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Microreactor-based mixing strategy suppresses product inhibition to enhance sugar yields in enzymatic hydrolysis for cellulosic biofuel production

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HIGHLIGHTS

- A novel microreactor-based energy-efficient process for cellulose hydrolysis.
- Macromixing in reactor for optimal time followed by no mixing in microreactor.
- Array of 200 μl and 400 μl microreactors used; 200 μl gives highest sugar yield.
- Upto 35% and 29% increase in glucose and reducing sugar yields, respectively.
- Linear calibration curve estimates product yield using liquid to solid height ratio.

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ABSTRACT

A novel microreactor-based energy-efficient process of using complete convective mixing in a macroreactor till an optimal mixing time followed by no mixing in 200–400 μl microreactors enhances glucose and reducing sugar yields by upto 35% and 29%, respectively, while saving 72–90% of the energy incurred on reactor mixing in the enzymatic hydrolysis of cellulose. Empirical exponential relations are provided for determining the optimal mixing time, during which convective mixing in the macroreactor promotes mass transport of the cellulase enzyme to the solid Avicel substrate, while the latter phase of no mixing in the microreactor suppresses product inhibition by preventing the inhibitors (glucose and cellobiose) from homogenizing across the reactor. Sugar yield increases linearly with liquid to solid height ratio (r_h), irrespective of substrate loading and microreactor size, since large r_h allows the inhibitors to diffuse in the liquid away from the solids, thus reducing product inhibition.

1. Introduction

One of the fascinating complexities of enzymatic hydrolysis of solid carbohydrates (Sun and Cheng, 2002) – the slowest and the rate-limiting step in the production of lignocellulosic fuels – is its multiscale and multiphase nature (Chakraborty et al., 2014). Soluble enzymes are convected from the bulk of the liquid phase to the solid-liquid interface and diffuse into the pores of the solids due to capillary action, where pore-scale adsorption (Várnai et al., 2011; Dutta and Chakraborty, 2016) of the cellulase's Carbohydrate Binding Domains (Gao et al., 2013) to the insoluble substrate occurs. This is followed by formation of the enzyme-substrate complex. Cleaving of the glycosidic linkages in the cellulose polymer occurs at the enzyme's Catalytic Domains, producing smaller carbohydrate chains, which, if soluble, diffuse out of the pores into the bulk liquid phase (Lee and Fan, 1982). These solid phase reactions are followed by liquid phase enzymatic hydrolysis of the soluble sugars to produce the monomer glucose. The solid and liquid phase hydrolyses are both inhibited by the soluble products – the monomer (glucose) and the dimer (cellobiose) (Ferchak and Pye, 1983). Thus, it is obvious that the true kinetics of depolymerization at the molecular scale is, most often than not, disguised by the mass transport at the pore scale and the reactor scale (Gaikwad and Chakraborty, 2013).

Of the three scales involved in this multiscale process, the length scales in lignocellulose deploymerization may often have a sequence unseen in classical multiscale systems: Reactor scale > Molecular scale > Pore scale (Dutta and Chakraborty, 2016). This altered sequence, however, holds until a critical hydrolysis time, since continuous deploymerization decreases molecular chain length and reactive dissolution from the pore surface increases pore size. After that critical reaction time, the hydrolysis

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system reverts to the usual sequence of length scales: Reactor scale > Pore scale > Molecular scale. However, convection usually continues to dominate the reactor scale mixing and mass transport, diffusion continues to govern the pore scale mass transfer, and reaction continues to drive chain cleaving and carbohydrate depolymerisation at the molecular scale.

After a certain amount of time has elapsed in hydrolysis, convection having performed the task it was deigned to – that of transporting enzymes from the bulk liquid phase to the vicinity of the solid-liquid interface, becomes superfluous and only remains a process that consumes electrical energy for fluid mixing in the reactor.

Many successful attempts have been made for increasing the hydrolysis yield by reducing mass transfer and inhibitory effects, by employing reactors with disparate mixing/shaking patterns (Ingesson et al., 2001; Roche et al., 2009), by varying impeller types (Kinnarinen et al., 2012; Lavenson et al., 2014) and reactor types: batch (Gaikwad and Chakraborty, 2013), semibatch (Gupta et al., 2012) and CSTR (Chakraborty et al., 2010), by exploring the effects of enzyme adsorption on the cellulose surface (Zhang and Lynd, 2004), by unfolding the multiscale dynamics of the system (Chakraborty et al., 2014), etc. The effects of high intensity mixing on hydrolysis of cellulose (Shamuniuk et al., 2011; Brethauer et al., 2011) and lignocellulose such as spruce (Palmqvist et al., 2011) have also been explored.

It turns out that there exists: (a) a complex interlocked dynamics between mass transfer (convection, diffusion and adsorption) and reaction kinetics (including product inhibition), (b) an intrinsic scale separation in the hydrolysis system (convection dominated macro-scale and diffusion dominated micro-scale). Therefore, in this paper two unasked questions are asked: is it possible to allow convection to govern the hydrolysis system when macromixing is essential, and accentuate micromixing or local diffusion when macromixing is redundant, or even detrimental? Can a physical separation of the dominant scales be engineered so that the reactants are homogenized when the enzyme adsorbs to the substrate, and the products are localized when product inhibition is potent?

In an attempt to answer these questions, microreactors are introduced as a new equipment for the process of enzymatic hydrolysis of cellulose for biofuel production. This new energy-efficient process uses an engineering combination of a macroreactor and an array of microreactors that exploit the intrinsic scale separation between the macro (convection) and micro (diffusion) scales to enhance the hydrolysis rate by reducing product inhibition along with a significant reduction in energy cost. This novel microreactor-based technology (Chakraborty and Singh, 2015) increases the rate of hydrolysis by minimizing the product inhibition as well as provides an energy-efficient process by optimizing the reactor mixing. This mixing strategy uses complete convective mixing in a macroreactor till an optimal mixing time followed by no mixing in 200–400 μl microreactors. Thus, the system is allowed to be dominated by the convection (macro) scale in the macroreactor till the optimal mixing time, after which the hydrolysis is governed by the diffusion (micro) scale in the microreactor. A scale-switching is enabled during the hydrolysis reaction: from macro to micro, from convection to diffusion, in order to affect a reduction in production inhibition, which, in turn, engenders significant leaps in glucose, reducing sugar and ethanol yields.

2. Material and methods

2.1. Material

Commercially available microcrystalline cellulose (Avicel PH101 of particle size 50 μm and density 0.6 g/cm^3 purchased from Sigma Aldrich Company, USA) is used as a substrate for enzymatic hydrolysis. Cellulase derived from *Trichoderma viride* with an activity of 1 U/mg of solid, purchased from Himedia Labs, Mumbai, India, containing all the three components of cellulase, (i.e., endoglucanase, exoglucanase and β-glucosidase), is used as an enzyme in the ratio of 1 mg of enzyme/20 mg substrate. The enzyme has a maximum activity at a pH range of 4.0–5.0 and a temperature of 40–50 °C against most cellulosic materials. 10 ml of 0.1 M sodium acetate is used as buffer for maintaining the pH of 5.0, suitable for enzymatic hydrolysis.

2.2. Methods

Batch experiments of enzymatic hydrolysis of pre-treated cellulose are performed in a 100 ml conical flask (i.e. macroreactor) with 10 ml reaction volume. Four types of reactor mixing strategies are used to study the kinetics of enzymatic hydrolysis. The strategies are (a) no mixing in a macroreactor for the total reaction time of 72 h, (b) continuous mixing in a macroreactor for the total reaction time of 72 h, (c) optimal macromixing, i.e., macromixing in the macroreactor for an optimum time, followed by no mixing in the macroreactor for the rest of the hydrolysis reaction, (d) optimal microreactor, i.e., mixing in a macroreactor till an optimal mixing time (t_{opt}), followed by the rest of the hydrolysis being performed in an array of unmixed microreactors. These microreactors are (i) 200 μl and (ii) 400 μl in volume, with L/D ratio of approximately 4:1, and are conical in shape in the bottom quarter and cylindrical in the top three quarters. The total reaction time of these two processes together is 72 h, and the switchover from macroreactor to an array of microreactors is done at an optimal mixing time (t_{opt}). The experiments are carried out in an incubator cum shaker under aseptic condition at a pH of 5.0, Temperature of 50 °C and a mixing speed of 150 RPM (only for the macroreactor), with substrate concentration varying from 2% to 5% wt./vol (i.e., 20 mg/ml to 50 mg/ml), with the 1% increase at a time, keeping all other parameters constant. (2% of substrate means 20 mg of solid substrate/ml of reaction volume = 200 mg/10 ml.)

Glucose concentration is measured by the GOD-POD test kit obtained from Accurex Biomedical Pvt. Ltd., Mumbai, India. Glucose oxidase (GOD) converts glucose to gluconic acid and hydrogen peroxide, following which the peroxide is oxidatively coupled with 4-aminoantipyrene and phenol in the presence of peroxidase to produce a red Quinoeimine dye. The corresponding absorbance is measured at 505 nm in UV Spectrophotometer (Agilent Technologies). The concentration of reducing sugar is estimated by the DNS method (Miller, 1959). DNS (3,5-Dinitrosalicylic acid) reagent reacts with reducing sugar to form 3-amino-5-nitrosalicylic acid. The corresponding absorbance is measured at 540 nm in UV Spectrophotometer (Agilent Technologies).

The dynamics of solid conversion for various initial concentrations ranging from 20 mg/ml to 50 mg/ml with 10 mg/ml increment each time are measured. After various intervals of time, the reaction is stopped and the reaction mixture is centrifuged and dried to remove all buffer solutions. The solid concentration is calculated by measuring its dry weight ($W_s(t)$). The temporal variation of solid porosity is calculated by measuring the average size of the solid cellulose particles sampled from the reactor at different times during the hydrolysis process. The initial porosity of the sample is measured using the formula

$$\in_0 = porosity\,at(t=0) = \frac{6 \times specificpore\ volume(at\ t=0)}{\overline{d}_p(t=0) \times specific\,surface\,area}, \qquad (1)$$

while, the solid porosity at all other times is calculated using the formula