Bioresource Technology 148 (2013) 611–614

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

A novel mixing strategy for maximizing yields of glucose and reducing sugar in enzymatic hydrolysis of cellulose

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HIGHLIGHTS

- Mixing effects on enzymatic hydrolysis of cellulose has been studied.
- Initial optimal mixing for few hours followed by no mixing maximizes product yield.
- Up to 31% increase in reducing yields reported for optimal mixing.
- Effect of solid loading on optimal mixing time has been quantified.
- Algebraic expression correlating optimal mixing time with solid loading obtained.

ARTICLE INFO

Article history:
Received 27 June 2013
Received in revised form 28 August 2013
Accepted 1 September 2013
Available online 12 September 2013

Keywords:
Cellulose
Cellulase
Enzymatic hydrolysis
Mixing
Bioethanol

ABSTRACT

This work explores the effects of mixing on enzymatic hydrolysis of cellulose to innovate a novel mixing strategy that maximizes glucose and reducing sugar yields for production of cellulosic ethanol while reducing the power required for reactor mixing. Batch experiments of cellulose hydrolysis are performed under aseptic conditions for 72 h at various substrate loading (2–6% wt./vol.), where the reactor mixing is terminated after different intervals of time ranging from 0 to 72 h. We find that initial mixing for a certain 'optimal mixing time' followed by no mixing for the rest of the reaction time maximizes glucose and reducing sugar yields. We report a maximum of 26% and 31% increase in glucose and reducing yields, respectively, in case of optimal mixing over continuous mixing for 2% substrate loading. We obtain an algebraic expression that predicts that the optimal mixing time increases exponentially with substrate loading.

1. Introduction

The commercial viability of the production of cellulosic ethanol lies on the effective conversion of cellulose to glucose by enzymatic hydrolysis, which is the rate limiting step in the bioethanol production process. The challenges are the slow rates of enzymatic reactions, deactivation of enzymes by mechanical shear and temperature (Reese and Ryu, 1980; Andreaus et al., 1999), product inhibition of enzymes (Ferchak and Pye, 1983), as well as the structural features of substrates (Zhu et al., 2008) which affect the reaction rate. Many researchers are trying to tackle these problems in different ways, such as by developing genetically modified bacteria and fungi which can secrete enzymes that are thermally stable, more specific towards substrates and have higher tolerance towards glucose and other products (Zhang et al., 2006), by optimizing enzyme composition (Berlin et al., 2007), by utilizing reactors with different shaking and/or mixing patterns (Roche et al., 2009), by adding additives to the reaction mixture (Ouyang et al., 2010), by using fed-batch bioreactors in which substrate and/or enzymes are added gradually to maintain low viscosity of the reaction mixture (Gupta et al., 2012), by recycling enzymes (Xue et al., 2012), by using different impellers in a reactor to change the mixing pattern so that the enzyme does not get deactivated by mechanical shear (Kinnarinen et al., 2012), etc. A recent study has shown that the quality of mixing is an important factor in enzymatic hydrolysis of cellulose (Lavenson et al., 2012). Taneda et al. have studied the effect of enzyme loading under static and agitated condition (Taneda et al., 2012). We have shown that micromixing limitations improve glucose and reducing sugar yields from cellulose in CSTRs (Chakraborty et al., 2010) and batch reactors (Gaikwad and Chakraborty, 2013) by preventing the two inhibitors, glucose and cellobiose, from coming in molecular contact with the enzymes and the substrates, thus reducing inhibition. In other words, the more local mixing in the reactor, the easier it is for the inhibitor to diffuse and bind to the active sites of the enzymes, forming enzyme-inhibitor/enzyme-inhibitor-substrate complexes (depending on whether the inhibition is competitive

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0960-8524/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2013.09.009

or non-competitive) that, in turn, decelerate the reaction rate by leaving fewer cellulase active sites available to the cellulose (substrate) molecules to bind.

This paper exploits this complex interlocked dynamics between chemical reactions and the convective and diffusive mass transport of cellulose and its hydrolysis products explored in our previous works (Chakraborty et al., 2010; Gaikwad and Chakraborty, 2013) to innovate a novel experimental strategy for maximizing glucose and reducing sugar yields from cellulose hydrolysis while reducing the power required for reactor mixing. Here, we also explore the effects of substrate loading on glucose and reducing sugar yields to study the effects of solid–liquid mixing vis-à-vis liquid–liquid mixing on the hydrolysis reaction. We obtain an algebraic expression that predicts that the optimal mixing time (for maximal glucose and reducing sugar production) increases exponentially with the substrate loading.

2. Methods

2.1. Materials

Commercially available microcrystalline cellulose, Avicel PH101, with average particle size of 50 μm and particle density of 0.600 g/cm^3, purchased from Sigma Aldrich Co. USA, has been used as substrate for our experiments. Dry solid purified cellulase enzyme with an activity of 1 U/mg of solid has been procured from HIMEDIA Labs, Mumbai, India, for the enzymatic saccharification of Avicel. The enzyme has been derived from *Trichoderma viride* and has a maximum activity at a pH range of 4.0–5.0 and a temperature of 40–50 °C against most cellulosic substrates. The purified enzyme contains all the three components i.e., endoglucanase, exoglucanase and β-glucosidase. For maintaining the pH of the reaction mixture, 0.1 M sodium acetate buffer (pH 5.0) has been used.

2.2. Experimental methods

The first part of this experimental study investigates the effects of partial initial mixing on glucose and reducing sugar production in enzymatic hydrolysis of cellulose in a batch reactor. For that, enzymatic hydrolysis experiments have been conducted for a period of 72 h for the following initial mixing durations: 0 h (no mixing), 1–8 h and 72 h (continuous mixing). The reactions are carried out in a 100 ml conical flask with a reaction mixture of 10 ml, kept in an incubator-cum-shaker under aseptic conditions and mixed at a shaking speed of 150 RPM with substrate concentration of 2% (wt./vol.) and enzyme loading of 1 mg/20 mg of substrate at a pH of 5.0 using 0.1 M sodium acetate buffer at 50 °C. All experiments have been duplicated to check the consistency of results. The effect of substrate loading on optimal mixing time has been studied by varying the solid loading from 2% to 6% with 1% increase at a time, keeping all other parameters constant.

2.3. Analytical methods

The glucose produced has been estimated by glucose oxidase (GOD) and peroxidase (POD) method using GOD–POD test kit obtained from Accurex Biomedical Pvt. Ltd., Mumbai, India. Glucose oxidase converts glucose to gluconic acid and hydrogen peroxide, following which the peroxide oxidatively couples with 4-aminoantipyrene and phenol in the presence of peroxidase to produce a red quinoeimine dye that absorbs light at 505 nm. The absorbance is directly proportional to the concentration of glucose in the sample:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\text{POD}} \text{Red quinoeimine dye} + H_2O \quad (2)$$

Reducing sugar has been measured by DNS method (Miller, 1959; Ghose, 1987). DNS reagent reacts with reducing sugars (and other reducing molecules) to form 3-amino-5-nitrosalicylic acid, which absorbs light at 540 nm.

3. Results and discussion

3.1. Effect of initial mixing on product yield

As already mentioned, the interplay of mass transfer and reaction kinetics plays an important role in determining the product yield and distribution in enzymatic hydrolysis of cellulose. Fig. 1 presents a comparison between no mixing and continuous mixing of enzymatic hydrolysis reaction mixture for 2% substrate loading. It may be observed from Fig. 1 that the initial rates of product (glucose and reducing sugars) formation are higher for continuous mixing than for no mixing but as the reaction time progresses, the rates of product formation decrease significantly, with no mixing producing more glucose and reducing sugars than continuous mixing. The initial higher rate in case of continuous mixing may be attributed to the fact that good mixing provides better mass transfer of enzymes to substrates, which ensures that the reaction that follows is not limited by mass transfer. However, after a certain reaction time, when enough products (glucose and cellobiose) have been formed to inhibit the reaction, no mixing gives better yield than continuous mixing due to reduced reaction inhibition. Mixing limitations reduce inhibition by taking advantage of the natural concentration heterogeneity in the reactor preventing the convection-mediated mass transfer of glucose and cellobiose from the regions in the reactor where the reaction has already occurred to the active sites of free enzymes in the reactor where the reaction is yet to occur. Therefore, we find no mixing to be better than complete mixing in case of enzymatic hydrolysis of cellulose.

Based on the understanding of this complex relationship between mixing, reaction and product inhibition, we conduct experiments where the reaction mixture is mixed initially for a certain period of reaction time, following which the mixing is terminated and the reaction is allowed to continue without any mixing for rest of the duration of 72 h. The initial period of mixing is varied from 0 to 8 h in increasing steps of 1 h, and we also examine the case of continuous mixing for 72 h. Fig. 2 shows that for 2% substrate loading, the glucose and cellobiose yields maximize when 4 h initial mixing followed by no mixing is used. The longer the reaction time, the higher the product yields. At the start of the reaction when

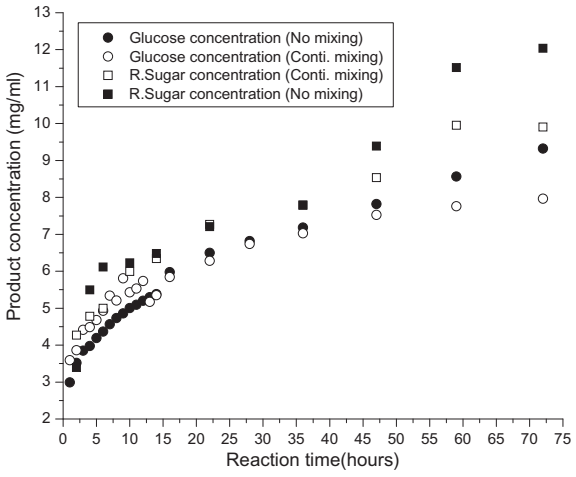

Fig. 1. Comparison of glucose and reducing sugar concentrations (mg/ml) for no mixing against continuous mixing at various hydrolysis times (hrs).