Bioresource Technology 164 (2014) 143–148

Contents lists available at ScienceDirect

Bioresource Technology

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

Relative extents of activity loss between enzyme–substrate interactions and combined environmental mechanisms

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HIGHLIGHTS

- Enzyme inactivation from substrate interactions and environmental factors were compared.
- 3 separate metrics showed enzyme–substrate interactions were dominant.
- Decrease in activity was a function of time and substrate concentration.
- Half-lives were lower for enzyme–substrate interactions.
- Inactivation rate constants were higher for enzyme–substrate interactions.

ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form 21 April 2014
Accepted 22 April 2014
Available online 6 May 2014

Keywords:
Enzymatic hydrolysis
Activity loss
Enzyme–substrate interactions
Environmental inactivation

ABSTRACT

Enzymatic hydrolysis of biomass undergoes a significant decrease in rate, which is often attributed to activity loss of enzyme during the incubation. Activity loss due to both interaction with substrate (for example inactivation of adsorbed enzyme) and all combined environmental mechanisms in a substrate free buffer solution were compared in this study. Enzyme–substrate interactions contributed more towards the overall activity loss than did the combined environmental sources as evidenced from three independent metrics. (1) Relative extents of inactivation were higher for enzyme–substrate interactions than for environmental mechanisms. (2) Apparent Half-lives (1.37–11.01 h) following interaction with substrate were relatively small compared to environmental inactivation, which was 21.5 h. (3) The inactivation rate constant for enzyme–substrate interactions (0.56 h^{-1}) was 46 times higher than that of environmental inactivation (0.0123 h^{-1}). These results suggest enzyme–substrate interaction is the main cause of cellulase activity loss and contributes significantly to the slow rate of hydrolysis.

1. Introduction

Currently, several technique barriers hamper the industrial development of ethanol from biomass, and enzymatic hydrolysis is the well-known rate liming step (Dasari and Berson, 2007; Dasari et al., 2009; Dunaway et al., 2010; Nidetzky and Steiner, 1993; Valjamae et al., 1998; Ye and Berson, 2011). Many studies focused on studying chemical properties of cellulose that limit fast

Abbreviations: A_0, inactivation extent of enzyme; $A_{0\text{-}environ}$, inactivation extent of enzyme due to environmental sources; A_{app}, apparent inactivation extent of enzyme due to enzyme–substrate interactions; $A_{E\text{–}S}$, normalized activity of cellulases at a certain time following interaction with substrate; A_{max}, the maximum adsorption sites per unit substrate (g/g); $A_{environ}$, normalized cellulase activity at a certain time during incubation in a substrate free buffer solution; C_1, glucose released in initial incubation period (g/L); C_2, the total glucose released after the initial and second incubation (g/L); $C_{2\text{-}0}$, glucose released after the second incubation without initial incubation (g/L); C_3, the glucose concentration increment in the control experiment during the second incubation (g/L); $(E)_0$, concentration of total enzyme (g/L); (ES), concentration of initial enzyme–substrate complex (g/L); $(ES)_{active}$, concentration of active enzyme–substrate complex (g/L); $(ES)_{inactive}$, concentration of inactive enzyme–substrate complex (g/L); K_d, the equilibrium constant of dissociation (g/L); $k_{f(ap)}$, the apparent inactivation rate constant (h^{-1}); $k_{f,E\text{–}S}$, the inactivation rate constant for adsorbed enzyme (h^{-1}); K_m, defined in Eq. 13 (g/L); $P_{E\text{–}S}$, relative extent of activity loss due to enzyme–substrate interactions; $P_{environ}$, relative extent of activity loss due to environmental mechanisms; (S), concentration of substrate (g/L); $t_{1/2}$, half life of enzyme (h); $t_{1/2,app}$, apparent half life of enzyme following enzyme–substrate interactions (h); $t_{1/2\text{-}environ}$, half life of enzyme following environmental inactivation (h); y_0, residue activity; $y_{0\text{-}environ}$, residue activity of enzyme following environmental inactivation; y_{app}, apparent residue activity of enzyme following enzyme–substrate interactions.

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http://dx.doi.org/10.1016/j.biortech.2014.04.080
0960-8524/

enzymatic conversion. However, these studies yielded contradicting results, as some findings suggested cellulose properties affected hydrolysis rate (Betrabet and Paralikar, 1977; Ooshima et al., 1983) while other results suggested cellulose properties did not affect hydrolysis (Lenz et al., 1990; Ohmine et al., 1983; Puls and Wood, 1991). Through "restart" experiment using new enzyme on partially hydrolyzed Avicel microcrystalline cellulose, it was found that reactivity of cellulose substrate did not change during the enzymatic hydrolysis process (Yang et al., 2006). Therefore, some factor other than the cellulose properties must limit the hydrolysis rate. It has been recently demonstrated using an AFM imaging technique that a "traffic jam" of cellobiohydrolase units on a substrate strip reduces hydrolytic efficiency (Igarashi et al., 2011). Our work also revealed that inactivation of adsorbed cellobiohydrolase1 (CBH1) is a major factor limiting the reaction rate (Ye and Berson, 2011; Ye, 2012).

Enzyme activity loss during the hydrolysis process has traditionally been associated with thermal, mechanical, and/or chemical mechanisms (Okino et al., 2013; Ye et al., 2012; Zhang et al., 2010). Due to the recent discovery of enzyme activity loss due to interaction with substrate, it is important to understand the relative contributions of enzyme–substrate interactions and all combined environmental sources (the net result of all contributions of the processing environment other than the substrate) to activity loss. The following experiments were designed to address this objective. First, activity loss was determined and normalized as a function of substrate loading, and compared with cellulase incubated without substrate. Then, three independent metrics were compared to quantify the relative extents of inactivation: (1) Relative activity loss from enzyme–substrate interactions and from environmental sources. (2) The apparent half-life and residual activity of enzyme following interaction with substrate and from environmental inactivation mechanisms. (3) The inactivation rate constant due to enzyme–substrate interactions and due to environmental sources.

2. Methods

2.1. Testing for relative extents of enzyme activity loss

Tests were run at 50 °C and 150 RPM in 250 mL flasks in an Innova 4230 incubator shaker. The pH of each flask was adjusted to 4.8 with citrate buffer. To prevent bacterial growth, 3 μL/mL of cycloheximide and 4 μL/mL of tetracycline was added to the slurry. The total operating volume of each test was 100 mL.

Relative extents of enzyme activity loss were examined using Solka Floc as the substrate, which is a commonly used cellulose model substrate (Zhang and Lynd, 2004) and has been extensively used to study cellulose hydrolysis kinetics (Bertrain and Dale, 1985; Sinitsyn et al., 1991; Ye and Berson, 2011). Solka Floc is a regenerated cellulose containing minor xylan contamination. Howell and Mangat (1978) reported that Solka-floc is nearly pure α-cellulose with just 0.2–0.4% lignin and 2.3% pentosans. The xylan and lignin content in is so small that Kádár et al. (2004) and Bansal et al. (2009) regarded Solka Floc as pure cellulose. Although it has been reported that xylooligomers could competitively inhibit cellulose hydrolysis, where 1.67 g/L resulted in 5–13% lower yield compared to hydrolysis of pure cellulose for example (Qing et al., 2010), the very low xylan content here on the order of just 0.1 g/L should not cause any pentose sugar inhibition.

To determine enzyme activity loss due to the combined environmental mechanisms, experiments were run where enzyme underwent two incubation periods. 0.6 mL of Spezyme CP cellulases was initially incubated for 2, 4, 8, 16, 24, 48 or 72 h without substrate, and then 2 g substrate was added for a second incubation period for 1 h as an assay to measure cellulase activity. These experiments were performed with duplicate samples and the measurements for each sample were repeated twice.

To determine enzyme activity loss from enzyme–substrate interactions, first 0.1, 0.2, 0.4, 0.8, and 1.2 g of Solka Floc substrate and 0.6 mL of Spezyme CP cellulases (104 mg/mL or equal to 50 FPU/mL) were added in the flasks and incubated for the same times listed above. This gave concentrations of 300, 150, 75, 37.5 and 25 FPU/g cellulose. The glucose released in this period was recorded as C_1. Each set of conditions was run in four flasks and duplicate measurements were made for each sample at each time point. A second one-hour incubation was then performed as an activity assay for the enzyme. During this second incubation, the total substrate amount was brought up to two grams. Substrate concentration was kept intentionally low to prevent inaccurate activity measurements due to mass transfer limitations, and is well below levels known to cause product inhibition. The total glucose released after the initial and second incubation was recorded as C_2. Of the original four flasks, two were used as a control and run without adding fresh substrate in the second incubation. The glucose concentration increment in the control experiment during the second incubation was recorded as C_3. The C_3 value was used to quantify the amount of sugar released from the original substrate during the second incubation. Enzyme's activity following interaction with substrate is defined as the glucose concentration increment (g/L) as a result of the freshly added substrate in the activity assay period, and is normalized by the 1-h glucose released ($C_{2\text{-}0}$) without initial incubation. Activity is defined here by: $(C_2 - C_1 - C_3)/C_{2\text{-}0}$. Fersht (1999) suggested that the activity of enzyme often varies from batch to batch. Normalization using a standard set of conditions can compensate for any variance in activity.

The procedure to determine the total activity loss as a function of incubating time is summarized in Table 1. A key assumption of this experiment is that substrate reactivity did not change. In addition to Yang et al., 2006 results indicating that substrate reactivity did not change, Ye, 2012 found that cellulose hydrolysis was not affected by variance of substrate properties such as crystallinity, pore size distribution, etc.

2.2. Glucose measurements

The liquid was tested for dissolved glucose content using a YSI 2700 Select Biochemistry Analyzer. For sampling, the slurry was stirred under the laminar flow hood and 1.5 mL was removed and heated above 85 °C for 10 min to stop the reaction. The sample was then centrifuged to separate the liquid out of the slurry.

3. Results and discussion

3.1. Relative extents of enzyme activity loss from enzyme–substrate Interactions and all combined environmental mechanisms

Normalized activity of cellulases following interaction with substrate is shown in Fig. 1. Activity decreased quickly up to ~24 h. For example, at 24 h, remaining activity was about 60% of the initial activity after interaction with 1 g/L substrate and only about 10% after interaction with 12 g/L substrate. This compares to about 80% remaining without any interaction with substrate (Fig. 2). After 24 h, activity of cellulases following interaction with substrate decreased more slowly, dropping to 45% and 10% of its original value over the next 48 h for the lowest, 1 g/L, and highest, 12 g/L, concentrations tested. By 72 h, the remaining activity with substrate was just 10–40% depending on substrate loading, and 60% without substrate. In summary, the more substrate present,