Nu	= Nusselt number ($=hD/\lambda$)	[—]	μ	= viscosity at bulk temperature	[kg/m·sec]
n	= rotational speed of impeller	[1/sec]	μ_w	= viscosity at wall temperature	[kg/m·sec]
Pr	= Prandtl number	[—]	λ	= thermal conductivity	[J/m·sec·°C]
p	= pitch of impeller	[m]	ρ	= density	[kg/m ³]
$Q, Q_1, Q_2, Q_3, Q_4, Q_5$	= volumetric flow rates	[m ³ /sec]	$\dot{q}$	= rate of heat transfer at the wall	[J/sec]
Q_z	= overall flow rate of axial circulation flow ($=Q_1+Q_2$)	[m ³ /sec]			
q	= average heat flux in Eq. (1)	[J/m ² ·sec]			
Re	= Reynolds number ($=d^2n\rho/\mu$)	[—]			
t	= local temperature	[°C]			
t_∞	= bulk temperature	[°C]			
t_w	= wall temperature	[°C]			
t_i, t_o	= inlet and outlet temperatures	[°C]			
$t_{(1)}$ to $t_{(20)}$	= local temperatures measured at locations 1 to 20 shown in Fig. 10	[°C]			
U	= velocity at $y=c$ in Fig. 8 (b)	[m/sec]			
u	= local velocity	[m/sec]			
y	= distance from tank wall	[m]			
z	= distance from tank bottom	[m]			
α	= thermal diffusivity	[m ² /sec]			
$\beta_1, \beta_3, \beta_4$	= adjustable constants in Eqs. (4), (5), (7) and (8)	[—]			
β_2	= adjustable constant in Eq. (6)	[1/m]			
γ	= adjustable constant in Eq. (2)	[—]			
δ_t	= thickness of thermal boundary layer	[m]			

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PRETREATMENT OF CELLULOSIC MATERIALS FOR DIGESTION BY *RUMINOCOCCUS ALBUS*

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The effects of chemical pretreatment on cellulose digestion by a cellulolytic anaerobe, *Ruminococcus albus*, were examined. Pretreatment with H_3PO_4 was the most effective for digestion of crystalline cellulose by *R. albus* of the four pretreatment methods investigated. The best condition for pretreatment of Avicel with H_3PO_4 was as follows; H_3PO_4 concentration: 85–100 (v/v)%; treatment time: over 5 min; treatment temperature: 0°C at 85 (v/v)% H_3PO_4 or 0–60°C at 95 (v/v)% H_3PO_4 ; cellulose concentration: 1–3 (w/w)%. Treatment efficiencies did not decrease after five repeated uses of H_3PO_4 . For effective digestion of newspaper or rice straw, delignification was essential in addition to swelling with H_3PO_4 . For newspaper and rice straw which were cryomilled, delignified and swollen with H_3PO_4 , specific cellulose digestion rates by *R. albus* were 0.081 and 0.099 g cellulose/g dry cells/hr, respectively.

Introduction

Cellulose is produced in most higher plants by the utilization of solar energy and comprises from 20 to 50% of their dry matter content. In the near future,

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cellulose may become an important carbon and energy resource for microbial growth if it can be hydrolyzed to glucose. So far, cellulases from fungi have been intensively applied to the hydrolysis of cellulosic wastes such as rice straw, newspaper and wood^{1,2,5,17,18}. However, such wastes are resistant to enzymatic attack because of the crystallinity of

cellulose and the presence of lignin. Many pretreatment methods for effective hydrolysis of cellulosic wastes have been developed, such as fine grinding or gamma ray irradiation and chemical treatment with acid, alkali or metal chelate cellulose solvent^{3,6-9,11}.

Apart from cellulases from fungi, anaerobic digestion of cellulose with cellulolytic rumen anaerobes have been studied by us¹²⁻¹⁵. In these experiments, ball-milled pure cellulose was excellently digested, but the use of newspaper or rice straw as a cellulose source for the anaerobes resulted in a low rate of digestion of cellulose, apparently attributable to the existence of impurities such as lignin.

In the present paper, the effect of chemical pretreatment on digestion of crystalline cellulose by *Ruminococcus albus* was investigated as well as reusability of the chemical reagents. Pretreatment methods to remove impurities such as lignin from cellulosic wastes were also studied.

1. Experimental

1.1 Cellulosic materials

Avicel (microcrystalline cellulose) was purchased from Asahi Kasei Co. Ltd. Newspaper and rice straw cut into 5×5 mm and 5 mm (length) pieces, respectively, were used as representative cellulosic wastes. Blank newsprint was mainly used except for the experiment to investigate the effect of printing ink on cellulose digestibility.

1.2 Methods of pretreatment for Avicel

Zinc chloride, sulfuric acid, phosphoric acid or cadoxen (a supernatant of 5 (w/w)% cadmium oxide in 30 (w/w)% ethylenediamine) was used as swelling or dissolving reagent¹¹. A given amount of Avicel was mixed with a swelling or dissolving reagent under the conditions described below. The slurry was mixed with 20 volumes of tap water. The precipitate was filtered under reduced pressure, thoroughly washed with tap water and weighed. A part of the cake was dried at 70°C for a day to determine the water content. The residual cake was used without drying as cellulose source of the medium.

In the experiment for recovery and reuse of phosphoric acid, the slurry was mixed with 20 volumes of acetone in place of tap water. After filtration, acetone and phosphoric acid were separated by simple batch distillation, at 65–75°C and then under reduced pressure. The undistilled fraction (mainly phosphoric acid) was reused to swell Avicel. The distillate (mainly acetone) was also reused. In this way, phosphoric acid and acetone were recovered and reused.

1.3 Methods of pretreatment for cellulosic wastes

Cryomilling was carried out at –60°C for newspaper and rice straw by courtesy of Hosokawa Iron

Works Ltd. Average powder sizes of newspaper and rice straw were about 300 μm (length: fibrous) and about 45 μm (diameter: particulate), respectively.

The procedures for removing lignin and hemicellulose were almost the same as reported by Timell¹⁶. For the delignification, cellulosic material (12.5 g) was suspended in 750 ml of distilled water at 70–80°C. Acetic acid (1 ml) and sodium chlorite (5 g) were added four times at intervals of 1 hr. The mixture was intermittently shaken without cooling. The cellulosic residue was collected by filtration with suction, washed thoroughly with tap water, and dried at 70°C overnight.

For the removal of hemicellulose, the sample (8.4 g) was shaken with 100 ml of 24 (w/w)% KOH at room temperature for 8 hr, and the mixture was allowed to stand overnight. The cellulosic residue was collected by filtration with suction and suspended in 5 l of tap water. After neutralization with acetic acid, the cellulosic residue was filtered with suction, thoroughly washed with tap water, and dried at 70°C overnight.

1.4 Microorganism and culture condition

R. albus, a representative cellulolytic anaerobe in rumen, was used. The medium used was the same as described in the previous papers^{14,15}. The cultivation was carried out in a test tube or a jar-fermentor (Iwashiyama Co., Type MB) under anaerobiosis at 37°C. The concentration of cellulosic material in the medium was prepared so as to be 10 g/l (test tube cultivation) or 15 g/l (jar-fermentor cultivation), which was estimated from the water content of wet cellulosic material. For test tube cultivation, 5 to 6 drops of the culture broth grown on 0.2% cellobiose medium for 24 hr were inoculated with a Pasteur pipette. For jar-fermentor cultivation about 50 ml of the seed culture, which was grown for 24 hr in the medium containing 10 g/l of ball-milled Avicel, was inoculated with a hypodermic syringe. The methods for preparing the medium and the culture conditions were described in detail previously^{12,13,15}.

1.5 Analysis

Amounts of ethanol, acetic acid, cellulose and dry cells were measured by the methods described previously^{12,13}. *R. albus* formed ethanol and acetic acid, mainly as a result of digesting cellulose^{12,13,15}. Because formation ratios of ethanol and acetic acid for cellulose did not vary so much, the amount of ethanol formed during 3-day cultivation was used as an indicator of digestibility of cellulosic material in some experiments.

X-ray diffraction analysis to measure crystallinity of cellulose was mainly carried out by the procedures reported by Tanaka *et al.*¹¹. Samples for Rigaku Denki X-ray diffractometer were compressed at 6 MPa for 3 min by an IR sample preparation press. From

■: Ethanol formed by using Avicel ball-milled for 3 days
 ▨: Ethanol formed by using Avicel without pretreatment

Swelling conditions;

A: H_2SO_4 , 4 (w/w) % Avicel, at 0°C , for 5 min

B: H_3PO_4 , 2 (w/w) % Avicel, at 0°C , for 30 min

Symbols: ethanol formed (□); recovery of cellulose (x)

Fig. 1 Pretreatment effects of H_2SO_4 and H_3PO_4 concentration on ethanol formation by cultivation of *R. albus*

Swelling conditions: 85 (v/v) % H_3PO_4 , 2 (w/w) % Avicel, at 0°C .

Symbols: identical with those in Fig. 1.

Fig. 2 Effects of treatment time of crystalline cellulose with phosphoric acid on ethanol formation by cultivation of *R. albus*

X-ray diffractograms, crystallinity indices were calculated¹⁰⁾.

Lignin content of cellulosic wastes was measured by the method with sulfuric acid¹⁹⁾.

2. Results and Discussion

2.1 Pretreatments for Avicel

Reagents and conditions for swelling cellulosic materials were determined by use of microcrystalline cellulose, Avicel.

1) Selection of swelling reagent Four reagents which have been reported¹¹⁾ to be effective for swelling crystalline cellulose were examined preliminarily: (a) 70 (w/w) % ZnCl_2 at 0°C for 10 min; (b) 60 (v/v) % H_2SO_4 at 0°C for 30 min; (c) 80 (v/v) % H_3PO_4 at 0°C for 10 min; and (d) cadoxen at 0°C for 60 min. The treatments with H_2SO_4 and H_3PO_4 had quite

■: Ethanol formed by using Avicel ball-milled for 3 days
 ▨: Ethanol formed by using Avicel without pretreatment

Swelling conditions: 85, 90 or 95 (v/v) % H_3PO_4 , 2 (w/w) % Avicel, for 30 min
 Symbols: 85 (v/v) % H_3PO_4 (■); 90 (v/v) % H_3PO_4 (▨); 95 (v/v) % H_3PO_4 (□).

Fig. 3 Effects of treatment temperature of crystalline cellulose with phosphoric acid on ethanol formation by cultivation of *R. albus*

Swelling conditions: 85 (v/v) % H_3PO_4 , at 0°C , for 30 min.

Symbols: identical with those in Fig. 1.

Fig. 4 Effects of Avicel concentration in treatment with phosphoric acid on ethanol formation by cultivation of *R. albus*

good effect on cellulose digestion by *R. albus*. On the other hand, Avicel treated with ZnCl_2 or cadoxen was hardly digested by *R. albus*. Takana *et al.*¹¹⁾ and Ladisch *et al.*⁴⁾ reported swelling effects of zinc chloride and cadoxen on enzymatic saccharification of cellulosic material, respectively. Therefore, the poor digestibility of Avicel treated with zinc chloride or cadoxen may be due to the inhibition of the residual zinc or cadmium ion for the growth of *R. albus*.

As shown in Fig. 1(A), treatment at higher H_2SO_4 concentration led to low recovery of cellulose owing to dissolution of cellulose, which was also indicated by Tanaka *et al.*¹¹⁾. On the contrary, the loss of cellulose with phosphoric acid treatment was negligible and swelling treatment was effectively achieved

Table 1 Crystallinity index of Avicel swollen with phosphoric acid at various temperatures

Treatment temperature [°C]	Crystallinity index [%]		
	95 (v/v)% H_3PO_4	90 (v/v)% H_3PO_4	85 (v/v)% H_3PO_4
0	36	31	34
20	31	47	75
40	35	73	81
100	78	79	81

Crystallinity index of original Avicel was 81%.

Table 2 Repeated use of phosphoric acid for swelling Avicel

Number of repeated uses	Recovery of Avicel [(w/w)%]	Recovery of acetone [(v/v)%]	Ethanol formed [g/l]
1	101	—	1.59
2	92	74	1.79
3	103	81	1.69
4	110	79	1.51
5	102	80	1.81
6	100	80	1.82

Swelling conditions: 2 (w/w)% Avicel, at 0°C, for 30 min. Amounts of ethanol formed by using Avicel ball-milled and Avicel without pretreatment were 1.74 g/l and 0.26 g/l, respectively.

at 85–100 (v/v)% H_3PO_4 concentration, as shown in Fig. 1(B). Moreover, sulfuric acid is more corrosive than phosphoric acid. In subsequent experiments, the conditions for treatment with phosphoric acid were investigated in detail.

2) Effect of treatment time on swelling of cellulose

Figure 2 shows that the crystalline cellulose was thoroughly swollen even by treatment for 5 min. On treatment for a short time (less than 10 min), however, a part of the Avicel powder sometimes formed pellets in H_3PO_4 solution and took longer to become a homogeneous suspension. Treatment for a longer time did not lead to decreased recovery of cellulose, as shown in Fig. 2. For larger particles than Avicel or for cellulosic materials containing lignin, penetration of phosphoric acid into particles may take a longer time. Thus, treatment for 30 min was adopted and the other conditions were examined in subsequent experiments.

3) Effect of treatment temperature and Avicel concentration on swelling of cellulose

As shown in **Fig. 3**, the temperature at which Avicel was treated had a remarkable effect on subsequent digestion by *R. albus*. When 85 (v/v)% H_3PO_4 was used, treatment efficiency was high only at 0°C and was very low at 20–100°C. On the other hand, Avicel treated with 95 (v/v)% H_3PO_4 at 0–60°C was fully digested by *R. albus*. These results were comparable with those for crystallinity index shown in **Table 1**: The temperature at which the crystallinity index changed from low to high value (Table 1) almost coincided with the tem-

Two (w/w)% Avicel was treated with 85 (v/v)% H_3PO_4 at 0°C for 30 min. Symbols: cellulose (●); dry cells (▲); ethanol (□); acetic acid (○).

Fig. 5 Digestion of pretreated crystalline cellulose by jar-fermentor cultivation

perature at which digestibility decreased from high to low value, and became high with increasing phosphoric acid concentration (Fig. 3). From these results, it was found that a concentration of phosphoric acid higher than 95 (v/v)% was desirable for treatment of crystalline cellulose at room temperature. In these treatments, recovery of cellulose was 95–100 (w/w)%.

Effect of Avicel concentration on swelling of cellulose is shown in **Fig. 4**. It was found that treatment at more than 4 (w/w)% Avicel concentration resulted in low treatment efficiency.

4) Reusability of phosphoric acid for the treatment

Recovery and reuse of phosphoric acid were attempted for the treatment of Avicel. In this case, initial concentration of phosphoric acid was 100 (v/v)% and reuse of phosphoric acid was investigated five times. **Table 2** shows the results for repeated use of phosphoric acid. Avicel was recovered completely in each run. Average recovery of acetone was about 80% and acetone recovered could be reused. If continuous multi-stage distillation is applied, recovery of acetone is improved. Amount of ethanol formed by the cultivation of *R. albus* was almost the same in each run. Quality of phosphoric acid recovered seems not to be deteriorated in repeated use. In the treatment of cellulosic materials such as newspaper, phosphoric acid and acetone likewise could be used repeatedly.

5) Digestion of pretreated Avicel by jar-fermentor cultivation

Using swollen Avicel as a carbon source, jar-fermentor cultivation was carried out as shown in **Fig. 5**. During 72 hr cultivation, 12.6 g cellulose/l was digested, and 2.3 g ethanol/l and 2.7 g acetic

Table 3 Effects of pretreatment on amounts of ethanol formed [g/l] in cultivation of *R. albus*

Pretreatment	Newspaper	Rice straw
1 no treatment	0.08 (0.14)	0.09
2 P	0.31	0.07
3 C	0.06	0.36
4 C+P	0.47	0.53
5 C+L	1.00	0.61
6 C+L+P	1.64	1.45
7 C+L+H	1.37	1.40
8 C+L+H+P	1.29	0.80
9 L	0.81 (0.65)	0.63
10 L+P	1.40 (1.67)	0.75

C: Cryomilling; L: Delignification, H: Removal of hemicellulose; P: Swelling of 2 (w/w)% cellulosic material with 85 (v/v)% H_3PO_4 at 0°C for 30 min.

Amounts of ethanol formed by using Avicel ball-milled and Avicel without pretreatment were 1.65 g/l and 0.15 g/l, respectively. Figures in parentheses show the results for printed newspaper.

acid/l were produced. Dry cell concentration reached about 2.0 g/l at 48 hr. Average specific cellulose digestion rate¹²⁾ was 0.101 g cellulose/g dry cells/hr: this value was almost the same as that in the case of using Avicel ball-milled, namely 0.090 g cellulose/g dry cells/hr. The cellulose digestion ratio was 76 (w/w)% at 72 hr cultivation time. Under similar cultivation conditions, Avicel ball-milled gave 79 (w/w)% of the cellulose digestion ratio.

2.2 Pretreatments for cellulosic wastes

As cellulosic wastes such as newspaper and rice straw contain impurities such as lignin, the effects of various pretreatments on digestion of cellulosic wastes by *R. albus* were examined in combination with the phosphoric acid treatment described above.

1) Effects of various pretreatments for newspaper and rice straw on digestion by *R. albus* As printing ink used for newspapers was found to have no effect on cellulose digestibility by *R. albus*, as shown in Table 3, blank newsprint was used in the following experiments. Combinations of cryomilling, delignification, removal of hemicellulose and swelling with phosphoric acid were employed for pretreatments for newspaper and rice straw. Results of the pretreatments for newspaper and rice straw are summarized in **Table 3**.

Cryomilling had a small effect on digestion of newspaper by *R. albus*, but had a great effect on digestion of rice straw (compare 1, 2, 9 and 10 with 3, 4, 5 and 6 in Table 3, respectively). Surface area of the cellulosic wastes was considerably increased by the cryomilling and became more easily attacked by cellulase from *R. albus* or by chemicals such as phosphoric acid. Increase in digestibility of rice straw by cryomilling may be explained by the increase of surface area. Sasaki *et al.*⁹⁾ also reported that the

crystallinity index of rice hulls was not changed by cryomilling but that cryomilled rice hulls were saccharified up to about 35% by cellulase from *Trichoderma viride*. On the other hand, cellulose from softwood combines more firmly with lignin compared with cellulose from rice straw or corn¹⁹⁾. Thus, the reason why cryomilling hardly promoted digestion of newspaper by *R. albus* may be explained by the structural difference of lignocellulose.

Cellulose digestibility was enhanced by delignification (compare 1 and 2 with 9 and 5 in Table 3, respectively). Phosphoric acid treatment after the delignification, in particular, resulted in newspaper and rice straw being digested satisfactorily (6 in Table 3).

Removal of hemicellulose, the main impurity other than lignin, appeared to have a negative effect (compare 6 with 8 in Table 3). Tanaka *et al.*¹¹⁾ indicated that crystallinity was increased by delignification with alkali (NaOH). In the present experiment, the crystallinity indices of newspaper and rice straw, which were 48% and 44% respectively, for 6 in Table 3, increased to 62% and 63% respectively by treatment with alkali (KOH) for the removal of hemicellulose.

From the above experiments, delignification and swelling with phosphoric acid were indispensable and cryomilling was effective for the effective digestion of newspaper. In the case of rice straw, cryomilling, delignification and swelling with phosphoric acid were essential pretreatments. Lignin contents of newspaper and rice straw were reduced to 4 (w/w)% and 9 (w/w)% respectively from 26 (w/w)% and 23 (w/w)% by the delignification.

2) Digestion of pretreated newspaper or rice straw by jar-fermentor cultivation As the processes of pretreatment, cryomilling, delignification and swelling with phosphoric acid (2 (w/w)% cellulosic material, 85 (v/v)% H_3PO_4 at 0°C for 30 min) were adopted. Jar-fermentor cultivation was carried out using the pretreated newspaper or rice straw as cellulose source.

In the case of pretreated newspaper, 9.3 g cellulose/l was digested, and 2.0 g ethanol/l and 2.3 g acetic acid/l were produced for 72 hr. Dry cell concentration reached about 2.3 g/l at 48 hr. Average specific cellulose digestion rate and cellulose digestion ratio were 0.081 g cellulose/g dry cells/hr and 70 (w/w)%, respectively.

On the other hand, 12.7 g/l of rice straw as cellulose was digested, and 2.0 g ethanol/l and 2.3 g acetic acid/l were produced for 72 hr as shown in **Fig. 6**. Dry cell concentration reached about 2.0 g/l at 48 hr. In this case, average specific cellulose digestion rate and cellulose digestion ratio were 0.099 g cellulose/g dry cells/hr and 75 (w/w)%, respectively. These values were comparable with those for Avicel swollen

by phosphoric acid. Cellulose digestion ratios of newspaper and rice straw which were ball-milled for 3 days were 55 (w/w)% (94 hr cultivation time) and 53 (w/w)% (96.5 hr cultivation time), respectively. The effectiveness of the combination of three pretreatments is obvious.

As shown in Figs. 5 and 6, cellulose digestion rate became very low after 72 hr. Crystallinity indices of Avicel swollen by phosphoric acid and pretreated rice straw were 34% and 44%, respectively. These may indicate that small amounts of crystalline cellulose still remain in these samples. If such cellulose were swollen, Avicel and rice straw would be digested completely.

In the present experiments, newspaper and rice straw were cryomilled and the surface area of the cellulosic wastes was considerably increased. Cryomilling is not essential for increasing surface area and may be replaced with the usual pulverization.

In conclusion, chemical pretreatments such as swelling with phosphoric acid and delignification of cellulosic materials stimulated their subsequent digestion by *R. albus*.

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Rice straw was cryomilled, delignified and then swollen with H_3PO_4 as described in the footnote in Table 3.

Symbols: identical with those in Fig. 5.

Fig. 6 Digestion of pretreated rice straw by jar-fermentor cultivation

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