s$ does not significantly impact the value of $\eta^*$ (Fig. 4). Although $S_i$ is simulated with a range of $K_s$ values in Fig. 4, only supernatant carbohydrate concentrations lower than 0.2 g L$^{-1}$ are typical in cellu-
lose fermentors, probably owing to the quick consumption by planktonic cells. The results from these simulations indicate that
the only possibility for $\eta^*$ to approach 1 within the typical $S_i$ range
is by a reduction in $J_m$ or with a thicker biofilm ($L_s$). A reduced rate of cellulose hydrolysis ($J_m < 10^{-5}$) is typically associated with less-
biodegradable substrates, such as crystalline cellulose (Fig. 4). Un-
der these conditions, the rate of substrate utilization may be con-
strained by the rate of substrate diffusion (Fig. 4).

4. Conclusions

The hydrolysate flux and utilization in a cellulolytic biofilm
formed on a cellulose surface was evaluated with the model sys-
tem developed in this study. The data indicate that a thin cellu-
lytic biofilm apparently must generate more hydrolysate than consumed. This is necessary to establish a soluble substrate con-
centration that is higher than that of the bulk solution, which
therefore supports biofilm growth. This concentration gradient is
maintained by a cellulose hydrolysis rate that is greater than the
utilization rate. In this sense, there exists a hydrolysate surplus
and thus, the hydrolysate utilization rate in a cellulolytic biofilm becomes its major growth-limiting factor. These results may also
apply to other microbes that form thin biofilms on insoluble sub-
strates, although this remains to be tested.

Acknowledgements

This work was supported by the BioEnergy Science Center (BESC), which is a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Re-
search in the DOE Office of Science. Oak Ridge National Laboratory
is managed by UT-Battelle, LLC, for the US Department of Energy
under contract DE-AC05-00OR22725.

References

American Public Health Association, Washington DC, USA.
enzyme systems. Prokaryotes 2, 578–617.
and hydrodynamic boundary layers over biofilms. Environ. Technol. 18, 375–
385.
CRC, 2003. Handbook of Chemistry and Physics, 84th ed. Chemical Rubber Company,
Cleveland, Ohio.
through a biofilm grown on activated carbon particles in a draft-tube three-
Treament, 2nd ed. Marcel Dekker, New York.
Hamilton-Brehm, S.D., Mosher, J.J., Vishnivetskaya, T., Podar, M., Carroll, S., Allman,
Nov., an anaerobic, extremely thermophilic, cellulolytic bacterium isolated from
1020.
enzyme systems for biomass conversion: emerging paradigms. Biofuels 1, 323–
341.
Jensen, P.D., Hardin, M.T., Clarke, W.P., 2009. Effect of biomass concentration and
inoculum source on the rate of anaerobic cellulose solubilization. Bioresour.
Technol. 100, 5219–5225.
Polysaccharides. The Effect of Ion Binding on the Molecular Properties of Low
Molecular Weight Polysaccharides. Project 2236, Report Four to the Pioneering
Research Committee, Pioneering Research Program. The institute of paper
chemistry, Appleton, Wisconsin.
production from cellulose hydrolysate produced via temperature-shifted
Lynd, L.R., Laser, M.S., Brandsby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M.,
577.
Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing
Miron, J., Ben-Chedalla, D., Morrison, M., 2001. Invited review: adhesion
of flow cell technology for monitoring biofilm development and cellulose
Park, Y.S., Yun, J.W., Song, S.K., 1998. Biofilm properties under different substrate


