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ORIGINAL PAPER

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Enzymatic hydrolysis and physical characterization of commercial celluloses and cellulose-based ion-exchange powdered mixed resins

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Abstract Commercial celluloses (BH20, Epicote, FC+) and their cellulose-containing powdered mixed resins (PMR) were evaluated using enzymatic and physical methods. Samples were hydrolyzed with purified *Trichoderma viride* cellulase extract and measured for released reducing sugar using the dinitrosalicylic acid method. Physical characterization was performed with gross specific surface areas (GSSA) and relative crystalline indices (RCI). In addition, FC+ was exposed to physical and chemical processing commonly encountered in spent PMR processing to determine potential effects on reducing sugar release in high intensity containers. Reducing sugar released from the celluloses by *T. viride* cellulase ranged from 135.37 to 244.48 mg day^{-1}; the celluloses were highly crystalline, ranging from 82.47 to 84.57%; and the GSSA medians for the celluloses ranged from 1,298.60 cm^2 g^{-1} to 2,493.20 cm^2 g^{-1}. Most processing treatments on the FC+ reduced the amount of reducing sugar released and increased RCI. Cellulose hydrolysis rates did not show a strong correlation with the physical characterization. These results suggest that (1) celluloses and PMR can serve as abundant sources of bioavailable carbon in water treatment systems, and (2) the use of correlative physical characteristics to evaluate a cellulose-based commercial product may not accurately predict microbial activity; a complementary microbial test such as cellulose hydrolysis with cellulase may prove useful.

Introduction

Many nuclear power plant liquid treatment systems utilize an ion-exchange powdered mixed resin (PMR) system to remove anions, cations, and particulate/flocculent crud that interfere with the fuel source, and cause localized corrosion and stress-corrosion cracking (Electrical Power Research Institute 1994). Cellulose is a cost-effective support fiber added to PMR to ensure a crack-free precoat and to act as a spacer that increases the effective surface area of the ion-exchange resins.

Spent low-level waste PMR (radioactive contamination <5 rem) is removed, dewatered and combined in high-intensity containers (HICs) for transport and burial. From 1990 to 1995, some HICs had heavy biogas production that was linked to a consortium of anaerobic and facultative microorganisms utilizing the cellulose in PMR as a carbon and energy source (Clesceri and Clarkin 1998). Although cellulose is biologically unavailable to most microorganisms, cellulase-producing microorganisms can hydrolyze it to soluble reducing sugars, which fermentative microorganisms can metabolize, producing biogas as an end-product or by-product. The hydrolysis rates have been shown to correlate with cellulose type, crystallinity (Weimer and Weston 1985), and gross specific surface area (GSSA) (Weimer et al. 1990), which can be affected by physical and chemical processing (Weimer et al. 1995).

The objectives of this study were: (1) to characterize three parent celluloses and their PMR by GSSA, relative crystalline indices (RCI), and cellulase-mediated cellulose hydrolysis, and (2) to evaluate the effects of physical and chemical HIC-processing on reducing sugar release.

Materials and methods

Cellulose hydrolysis with *Trichoderma viride* cellulase

T. viride crude cellulase (10 g; Miles Laboratories, Elkhart, Ind.) was centrifuged with 150 ml cold sodium phosphate buffer (50 mM, pH 7.0) at 16,000 *g* for 20 min at 4°C. Fifty grams of $(NH_4)_2SO_4$ was added to the supernatant and the pellet was discarded. The solution was recentrifuged, the pellet saved, and the process repeated. The two pellets were combined and resuspended in 100 ml 100 mM sodium acetate buffer (pH 4.8). The cellulase stock solution was determined to have a CMC (endoglucanase) activity of 0.289 international units (IU) and a filter paper (overall cellulase) activity of 0.0911 IU (Saunders 1984).

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Cellulose hydrolysis rates were determined for three parent celluloses and their PMR (Table 1). Cellulose (0.5 g), 49 ml citrate buffer (0.05 M, pH 4.9), and 1 ml cellulase stock solution were placed in 125 ml Erlenmeyer flasks at 45°C and 150 rpm. Samples (2 ml) were taken at 0 (contact hydrolysis), 10, 20, 30, 40, 50, 60, 90, 120, 240, and 1,440 min (24-h accumulated reducing sugar), placed in vials containing 0.2 ml cold 1 N phosphoric acid and refrigerated until measurement of reducing sugar. The 24-h incubation limit was chosen to minimize contamination, evaporation (Saunders 1984), and loss of CMCase activity (Ohmine et al. 1983).

Reducing sugars were measured by a modified version of the Miller procedure (Miller 1959). A dinitrosalicylic acid (DNS) solution (1% DNS, 0.2% phenol, 1% NaOH) was gassed with oxygen-stripped nitrogen, 0.05% Na_2SO_3 was added, and the bottles were crimped and stored at room temperature in the dark. Samples (1 ml supernatant from incubations) and 3 ml DNS solution were boiled for 5 min. Rochelle salt (1 ml; 40%) was added, and the mixture was allowed to cool for 15 min. Absorbance was measured at 575 nm on a Bausch and Lomb Spectronic 20 and converted to reducing sugar on a standard curve of dextrose ranging from 0.075 to 0.500 g l^{-1}. Reducing sugar concentrations were corrected for changing volume and removed reducing sugar in the incubation flasks. A standard and a quality control sample were run with each analysis.

Physical characterization of parent celluloses

RCI was measured by acid hydrolysis for Epicote, BH20, and FC+. Cellulose (50 mg) and 3 ml 6 N HCl were boiled for 7 h with duplicate samples taken every hour, filtered through tared 3 μm polycarbonate filters, washed with three 5-ml volumes of deionized water, and dried at 105°C. The percentage of remaining cellulose was adjusted for dry weight and plotted versus time on semilog paper. The line was back-extrapolated to determine the RCI. GSSA was calculated by measuring lengths and widths of 100 fully-hydrated fibers using an optical micrometer (Klarman and Ruling, Litchfield, N.H.) with either a 20× or 45× objective lens and applying a cellulose fiber model (Weimer et al. 1990).

Effects of physical and chemical HIC-processing treatments

FC+ cellulose was placed in 60-ml serum bottles with deionized water and (1) autoclaved at 121°C for 30 min, (2) frozen overnight, (3) dried at 105°C and rehydrated, or (4) left at room temperature overnight to hydrate (untreated control). The samples were then analyzed for reducing sugar released by the treatment, GSSA, RCI, and cellulose hydrolysis by cellulase.

For germicide studies, 0.5 g FC+ was placed in a 60-ml serum bottle with 20 ml 10% glutaraldehyde or 20 ml 30% H_2O_2. The solution was left overnight and tested as above. A second test with 10% glutaraldehyde was subjected to an additional washing.

Results

Physical characterization of parent celluloses

Epicote, BH20, and FC+ had RCI >80% and were not significantly different (Table 1). A control boiled with deionized water instead of HCl showed little loss of cellulose mass after 7 h, with recoveries exceeding 90%.

The distribution of individual fiber GSSA for all three celluloses was similar, with many small GSSA, and a few large GSSA that highly influenced means and resulted in large standard deviations. Replication did not result in reduced variation. The assumption of normality for all three GSSA data sets was rejected at a significance level of 0.05 with the Kolmogorov-Smirnov normality test. Log transformations of GSSA resulted in distributions that could not be rejected as normal for BH20 and FC+, but was rejected for Epicote. One-way analysis of variance of log-transformed GSSA of BH20 and FC+ showed that BH20 was significantly greater (P =0.06). All three sets

Table 1 Information and physical characterization of commercial parent celluloses and their powdered mixed resin (PMR) derivatives often used in water treatment systems and used in this study[a]. The relative crystalline indices (RCI) and gross specific surface areas (GSSA) refer only to the parent celluloses

Producer (address)	Parent cellulose	PMR	RCI (% crystallinity)	Median GSSA[b] (cm^2 g^{-1}); GSSA rank[c]
Epicor (Linden, N.J.)	Epicote	Epifloc-21H, Epifloc-21NH	84.57	1,298.60; 3
Graver Chemical (Newark, N.J.)	BH20	P201H, P202H, X203H	83.76	2,493.20; 1
Purolite International (Bala Cynwyd, Pa.)	FC+	CG12H	82.47	2,072.00; 2

[a] Use of brand names does not imply endorsement of these products by the authors
[b] n=100
[c] Significant as determined by Kruskal-Wallis analysis of variance by ranks and Mann-Whitney tests

Table 2 Reducing sugar released by enzymatic hydrolysis using *Trichoderma viride* cellulase for parent celluloses and PMR

Parent cellulose	PMR	24-h Reducing sugar (mg)[a]	Contact hydrolysis (mg)[a]
Epicote		135.37±7.39	8.03±2.72
	21-H	78.26±1.17	4.54±0.19
	21-NH	69.53±1.38	4.73±0.08
BH20		161.27±7.51	6.66±2.72
	X203H	121.51±6.72	4.68±0.16
	P201H	93.93±0.00	5.14±0.04
	P202H	100.05±5.29	5.28±1.63
FC+		244.48±10.32	13.60±4.53
	CG12H	109.64±3.47	6.90±0.35

[a] Average of two duplicate samples ±SD

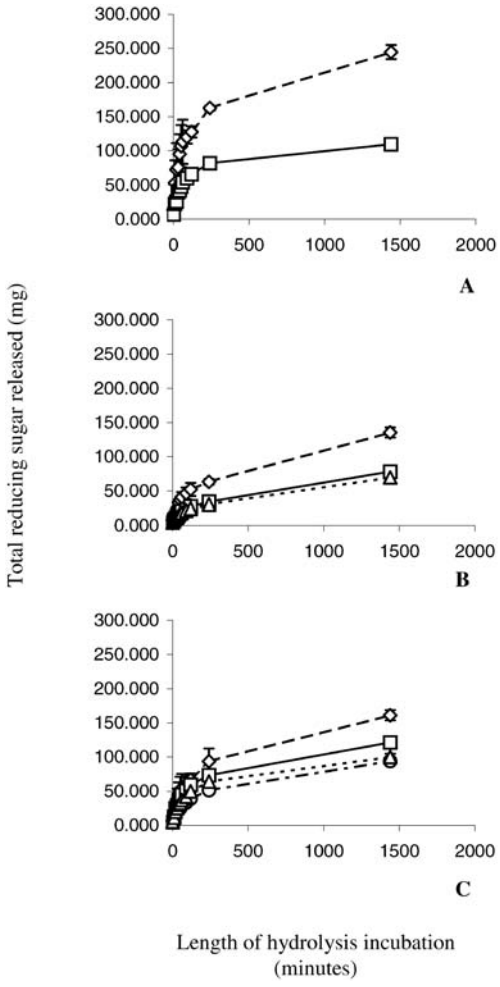

Fig. 1A–C Cellulose hydrolyses by *Trichoderma viride* cellulase for parent cellulose fibers and their powdered mixed resin (PMR) derivatives. **A** *Diamonds* FC+, *squares* CG12H. **B** *Diamonds* Epicote, *squares* 21H, *triangles* 21NH. **C** *Diamonds* BH20, *squares* X203H, *circles* P201H, *triangles* P202H. Each point is the average of two duplicate analyses ±SD

were then compared with the nonparametric Krukal-Wallis analysis of variance by ranks test and the GSSA were shown to be significantly different with BH20 > FC+ >Epicote (Table 1).

Hydrolysis of parent celluloses and PMR with *T. viride* cellulase extract

All parent celluloses and PMR released significant reducing sugar and had similar hydrolysis patterns (Table 2). Hydrolysis was immediate and proceeded quickly for the first 2 h followed by slower rates up to 24 h (Fig. 1A–C). FC+ had the highest contact hydrolysis reducing sugar and the largest total 24-h released reducing sugar (Table 2; Fig. 1A). Epicote (Fig. 1B) and BH20 (Fig. 1C) were significantly lower in both 24-h released reducing sugar and contact hydrolysis. The FC+ derivative (CG12H) and the BH20 derivatives (X203H, P201H, and P202H) released similar amounts of reducing sugar after contact and 24 h, but the Epicote derivatives (21-H and 21-NH) were modestly lower (Table 2; Fig. 1A–C).

Physically and chemically processed FC+

Untreated and treated FC+ cellulose released reducing sugar when hydrolyzed with *T. viride* cellulase extract. Autoclaved, frozen and rehydrated FC+ cellulose released less reducing sugar after 24 h and had higher RCI than the untreated FC+ (Table 3). Slight, but not significant, decreases in GSSA were seen in the temperature-treated FC+ cellulose.

FC+ cellulose exposed to chemical treatments of 10% glutaraldehyde or 30% H_2O_2 showed slight reductions in released reducing sugar when compared to untreated FC+ cellulose (Table 3). The RCI of glutaraldehyde-treated FC+ could not be determined due to incomplete removal of the glutaraldehyde.

Discussion

Three celluloses and their PMR used in nuclear power plant water treatment systems were characterized by RCI, GSSA, and enzymatic hydrolysis. Each cellulose and PMR had immediate and large releases of reducing sugar. The physical characterizations did not correlate with the enzymatic hydrolyses.

Physical characteristics did not serve as predictors for biological hydrolysis of the cellulose materials. Wide RCI ranges have been inversely correlated with cellulose

Table 3 RCI and reducing sugar released by cellulose hydrolysis with *T. viride* cellulase for FC+ and chemically and physically processed FC+

Treatment	RCI (% crystallinity)	24-h Reducing sugar (mg)	Contact hydrolysis	Median GSSA[a] ($cm^2\ g^{-1}$)
Untreated	80.72	200.72	6.93	2,153.4
Autoclaved	92.56	164.03	7.04	2,056.0
Frozen	92.77	165.67	6.49	2,033.0
Rehydrated	ND[b]	157.36	6.00	ND
30% Peroxide	89.13	187.43	9.96	2,075.0
10% Glutaraldehyde	ND	178.43	9.30	2,052.3

[a] n=100
[b] Not determined

hydrolysis by cellulase (Weimer and Weston 1985) but, in this study, the narrow range of RCI precluded RCI-measured crystallinity as an explanation for the large differences in cellulase-mediated hydrolysis rates. The acid hydrolysis was not carried out with PMR because of uncertainty about effects on resins, possible health hazards, and uncertainty about the total cellulose contained. GSSA has been shown to be directly correlated to bacterial degradation of cellulose-containing materials (Weimer et al. 1990) but in this study the cellulose with the highest GSSA (BH20) released considerably less reducing sugar than FC+.

Hydrolysis of FC+ released the most reducing sugar of the parent celluloses, but its PMR were comparable to those of BH20 in reducing sugar released in a 24-h period. All PMR had considerably lower sugar release than their parent celluloses, but the tests measured the amount of sugar released per 0.5 g and did not account for ratios of cellulose to ion-exchange resin. An additional complication was that after the early incubation stages it was unclear how much increased measured reducing sugar was from further cellulose degradation and how much resulted from further degradation of soluble glucose polymers. A continuous flow setup that removes soluble sugars could be used to address this question.

The pattern of reducing sugar release shown by the parent celluloses and PMR can be explained as fast hydrolysis of readily-hydrolysable amorphous regions followed by slower hydrolysis of less accessible, less readily-hydrolysable crystalline regions. Limited pre-exposure of parent celluloses or PMR to cellulase could reduce accessible cleavage sites and increase crystallinity, thereby attenuating microbial hydrolysis of the cellulose and soluble carbon release in PMR systems and HICs.

FC+ was used to evaluate HIC-processing treatments because it had the highest hydrolysis rates and released the most reducing sugar of the three parent celluloses. Freezing or autoclaving resulted in marked RCI increases with concomitant decreases (17–18%) in released reducing sugar when compared to the untreated FC+ (Table 3). This is in agreement with previous studies that have shown that temperature treatments can increase RCI (Weimer et al. 1995), and that decreases in cellulase-mediated cellulose hydrolysis rates often correlate with changes in cellulose type or increased crystallinity (Weimer et al. 1990). These findings suggest that, in addition to the direct effects that extreme temperatures have in controlling microbial activity in HICs, there may also be a reduction in the bioavailability of the cellulose as a carbon source because of increased crystallinity and reduced rates of microbial cellulose hydrolysis.

Glutaraldehyde and stabilized H_2O_2 are antimicrobials used in HICs to inhibit microbial gas production during transport and storage. They were added at high concentrations to determine their effects on cellulose hydrolysis by extracellular cellulases. The cellulose hydrolysis of glutaraldehyde-treated FC+ was inhibited early in the incubations, but total reducing sugar released was almost the same as that of untreated FC+ after 24 h. Glutaraldehyde-treated FC+ with an additional washing did not show the initial attenuation of hydrolysis seen in the unwashed sample. The slow equilibration into the test medium by cellulose-adhered glutaraldehyde in the unwashed sample may explain this initial attenuation. This could be beneficial in bioremediation attempts as static high concentrations of a biocide could inhibit microbial activity and then be diluted or removed later to allow cellulose fermentation for mass reduction. Overall, the reduction in released reducing sugar after 24 h was less for the chemical treatments than it was for the physical treatments.

Cellulose hydrolysis using *T. viride* cellulase provided repeatable, comparable results, and avoided the complications of mixed microbial population studies. Physical characterization did not provide a good prediction of biological cellulose hydrolysis indicating that biological tests, e.g., hydrolyses using enzyme extracts or defined microbial methods, may be more useful when evaluating the biostability of cellulose-based industrial products; this is especially true when the products have similar physical characteristics (e.g., RCI) or are of the same allomorphic type. These results are of interest to nuclear power plant operators and others involved with water treatment because they show the fast and considerable release of solubilized bioavailable carbon from the breakdown of cellulose-containing PMR by ubiquitous fungal enzymes.

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