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Influence of particle size and pH on anaerobic degradation of cellulose by ruminal microbes

Zhen-Hu Hu, Han-Qing Yu*, Ren-Fa Zhu

Laboratory of Environmental Biotechnology, School of Chemistry, Department of Applied Chemistry, The University of Science and Technology of China, Hefei, Anhui 230026, China

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Abstract

Batch experiments were performed to investigate the influence of cellulose particle size and pH on the anaerobic degradation of crystalline cellulose by ruminal microbes. At a particle size of 50 μm there was a higher hydrolysis and acidogenesis rate, and a reduced degradation time, than for 100-μm particles. Reduction in cellulose particle size resulted in decreased methane production, but an increase of soluble products. Cellulose degradation increased with pH from pH 6.0 to 7.5, whereas at pH $\leqslant$ 5.5 there was no degradation. The inhibitory effect of low pH ($\leqslant$5.5) on ruminal microbes was not completely remedied even when the pH of the medium was adjusted to a neutral range. In an anaerobic cellulosic waste degrading system inoculated with ruminal microbes the fermentation system should therefore be maintained above pH 6.0. In all cases, volatile fatty acids were the major water-soluble products of cellulose degradation; acetate and propionate accounted for more than 90% of the volatile fatty acid total.

Keywords: Anaerobic degradation; Cellulose; Inhibitory effect; Particle size; pH; Ruminal culture

1. Introduction

Cellulosic wastes are generated in significant amounts from both industrial and agricultural/forestry activities, and are important potential renewable energy resources (Thanakoses et al., 2003). The conversion of cellulosic wastes to useful form, as feedstuff or fuels, using chemical or biological methods has been proposed and explored. Biological conversion is attractive for its environmental benefits, such as reducing the greenhouse effect and increasing resource availability (Thanakoses et al., 2003). Owing to the refractory structure of cellulose, hydrolysis is the key process for the biological conversion of cellulosic wastes (Noike et al., 1985; Pavlostathis et al., 1988).

Many attempts have been made to determine the nature of the substrate characteristics affecting the hydrolysis of particulate organic substrates by anaerobic cultures. The surface area and particle size of cellulose were found to be important substrate characteristics in determining the initial rates of hydrolysis (Eriksson et al., 2002). Degradation with anaerobic sludge indicated that cellulose with a particle size of 20-μm resulted in a higher conversion efficiency than that with 50-μm particle size (Chyi and Dague, 1994). Owing to crystalline regions of cellulose tightly held together by hydrogen bonding, crystalline cellulose is less accessible to enzymes (Eriksson et al., 2002). Rumen microbes have shown high efficiency in conversion of cellulose because of their high cellulase activity, by which even crystalline cellulose can be hydrolyzed (Gijzen et al., 1987). However, information about the effect of particle size on the hydrolysis and acidogenesis of particulate organic substrates by ruminal microbes is still limited.

*Corresponding author. Tel.: +86 551 360 7592; fax: +86 551 360 1592.
E-mail address: hqyu@ustc.edu.cn (H.-Q. Yu).

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In an artificial rumen continuous- or semi-continuous-flow bioreactor for cellulosic waste degradation, the pH value in the reactor is likely to decline to a very low level, because of formation of excess volatile fatty acids (VFAs), whereas in a natural rumen system, the pH is in the range of 6.1–7.1. The inhibitory effect of low pH on the anaerobic degradation of cellulose is as yet little reported in the literature. However, information on this would be helpful for designing and operating artificial rumen reactors for converting cellulosic wastes to valuable products.

The aim of this work was to determine the effects of particle size on the hydrolysis and acidogenesis of cellulose with ruminal microbes, and to find out the optimum pH range for cellulose degradation. Furthermore, the inhibitory effect of low pH to the artificial rumen degradation was also explored.

2. Materials and methods

2.1. Culture, substrate and media

Rumen digesta obtained from a fistulated goat were immediately transferred to the laboratory and squeezed through four-layer cheesecloth. The rumen fluid was used as a source of seed microorganisms.

Crystalline cellulose with average particle sizes of 50 and 100 μm (Avicel PH 101 and 102, Ajiao Co., China) was used as the sole carbon and energy source. The medium used in these experiments contained the following ($g\,L^{-1}$): $NaHCO_3$, 8; KH_2PO_4, 1; K_2HPO_4, 3; $CaCl_2 \cdot 2H_2O$, 0.03; $MgCl_2 \cdot 6H_2O$, 0.08; NH_4Cl, 0.18; L-cysteine-HCl, 0.17; modified Pfennig metal solution, 1 ml; B vitamin solution, 5 ml. Pfennig metal solution had the following composition ($g\,L^{-1}$): $ZnSO_4 \cdot 7H_2O$, 0.10; $MnCl_3 \cdot 4H_2O$, 0.03; H_3BO_3, 0.30; $CoCl_2 \cdot 6H_2O$, 0.20; $CuCl_2 \cdot 2H_2O$, 0.01; $NiCl_2 \cdot 6H_2O$, 0.02; $NaMoO_4 \cdot 2H_2O$, 0.03; $FeCl_2 \cdot 4H_2O$, 1.50. B vitamin solution had the following composition ($mg\,L^{-1}$): nicotinic acid, 20; cyanocobalamin, 20; thiamine, 10; *p*-aminobenzoic acid, 10; pyridoxine, 50; and pantothenic acid, 5.

2.2. Experimental conditions

In vitro anaerobic degradation tests were carried out in 250-ml serum bottles filled with rumen fluid (20 ml) and the medium described above (80 ml). Crystalline cellulose at a rate of 4 and 8 $g\,L^{-1}$ was used in particle-size experiments, while 10 $g\,L^{-1}$ was used in pH experiments. Two particle sizes (average diameter 50 and 100 μm) and seven pH levels (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) were tested. After adjustment with 2 N NaOH and 2 N HCI to the predetermined pH values, the headspace was subsequently flushed with N_2 gas for 2 min to create anaerobic conditions. The bottles were then sealed with butyl rubber stoppers and aluminum crimp seals and incubated at $39.5 \pm 1\,^{\circ}C$ in a shaking incubator.

After incubation for 132 h in pH experiments, all bottles were opened under N_2 and 1.0 g $NaHCO_3$ was added, after which 2 N NaOH or 2 N HCI was then added to adjust the pH to 6.7–6.9 before the headspace was flushed with N_2 and the bottles were resealed. The pH values after adjustment were 6.75, 6.74, 6.78, 6.76, 6.81, 6.79, and 6.74, respectively. The incubation conditions were the same as before.

2.3. Analytical methods

Cellulose, reducing sugars, VFAs and soluble total organic carbon (TOC) were determined as described by Hu et al. (2004). Soluble TOC was measured using a TOC analyzer (TOC-V_{CPN}, Shimadzu Co., Japan), whereas VFAs was determined by a gas chromatograph (GC-6890N, Agilent Inc., USA) equipped with a flame ionization detector and a 30 m × 0.25 mm × 0.25 μm fused-silica capillary column (DB-FFAP). Biogas production was measured by a water displacement method and analyzed by a gas chromatograph (SP-6800A, Lunan Instrument Co., China) equipped with a thermal conductivity detector and a 1.5 m × 2 mm stainless-steel column packed with Porapak T (50–80 mesh).

3. Results and discussion

3.1. Effects of particle size

3.1.1. Cellulose degradation

When degradation of cellulose at the two substrate levels was investigated, at 4 $g\,L^{-1}$ (Fig. 1a) the degradation efficiency achieved within 216 h was 93% for cellulose with a particle size of 50 μm, and approx. 92% at a particle size of 100 μm, indicating that degradation efficiency was not substantially affected by the change of particle size. However, as shown in Fig. 1a, up to 144 h, 50-μm cellulose had a steeper degradation curve than 100-μm. This suggests that the reduction of particle size was beneficial to the hydrolysis of cellulose. This difference could partially be attributed to the enlargement of the available specific surface area that microorganisms could reach and adhere (Sanders et al., 2000). Palmowski and Müller (2003) have investigated the significance of the surface area in anaerobic degradation of particulate substrates through a kinetic model. They have found that the hydrolysis rate was mainly based on the sample surface area. A good agreement between the measured and calculated was observed for the samples of various specific surface areas. These results reinforced the significance of

cellulose particle size in anaerobic degradation processes.

With the substrate at 8 g L^{-1}, the degradation efficiency of cellulose of particle size 50 μm was 89%, slightly higher than the 86% for 100-μm particles. However, the degradation curves for the two particle sizes were similar (Fig. 1b), so that cellulose degradation efficiency was unaffected by particle size at this range. Cellulose degradation efficiency for 4 g L^{-1} was slightly higher than with 8 g L^{-1}.

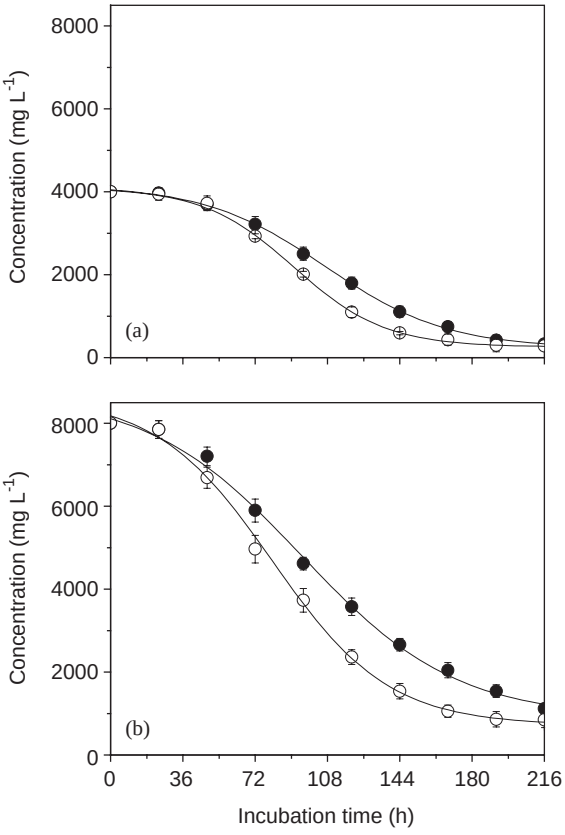

Fig. 1. Degradation of cellulose by ruminal microbes from goat. Substrate level: (a) 4 g L^{-1} and (b) 8 g L^{-1}; cellulose particle size: ○ 50-μm; ● 100-μm.

3.1.2. VFA and reducing sugar production

As shown in Table 1, a significant amount of VFAs was produced during degradation of cellulose. Acetate and propionate were found to be the two main VFAs for both particle sizes. In addition, butyrate and iso-butyrate were also formed, but in much smaller amounts. With 4 g cellulose L^{-1}, the amounts of VFAs were 2047 mg L^{-1} for 50-μm and 2102 mg L^{-1} for 100-μm particles, respectively, accounting for 77% and 69% of total soluble metabolites. With 8 g cellulose L^{-1}, the corresponding amounts were 4598 and 3815 mg L^{-1}. Their contributions to the total soluble metabolites were 84% and 78% from 50- and 100-μm cellulose particles, respectively.

The reducing sugar concentration was 49–74 mg L^{-1} for the four treatments (Table 1). The fraction of reducing sugars in soluble TOC (1248–2417 mg L^{-1} as organic carbon) was only <2%. Since reducing sugars are readily utilized by anaerobic microorganisms, a low concentration of reducing sugars indicates that there was no accumulation of hydrolytic products in the anaerobic fermentation of cellulose by ruminal cultures (Chyi and Dague, 1994). This suggests that the acidogenesis of hydrolytic products was much faster than the hydrolysis of crystalline cellulose by ruminal cultures.

3.1.3. Biogas production

At 4 g cellulose L^{-1}, approx. 400 ml methane L^{-1} reactor was produced for both particle sizes (Fig. 2a). However, there was considerable difference between the results for the two particle sizes at 8 g substrate L^{-1}, with 110 ml methane L^{-1} reactor for 50-μm particles and 310 ml L^{-1} for 100-μm being produced. Since the methanogens are far more sensitive to the accumulation of VFAs than the acidogens (Veeken and Hamelers, 1999), the reduction of methane production might be attributed to the increase of available adsorption sites, i.e. with smaller particle size and higher substrate concentration. This might result in an enhancement of hydrolytic rate and VFA accumulation (Table 1).

Table 1
Products of hydrolysis and acidogenesis from cellulose at particle sizes of 50 and 100-μm

Substrate level (g L^{-1})	Particle size (μm)	VFAs (mg L^{-1})	HAc (mg L^{-1})	HPr (mg L^{-1})	HBu (mg L^{-1})	i-HBu (mg L^{-1})	TOC (mg L^{-1})	VFAs/TOC	Reducing sugars (mg L^{-1})
4	50	2047	1064	881	121	36	1291	0.77	57
	100	2102	1334	529	153	31	1248	0.69	49
8	50	4598	2496	1750	305	47	2417	0.84	74
	100	3815	1944	1509	258	67	1711	0.78	62

Note: HAc = acetate; HPr = propionate; HBu = butyrate; i-HBu = iso-butyrate.

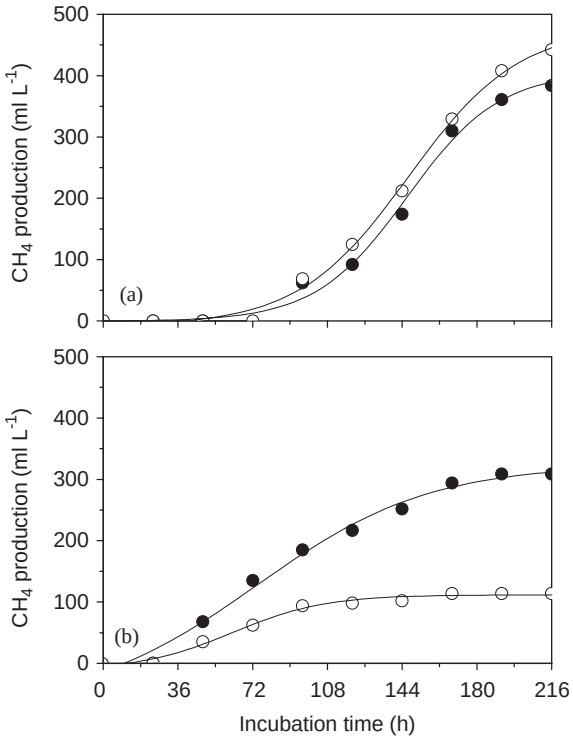

Fig. 2. Methane production from cellulose by ruminal microbes from goat. Substrate level: (a) 4 g L^{-1} and (b) 8 g L^{-1}; cellulose particle size: ○ 50-μm; ● 100-μm.

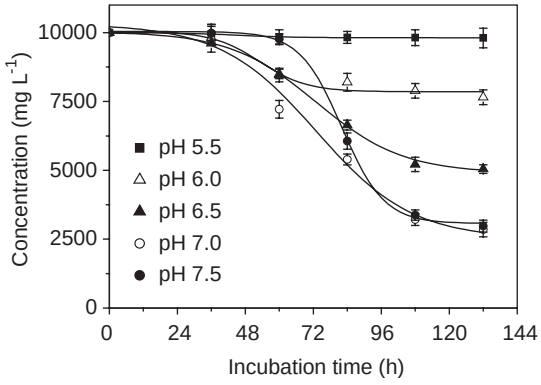

Fig. 3. Effect of pH on cellulose degradation profiles.

3.2. Effects of pH

3.2.1. Cellulose degradation

When the effect of pH on fermentation patterns was investigated (Fig. 3), at pH 7.0 the shortest lag-time was observed, with cellulose hydrolysis beginning 24 h after inoculation. Any decrease in pH below pH 7.0 resulted in a longer lag-time and a reduction in hydrolysis rate. However, as pH increased from 7.0–7.5, while a longer lag-time was also observed, total cellulose hydrolysis was not affected. Cellulose degradation efficiency increased with pH (Fig. 3). The highest cellulose degradation efficiency, 71%, was achieved at pH 7.0 and 7.5, but cellulose degradation did not occur at pH $\leqslant$ 5.5. The decline of cellulose degradation efficiency at lower pH could be attributed to the inhibition of cellulolytic bacteria, since most ruminal cellulolytic bacteria are pH-sensitive (Russell and Wilson, 1996). The pH sensitivity of cellulolytic bacteria can be explained by the differences in intracellular pH regulation (Russell and Rychlik, 2001). When the extracellular pH of acid-sensitive bacteria declines, the intracellular pH is relatively stable, but the increase in the transmembrane pH gradient causes a logarithmic accumulation of intracellular fermentation acid anions and hence leads to anion toxicity and products inhibition (Russell and Wilson, 1996). In contrast, pH-resistant ruminal bacteria, e.g. non-cellulolytic bacteria, allow their intracellular pH to decline. This protects them from the influx and accumulation of fermentation acid anions (Russell, 1991), but cellulolytic bacteria cannot grow with a low intracellular pH (Russell and Wilson, 1996).

3.2.2. VFA and soluble carbohydrate production

Acetate and propionate were the two major water-soluble products of cellulose fermentation at all pH levels tested, except where there was no cellulose degradation and no VFAs or reducing sugars were produced, i.e. at pH $\leqslant$ 5.5 (Table 2). The concentrations of acetate and propionate both increased with pH, and were greatest at 2033 and 1805 mg L^{-1} at pH 7.5. The contribution of VFAs to soluble products was 72–74%. The reducing sugar concentration was 62–80 mg L^{-1}. They comprised not less than 2% of TOC at all pH values, suggesting that acidogenesis of hydrolytic products, i.e. reducing sugars, was little affected by pH shift in the range pH 6.0–7.5.

3.2.3. Inhibitory effect of low pH on cellulose degradation

After pH was adjusted to a neutral pH range (pH 6.5–7.0), inhibition of cellulose degradation by low pH was partially overcome. Cellulose degradation efficiencies at initial pH of 4.5 and 5.0 increased to 67.9% and 70.3%, respectively, after the pH adjustment (Fig. 4). This indicates that operation at low pH, i.e. pH 4.5–5.5, for one week inhibited the cellulose degrading activity of ruminal microbes. No significant inhibition was observed for the tests at initial pH $\geqslant$ 6.0, suggesting that pH 6.0–7.5 was appropriate for cellulose degradation with ruminal microbes. This study implies that in a cellulose-degrading system inoculated with ruminal microbes, the medium pH should be kept above 6.0.

Of acidogenic products at the end of fermentation VFAs were the major aqueous products and they amounted to approx. 75% of the soluble TOC at the pH values tested. Acetate and propionate were the two main acids produced (Fig. 5), accounting for >90% total VFAs. The highest concentration of acetate,

Table 2
Products of hydrolysis and acidogenesis from cellulose at various pH values

PH	VFAs (mg L^{-1})	HAc (mg L^{-1})	HPr (mg L^{-1})	HBu (mg L^{-1})	i-HBu (mg L^{-1})	TOC (mg L^{-1})	VFAs/TOC	Reducing sugars (mg L^{-1})
7.5	4042	2033	1805	176	28	2468	0.730	89
7.0	3751	1836	1709	189	17	2317	0.724	62
6.5	2620	1505	817	287	11	1532	0.758	80
6.0	1326	751	483	92	0	781	0.750	64

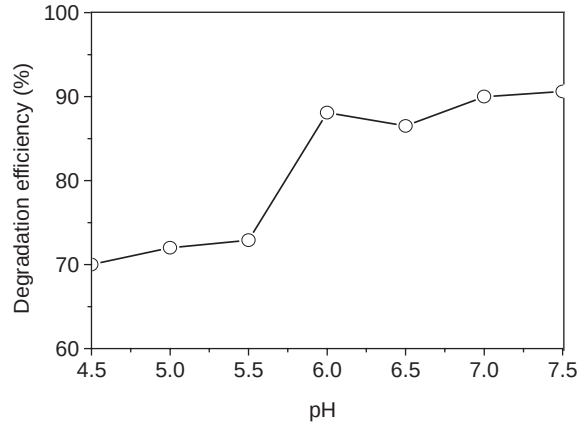

Fig. 4. Cellulose degradation efficiency after adjustment of pH to a neutral range (pH 6.5–7.0).

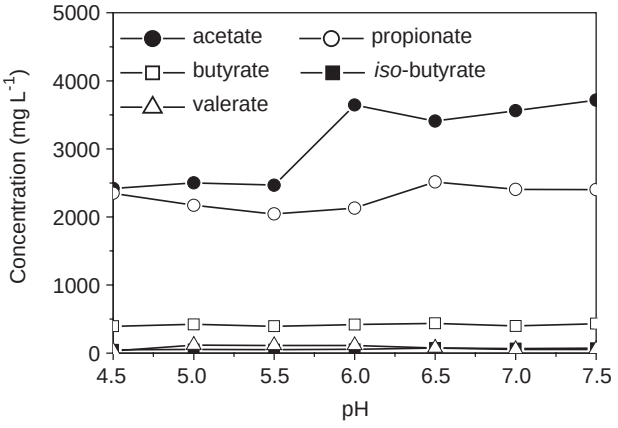

Fig. 5. Effect of pH on production of individual VFAs.

3918 mg L^{-1}, occurred at pH 7.5, but at pH 4.5 the corresponding value was only 2500 mg L^{-1}. Compared with acetate, amounts of propionate were similar at all pH values close to 2300 mg L^{-1}. In addition, the concentrations of longer VFAs, such as butyrate, iso-butyrate and valerate, were not affected considerably by the pH shift (Fig. 5). This implies that the inhibition of propionate production by low pH could be completely remedied, but it was not the same for acetate. This might be due to the fact that the propionate-producing bacteria in ruminal cultures had a stronger tolerance to low pH than acetate-producing bacteria. It also suggests that the inhibition of ruminal microbes under

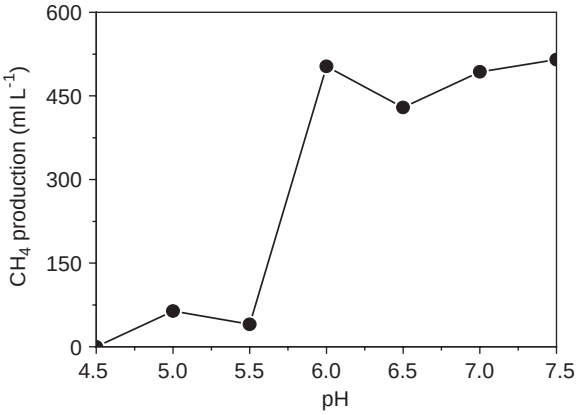

Fig. 6. Effect of pH on methane production by ruminal microbes from goat.

low pH conditions, i.e. at pH $\leqslant$ 5.5, could not be completely remedied in an anaerobic system for cellulosic wastes degradation.

Only a small amount of methane was produced at pH 4.5–5.5 (Fig. 6), indicating that methane production was inhibited at pH $\leqslant$ 5.5, whereas at pH 6.0–7.5, approx. 480 ml methane L^{-1} reactor was produced. The inhibition of methane production could not be remedied even when medium pH was raised to the optimum range.

4. Conclusions

Reduced particle size was beneficial to hydrolysis of cellulose by ruminal cultures. Compared with a particle size of 100 μm, there were increased rates of hydrolysis and acidogenesis and reduced degradation time for cellulose of particle size 50 μm. Cellulose degradation increased with pH in the test range, but at pH $\leqslant$ 5.5, no cellulose degradation occurred. This inhibition of ruminal microbes at low pH was not completely remedied even when the pH of the medium was adjusted to neutral, so that the fermentation media in an anaerobic cellulosic-waste-degrading system inoculated with ruminal microbes should be kept above pH 6.0. In all cases, VFAs were the major water-soluble product from cellulose degradation, with acetate and propionate accounting for $>$90% of the total VFAs.

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