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Combination effect of pH and acetate on enzymatic cellulose hydrolysis

ROMSAIYUD Angsana[1], SONGKASIRI Warinthorn[2], NOPHARATANA Annop[3], CHAIPRASERT Pawinee[1,*]

1. Division of Biotechnology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Thakham, Bangkhuntien, Bangkok 10150, Thailand. E-mail: romsaiyud@yahoo.com
2. Excellent Center of Waste Utilization and Management, National Center for Genetic Engineering and Biotechnology, Bangkhuntien, Bangkok 10150, Thailand
3. Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi, Bangkhuntien, Bangkok 10150, Thailand

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Abstract

The productivity and efficiency of cellulase are significant in cellulose hydrolysis. With the accumulation of volatile fatty acids (VFAs), the pH value in anaerobic digestion system is reduced. Therefore, this study will find out how the pH and the amount of acetate influence the enzymatic hydrolysis of cellulose. The effects of pH and acetate on cellulase produced from *Bacillus coagulans* were studied at various pH 5–8, and acetate concentrations (0–60 mmol/L). A batch kinetic model for enzymatic cellulose hydrolysis was constructed from experimental data and performed. The base hypothesis was as follows: the rates of enzymatic cellulose hydrolysis rely on pH and acetate concentration. The results showed that the suitable pH range for cellulase production and cellulose hydrolysis (represents efficiency of cellulase) was 2.6–7.5, and 5.3–8.3, respectively. Moreover, acetate in the culture medium had an effect on cellulase production (K_I = 49.50 mmol/L, n = 1.7) less than cellulose hydrolysis (K_I = 37.85 mmol/L, n = 2.0). The results indicated that both the pH of suspension and acidogenic products influence the enzymatic hydrolysis of cellulose in an anaerobic environment. To enhance the cellulose hydrolysis rate, the accumulated acetate concentration should be lower than 25 mmol/L, and pH should be maintained at 7.

Key words: acetate; *Bacillus coagulans*; cellulase; cellulose; hydrolysis kinetics
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Introduction

For anaerobic digestion of cellulosic waste, cellulose hydrolysis is achieved with extracellular hydrolytic enzymes which are excreted by fermentative microorganisms. A group of enzymes work in a synergistic manner to hydrolyze cellulose called cellulase. However, due to the low cellulase activities in swamps, the rate of enzymatic cellulose hydrolysis is low (Noike *et al*., 1985; Pavlostathis *et al*., 1988; Hu *et al*., 2004). Therefore, various strategies have been used to try to increase the rate of this step. The enzymatic hydrolysis is a very complex system with multiple factors generated in the processes, rendering it difficult for operation and control (Gan *et al*., 2003). The optimization of these enzymes will require a more detailed understanding of their regulation and activity as a tightly controlled, highly organized system.

The world-wide attention for the production of cellulase under anaerobic conditions has been attracted due to the possibility of using this enzyme complex to convert cellulosic waste into fuel or feedstuff (Handelsman *et al*., 2004; Bayer *et al*., 2007; Guo *et al*., 2008). However, in anaerobic digestion systems, hydrolysis often occurs together with acidogenesis, therefore byproducts especially volatile fatty acids (VFAs) from the acidogenic step are found to inhibit the hydrolysis of cellulose (Zoetemeyer *et al*., 1982; Gottschalk, 1986). In addition, the rise and accumulation in the VFAs concentration gave a corresponding drop in the suspension's pH (Angelidaki *et al*., 1993; Florencio *et al*., 1995; Lata *et al*., 2002; Pind *et al*., 2003; He *et al*., 2007). Thus, both VFAs and the suspension's pH influence hydrolytic microbial activity in an anaerobic environment (Barredo and Evison, 1991; Aguilar *et al*., 1995; Mosche and Jordening, 1998; Batstone *et al*., 2002; Pind *et al*., 2003). The highly complex nature of interactions between VFAs and pH to cellulose hydrolysis makes predicting the inhibitory levels in cellulase difficult. Some studies have addressed the effects of pH and VFAs on the enzymatic hydrolysis of cellulose (Veeken *et al*., 2000; Babel *et al*., 2004; He *et al*., 2007), but it is still largely unknown how pH and VFAs affect the cellulase. Furthermore, acetate seems to be a main intermediate and often found in the anaerobic fermentation of cellulose, but the action of weak acids on cellulase has not been investigated to the same

* Corresponding author. E-mail: pawinee.cha@kmutt.ac.th

extent as that of strong acids. In addition, acetate is also the main substrate for biomethanation from the anaerobic digestion of cellulosic waste.

Therefore, in this study, it is expected to figure out how the amount and inhibition degree of pH and acetate influence the productivity or efficiency of cellulase. The approach used in this study will aid further development of a kinetic model and understanding of the enzymatic hydrolysis of cellulose system. Eventually, a good understanding of these mechanisms is essential for the rational control in anaerobic biological waste treatment.

1 Materials and methods

1.1 Microorganism and inoculum preparation

This study used *Bacillus coagulans*, which is dominant cellulose degradation bacterium, isolated from mesophilic anaerobic digester-treated pineapple peel obtained from Excellent Center of Waste Utilization and Management, King Mongkut's University of Technology Thonburi, Thailand. This strain was acclimatized by the same substrate for more than five years because of its ability to utilize cellulose, xylose, xylan and glucose for producing VFAs, such as acetate, butyrate and propionate.

B. coagulans was cultivated in 2-L media bottle (Pyrex) containing basal medium (Li and Noike, 1992) supplemented with 1 g/L cellulose, under anaerobic growth conditions by using an anaerobic chamber (Bactron Anaerobic Chamber, Model I, USA).

1.2 Culture medium and preparation

The culture medium described by Li and Noike (1992) was used in all experiments. The basal medium contains the following ingredients (g/L): KH_2PO_4, 0.4; K_2HPO_4, 0.4; $MgCl_2$, 0.1; NH_4Cl, 1; and yeast extract, 2. After having been autoclaved at 121°C for 15 min and allowed to cool at room temperature, the following constituents were added (g/L): L-cysteine HCl, 0.5; $Na_2S{\cdot}9H_2O$, 0.5; $NaHCO_3$, 5; with 10 mL mineral solution, and 10 mL vitamin solution. The mineral solution contained (g/L): nitrotriacetic acid, 4.5; $FeCl_2{\cdot}4H_2O$, 0.4; $CoCl_2{\cdot}6H_2O$, 0.12; $AlK(SO_4)_2$, 0.01; NaCl, 1.0; $CaCl_2$, 0.02; Na_2MoO_4, 0.01; $MnCl_2{\cdot}4H_2O$, 0.10; $CuSO_4{\cdot}5H_2O$, 0.01; and $NiCl_2{\cdot}6H_2O$, 0.02. The vitamin solution contained (g/L): biotin, 2; folic acid, 2; pyridoxine HCl, 10; thiamine HCl, 5; riboflavin, 5; nicotinic acid, 5; DL-calcium pantothenate, 5; vitamin B12, 0.1; *p*-aminobenzoine, 5; and lipoic acid, 0.5. The medium contained cellulose powder (α-cellulose; 38–106 μm, Sigma-Aldrich C2008, USA) as the substrate at concentration of 4 g/L. The substrate was added separately and aseptically after autoclaving.

1.3 Hydrolysis test

The hydrolysis of cellulose was conducted by batch experiment using a 120-mL serum vial containing 80 mL of basal medium (Li and Noike, 1992) supplemented with 400 mg of fixed cellulose and 20 mL of inoculum (0.136 g VSS/L). This work was divided into two parts. In part I, productivity and the efficiency of the enzyme was investigated as a function of pH. The pH was adjusted to its initial values ranging from 5.0 to 8.0 in the phosphate-sodium buffer. Part II, the effect of acetate concentration was investigated. Various volumes of 2 mol/L acetate concentration (stock solution) were added to the culture medium for initial reaction. Acetate concentration was 10, 30, 40 or 60 mmol/L, pH was maintained at 7 ± 0.2 by 1 mol/L phosphate buffer. All tests were done under anaerobic condition using an anaerobic chamber. The vial bottles then were closed with rubber-stoppers and sealed with aluminum caps before incubated at 37°C. The control was performed as the same experiment without acetate addition. Experimentation was carried out for 24 h in triplicates.

1.4 Analytical methods

VFAs, alcohols, cellulase activity, glucose and cellulose residue were analyzed from the total content of each vial. Twenty-five milliliter culture sample from each vial was centrifuged at 10000 ×*g* for 15 min, then collected solid sediment and supernatant for analysis. Insoluble cellulose remaining in sediment was analyzed by Fibretherm (Gerhardt, Germany). The changes in concentration of insoluble cellulose over the course of the batch enzymatic hydrolysis were calculated for enzymatic efficiency estimation. The supernatant was retained for the determination of VFAs (acetate, propionate, butyrate, etc.) and alcohol using gas chromatography (GC-14B; Shimadzu, Japan) equipped with a flame ionization detector (FID) and Carbopack B-DA/4% Carbowax 20M column. Glucose and Lactate Analyzer (YSI 2300 STAT Plus, USA) was used for detecting glucose and lactate in the supernatant solution.

Cellulase production was determined by measuring the activity of cellulase over the time period. Carboxymethyl cellulase (CMCase) activity was assayed using a 1% (*W/V*) carboxymethyl cellulose (CMC) solution (Sigma Chemical Co., USA) in 0.1 mol/L phosphate buffer (pH 7.0) as substrate. The reaction mixture contained 0.5 mL of substrate and 0.5 mL of supernatant. This mixture was incubated at 50°C for 10 min (Cohen *et al*., 2005). After incubation, the CMC enzyme-mixed solution stopped the reaction by adding DNS followed boiling for 5 min (Miller, 1959). The developed color was read at 540 nm using spectrophotometer (Hach DR 12500, USA). One unit of cellulase (U) was defined as the amount of enzyme which produced 1 μmol glucose equivalent per min under the assay condition.

2 Results and discussion

2.1 Effect of pH on enzymatic cellulose hydrolysis

Four different initial pH values 5, 6, 7 and 8 were used during this part of the study. The cellulose degradation patterns and cellulase production profiles at various pH values are illustrated in Fig. 1. At pH 7, the amount of hydrolyzed cellulose was the highest (pH 7 > pH 6 > pH 5

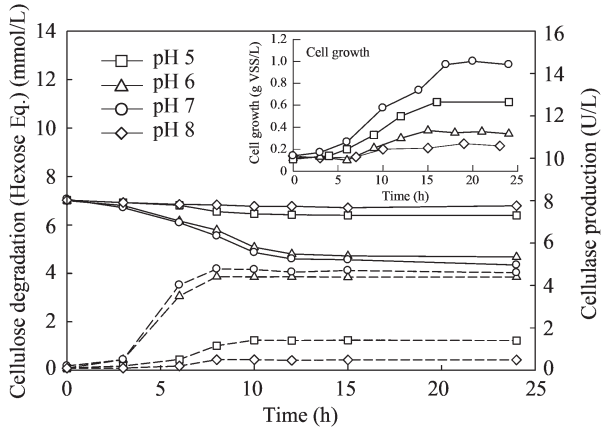

Fig. 1 Time course of cellulose degradations (solid line) and cellulase production (dotted line) by *B. coagulans* in batch culture at different pH values.

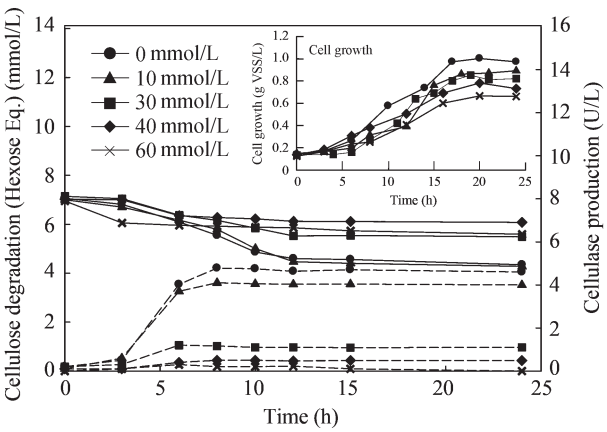

Fig. 2 Time courses of cellulose degradation (solid line) and cellulase production (dotted line) by *B. coagulans* in batch culture at different acetate concentrations.

> pH 8). It is clearly seen that at pH 7, cellulase obtained from *B. coagulans* were optimized for their activity. The shift of pH values above or below pH 7 was followed by the rapid decrease of hydrolyzed cellulose. From this result it can be considered that cellulase production and/or its efficiency was decreased when pH within the vial was shifted. pH will effect the total net charge of enzymes. Thus, when enzyme charges are disrupted, the enzyme undergoes changes in its 3D shape and hence becomes denatured and unable to catalyze chemical reactions. On the other hand, the shift of pH values could be partially attributed to the limited growth rate of cellulose-degrading microorganisms, resulting in the production of cellulase production and its activity decreased with cell amount decreasing (Hu *et al*., 2004; Ariffin *et al*., 2006).

2.2 Effect of acetate on enzymatic cellulose hydrolysis

Figure 2 illustrates the initial effect of acetate concentration on cellulose degradation and cellulase production (initial pH value was maintained at pH 7). A control experiment (without added acetate) gives the highest amount of hydrolyzed cellulose (2.72 mmol/L), when increased initial acetate concentration resulted in the reduction of cellulose hydrolysis. However, when 10 mmol/L acetate was added in the culture it had a slight effect on cellulose hydrolysis. As acetate concentration was increased to over 30 mmol/L, a rapidly decreasing amount of hydrolyzed cellulose was detected. It might be feedback inhibitory of the enzyme by acetate concentration (Srinivasan *et al*., 2001). Moreover, undissociated acids might affect the growth of the bacteria. Wang and Wang (1984) reported that the unionized acetic acid was much more inhibitory than the ionized acetate ion. They mentioned that complete growth inhibition occurred when the unionized acetic acid concentration was in the range of 40–50 mmol/L. This value is quite similar to our experimental result. The complete inhibition of enzyme activity (related with growth) was about 40 to 60 mmol/L.

2.3 Products formation of cellulose hydrolysis

The data for pH 7 without acetate were used to compare the cellulose degradation efficiency (Table 1). The percentage of cellulose degradation was reduced when pH within the vial was changed from pH 7. Comparing with pH 7 when suspension pH was increased up to 8 (alkaline condition), the relative efficiency of cellulose degradation lost over 90%; when the pH of the culture medium decrease to 5 (acidic condition), 77% of the relative efficiency of cellulose degradation decreased. In addition, an increase in the acetate concentration of 3 fold resulted in the reduction of cellulose degradation efficiency to 40%. Moreover, at 60 mmol/L of acetate added, cellulose degradation was also found together with glucose remaining in the medium (0.40 mmol/L), whereas the cellulase activities was close

Table 1 Effect of pH and acetate concentration on cellulose degradation, glucose residue and product formation at the end of culture period (24 h)

Condition	Cellulose degradation (%)	Glucose residue (mmol/L)	Production yield ((mmol/L)/(mmol/L cellulose) (Hexose Eq.))		
			Ethanol	Acetate	Butyrate
Initial pH					
5	8.87 (23.05) a	0.13	0.03	0.001	ND
6	33.18 (86.21) b	0.10	0.20	0.29	0.01
7	38.48 (100.00) b	0.09	0.43	0.56	0.10
8	3.32 (8.62) c	0.13	0.18	0.19	0.04
Acetate conc. (mmol/L)					
0	38.48 (100.00) a	0.09	0.43	0.56	0.10
10	38.90 (101.08) a	0.14	0.44	0.54	0.11
30	23.13 (60.11) b	0.14	0.56	0.43	0.09
40	13.43 (34.89) c	0.15	0.61	0.32	0.12
60	19.59 (50.90) b	0.40	0.78	0.19	0.11

Data in parenthesis are relative efficiency of cellulose degradation. Data with different letters in columns mean difference ($P < 0.05$). ND: not determined.

to zero. This illustrates that the acetate itself can hydrolyze partially the cellulose structure into glucose. Thus, only glucose accumulation was detected and no activity of cellulase. This was confirmed by setting an experiment without inoculation of *B. coagulans* (data not shown). The amount of formation has decreased due to the change of culture condition from the optimum environment. The detected products included acetate, butyrate, lactate and propionate, while ethanol was the sole alcohol detected. Among them, ethanol, acetate and butyrate were the major products. The yield of products (ethanol, acetate and butyrate) was determined by the product formation and the corresponding substrate degradation during the fermentation. The yields of ethanol and butyrate were raised with increasing acetate concentration. But also, the products ratio of ethanol:acetate:butyrate almost standing as 1:1:0.2, when suspension pH in the culture medium was shifted.

2.4 Kinetic of enzymatic cellulose hydrolysis

The basic hypothesis of cellulose degradation as proposed is illustrated in Fig. 3. Cellulose degrading into glucose is brought about mainly by cellulase that is produced from bacteria. The produced glucose is then taken up by cell and used for growth and products formation (VFAs and alcohols). As confirmed by Table 1, the low concentration of glucose (< 0.2 mmol/L) remaining in the supernatant. Therefore, the rate of cellulose degradation depended on cellulase, namely efficiency and production of enzymes. It is possible to determine the cellulase efficiency from rate of cellulose degradation. The cellulase activities measured in the supernatant of fermentation broth were defined as cellulase production in previous studies (Lee and Blackburn, 1975; Szengyel and Zacchi, 2000; Ibrahim and El-diwany, 2007; Femi-Ola and Aderibigbe, 2008). In these studies the effects of pH and acetate on both criteria have been considered.

2.4.1 Effect of pH

When cellulose degradation was inhibited by the suspension's pH, the original Michaelis-Menten model became unsatisfactory. The effect of pH on enzyme was analyzed by the Michaelis pH function (Angelidaki *et al*., 1993), as shown in Eq. (1). Parameters, pH_{OH} and pH_H determine the location and shape of the pH inhibition curves.

$$V_{max,\,app} = \frac{V_{max} \times C_{H^+}}{C_{H^+} + pH_{OH} + \frac{C_{H^+}}{pH_H}} \quad (1)$$

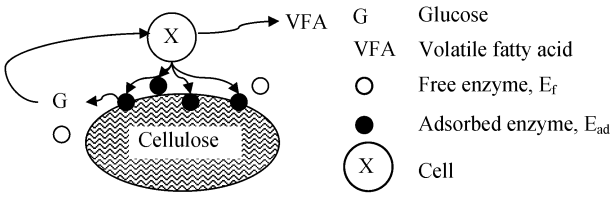

Fig. 3 Schematic for the proposed basic mechanism of the enzymatic hydrolysis of cellulose.

where, $V_{max,app}$ (U/(L·h)) is the apparent maximum rate, V_{max} (U/(L·h)) is the maximum rate, pH_{OH} and pH_H are the parameters due to the pH inhibition effect. As can be seen from Table 2, the rates of cellulose degradation are more pH sensitive than cellulase production rate.

Table 2 Kinetic parameters of Michaelis pH function on enzymatic cellulose hydrolysis for the rate of cellulase production and cellulose hydrolysis

Rate condition	pH_{OH}	pH_H	R^2
Cellulase production (U/(L·h))	7.5	2.6	0.93
Cellulose hydrolysis (mmol/(L cellulose·h))	8.3	5.3	0.95

pH_{OH} and pH_H, parameter due to pH inhibition effect; R^2: correlation coefficient.

2.4.2 Effect of acetate

The model for hydrolysis inhibition by acetate was modeled by a non-competitive enzyme inhibition model (Eq. (2)). This model could describe the reaction kinetics with a degree of confidence higher than 99.9% according to the square test.

$$V_{max,\,app} = \frac{V_{max}}{(1 + (\frac{A_a}{K_I})^n)} \quad (2)$$

where, A_a (mmol/L) is an acetate concentration, K_I (mmol/L) is the inhibition constant for cellulase by acetate, and n is degree of inhibition on cellulase by acetate. Followed by Eq. (2) the kinetic parameters of cellulase production and cellulose hydrolysis rate were estimated (Table 3).

The inhibition constant for the cellulose degradation rate was 37.85 mmol/L, which was lower than the cellulase production rate (49.50 mmol/L), suggesting that for *B. coagulans*, acetate inhibits the ability of cellulase for hydrolyzing cellulose more strongly than inhibiting cells for cellulase production. The inhibition effects might be based on the assumption that undissociated fraction of acids affect the enzyme structure and inhibits the activity of bacteria (Szengyel and Zacchi 2000, Babel *et al*., 2004). This is quite different to the results reported by Schwartz and Keller (1982) that the maximum undissociated acetate concentrations obtained from acidogenesis were 2.12 and 2.44 mmol/L at pH 6 and 7, respectively, because they worked with *Clostridium thermoaceticum*. However, we all agree that that undissociated fraction of acids inhibits the activity of bacteria.

Table 3 Kinetic parameters of the non-competitive enzyme inhibition model by acetate for the rate of cellulase production and cellulose hydrolysis

Rate condition	K_I (mmol/L)	n	R^2
Cellulase production (U/(L·h))	49.50 (0.94)	1.7	0.99
Cellulose hydrolysis (mmol/(L cellulose·h))	37.85 (0.83)	2.0	0.99

K_I: the inhibition constant for cellulase by acetate; n: the degree of inhibition on cellulase by acetate; R^2: correlation coefficient. Data in parenthesis are undissociated acetate concentration at pH 7.

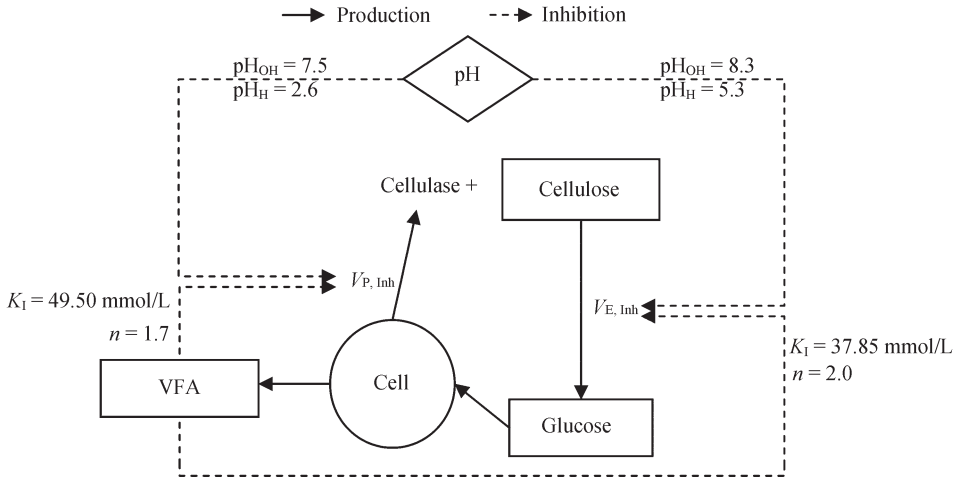

Fig. 4 Summarized overall diagrams of cellulose hydrolysis by cellulase from *B. coagulans* including affect of pH and acetate. $V_{E, Inh}$ and $V_{P, Inh}$ (u/(L·h)) are inhibition rate of cellulose hydrolysis and cellulase production, respectively.

3 Conclusions

The experimental results showed that the rate of enzymatic cellulose hydrolysis depended on suspension's pH and accumulation of acetate in the digester. The shift of pH (from 7) and over 30 mmol/L acetate accumulation led to a decrease of both cellulase production and cellulose hydrolysis. A batch kinetic model of enzymatic cellulose hydrolysis encompassing the effect of pH and acetate has been constructed. Thus, it can be the reaction involved in cellulose degradation while considering the effects of pH and acetate concentration as shown in Fig. 4.

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