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## Solid residues from *Ruminococcus* cellulose fermentations as components of wood adhesive formulations

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**Abstract** Residues from the fermentation of cellulose by the anaerobic bacteria *Ruminococcus albus* (strain 7) or *Ruminococcus flavefaciens* (strains FD-1 or B34b) containing residual cellulose, bacterial cells and their associated adhesins, were examined for their ability to serve as components of adhesives for plywood fabrication. The residues contained differing amounts of protein (0.4–4.2% of dry weight), but the ratios of monosaccharides recovered following two-stage treatment of the residue with detergent (pH 7) and TFA were similar for all three strains (0.71 glucose:0.18 xylose:0.08 mannose:0.02 galactose), suggesting similarities in exopolysaccharide composition. Three-ply aspen panels prepared with fermentation residues (FR) displayed better shear strength and wood failure under dry conditions than following a vacuum/pressure/soak/dry treatment, but adhesive properties were inferior to those prepared with conventional phenol-formaldehyde (PF) adhesives. However, panels prepared by incorporating the *R. albus* 7 FR into PF formulation, at 73% by weight of the total adhesive, exhibited shear strength and wood failure similar to that

obtained with PF adhesive alone. Use of residues from fermentations by these bacteria as components of adhesives may add value to biomass fermentations aimed primarily at producing ethanol and other chemical products.

### Introduction

Over the last several decades renewable resources have contributed an increasing share of fuel and chemical production in developed countries. One of the largest of these contributors has been ethanol produced by fermentation and used as a gasoline additive. Commercial ethanol is produced almost exclusively by saccharification of starch (usually from corn) and subsequent fermentation of the sugars by *Saccharomyces* yeast. The development of fermentations based on cellulosic biomass, instead of on starch, has remained attractive because of the low cost and great abundance of cellulosic materials, either directly from biomass energy crops, or from agroforestry wastes (Lynd et al. 1999).

While research on bioconversion of cellulosic materials to ethanol has largely focused on chemical or enzymatic hydrolysis of biomass with subsequent fermentation of sugars by yeast, the process is not economically viable for a variety of reasons (Lynd et al. 1999). The chemical hydrolysis route suffers from a requirement for postprocessing (e.g., neutralization of the hydrolyzate, the costly handling of waste products, and the removal or treatment of fermentation inhibitors formed during hydrolysis). The enzymatic route involves high costs associated with producing fungal enzyme with low inherent specific activities. A potential alternative route for cellulose bioconversion involves processes in which enzyme production, enzymatic hydrolysis, and sugar fermentation occur in a single bioreactor (Lynd et al. 1999, 2002). There is little doubt that the economic viability of biomass conversion processes will ultimately depend on the marketability of co-products produced during the bioconversion process. This is implicit in the

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modern notion of a “biorefinery” that is envisioned to ultimately produce a suite of biologically derived commercial products (Lynd et al. 1999).

The ruminal cellulolytic bacterium *Ruminococcus albus* can ferment cellulose, some hemicelluloses (e.g., xylans and glucomannans) and pectin, to produce a mixture of ethanol, acetic acid, H<sub>2</sub> and CO<sub>2</sub> (Hungate 1966; Pavlostathis et al. 1988). A necessary prerequisite of the *R. albus* cellulose fermentation is adherence of the bacteria to cellulose, which is mediated by a variety of adhesins that include cellulose-binding domains of cellulolytic enzymes; components of polycellulosomal organelles; pilin-like proteins and exopolysaccharide-containing glycocalyx materials (Weimer 1996; Miron et al. 2001). The glycocalyx is relatively resistant to disruption by the physical and chemical forces normally encountered by the organism in culture or in the rumen environment. The tenacity of the association between the bacterium and its cellulosic substrate has led us to examine the fermentation residue (FR), containing adherent microbial cells and glycocalyx, as a novel bioadhesive. In these experiments, the adhesive properties of residues of several *Ruminococcus* cellulose fermentations have been compared to those of phenol-formaldehyde (PF, the most common adhesive used in the manufacture of plywood) and to mixtures of FR and PF. Our initial experiments have focused on cellulose-grown cells, because the simplicity of cellulose composition facilitates more accurate chemical analysis of the glycocalyx material adherent to the cellulose.

## Materials and methods

### Preparation of FR containing bioadhesive

*Ruminococcus albus* (strain 7) and *Ruminococcus flavefaciens* (strains B34b and FD-1) were revived from -80°C glycerol stocks, and were grown at 39°C under a CO<sub>2</sub> atmosphere. The medium—a modified Dehority medium (MDM)—contained the following (per liter): 4 g Sigmacell 50 microcrystalline cellulose (SC50), 0.9 g KH<sub>2</sub>PO<sub>4</sub>, 3.2 g Na<sub>2</sub>CO<sub>3</sub>, 0.90 g NaCl, 0.73 g NH<sub>4</sub>Cl, 0.085 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.066 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.028 g MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.02 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g ZnCl<sub>2</sub>, 0.002 g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.002 g resazurin, 0.5 g yeast extract, 1.0 g cysteine HCl, 10 ml Schaefer’s vitamin mixture (Schaefer et al. 1980, but amended with 0.125 mg tetrahydrofolic acid per liter of vitamin mix) and 0.067 ml each of isobutyric, 2-methylbutyric, *n*-valeric and isovaleric acids. For *R. albus* 7, the medium was also amended with 25 μM 3-phenylpropanoic acid (PPA; Morrison et al. 1990). Additional Na<sub>2</sub>CO<sub>3</sub> was added from a saturated solution to adjust the initial pH of the medium to 6.9.

Fermentations to produce the residues for adhesive testing were carried out in 45 l glass carboys containing 40 l of the above medium, except that the cellulose was either SC50 (3 g/l) or long fibrous cellulose CF1 (Sigma, 4 g/l) per liter (Table 1). The medium (without cellulose) was filter-sterilized into carboys that contained the cellulose and enough water to hydrate the solids, and that had then been sterilized by autoclaving (121°C, 60 min). Carboys were warmed to 39°C, gassed with CO<sub>2</sub> and illuminated with a bright incandescent light (Fukushima et al. 2002) until the medium was fully anaerobic (as revealed by decolorization of the resazurin). Carboys were then inoculated with 200 ml late exponential-phase, cellulose-grown culture, and were vigorously

swirled once or twice daily to suspend the cellulose particles and facilitate their complete colonization by the cells. After 88–108 h of incubation, the liquid phase was removed by siphoning, and the rather sticky sediment layer (containing glycocalyx, embedded cells, and residual cellulose) was resuspended in a small volume of deionized water. The resuspended material was centrifuged at 15,000 g for 45 min, and the supernatant discarded. Centrifugation always resulted in a small amount (<5% by volume) of a grey-colored layer of cells that sedimented atop the yellow glycocalyx; this layer was removed by careful scraping with a stainless steel spatula. The pellet, which contained primarily residual cellulose along with variable amounts of glycocalyx material and adherent cells, was lyophilized; these materials are designated LFR (lyophilized FR). In one case, a portion of the pellet was incorporated into the adhesive formulation while still wet, for comparison to the lyophilized material; this material was designated WFR (wet FR).

### Composition of FR

LFR was analyzed for protein and for alkali-soluble carbohydrate after treating ~10 mg (weighed to 0.001 mg) of residue with 0.50 ml 1 N NaOH at 70°C for 1 h. Treated samples were neutralized by addition of 0.50 ml 1 N HCl, and centrifuged (12,000 g, 5 min). The supernatants were assayed for protein by the method of Bradford (1976), using Coomassie Plus reagent (Bio-Rad, Hercules, Calif.) with lysozyme as protein standard, and were assayed for alkali-soluble carbohydrate by the phenol-sulfuric acid method (Dubois et al. 1956) with glucose as standard.

To remove cellular material for subsequent characterization of the glycocalyx, residues (1 g) were autoclaved (121°C, 45 min) in 100 ml neutral detergent solution (Goering and Van Soest 1970). The solid residue was filtered onto 47 mm-diameter polycarbonate membranes (3 μm pore diameter; Poretics, Livermore, Calif.) and rinsed exhaustively with hot deionized water prior to lyophilization. Subsamples (10 mg) of the lyophilized neutral detergent fiber (NDF) were treated with 1 ml 2 N trifluoroacetic acid (TFA) at 120°C for 1.5 h, dried under an air stream, resuspended in 1 ml deionized water, and passed through Supelclean SAX anion exchange columns (Supelco, Bellefonte, Pa.). Neutral sugars were determined by ion chromatography (Hatfield and Weimer 1995).

### Adhesive

The following adhesive sources were used for the construction of plywood panels: PF (42% solids; Neste Resins, Springfield, Ore.); WFR from *R. albus* 7 fermentation (33% solids in water, never dried); and LFR from four separate *Ruminococcus* fermentations (each mixed with water to 33% solids). The adhesives were formulated according to Table 2. When mixing LFR and PF together, we initially mixed LFR with water until smooth, and then PF was added and mixed well. PF, when used without FR, was supplemented with GLU-X (Robertson, Brownstown, Ind.), a wheat-derived protein and starch product commonly used as a glue extender.

### Plywood panels

Aspen veneer, 178×178×3 mm (7×7×1/8 inch) thick was conditioned to equilibrium moisture content at 27°C, 30% relative humidity (RH). Adhesive was weighed onto veneers as required for the construction of three-ply panels and spread evenly across the veneer with a spatula. Veneer sheets were arranged in a cross-ply pattern (i.e., the wood grain in the middle sheet was oriented perpendicular to the grain of the outer sheets) and pressed at 180°C and 1.125 MPa (163 lb/in<sup>2</sup>). The adhesives used, singly or in combination, along with pressing times, are shown in Table 2.

**Table 1** Growth conditions for generating fermentation residues (FR). *CF1* Long fibrous cellulose, *SC50* Sigmacell 50 microcrystalline cellulose

Residue <sup>a</sup>	Bacterium	Cellulose source and amount (g)	Incubation time (h)	Residue dry weight (g)
Ra7 FR1	<i>Ruminococcus albus</i> 7	CF1 (160)	88	NT <sup>b</sup>
Ra7 LFR 2	<i>R. albus</i> 7	SC50 (120)	108	25.6
RfB34b LFR	<i>Ruminococcus flavefaciens</i> FD-1	SC50 (120)	100	79.5
RfFD-1 LFR	<i>R. flavefaciens</i> B34b	SC50 (120)	96	80.2

<sup>a</sup> FR was tested either wet (WFR) or lyophilized and rehydrated (LFR)

<sup>b</sup> Not tested

**Table 2** Adhesive formulations and pressing times used to produce aspen plywood sheets. *PF* Phenol-formaldehyde, *GLU-X* glue extender (see Materials and methods), *Ra7* *R. albus* 7, *RfB34b* *R. flavefaciens* B34b, *RfFD-1* *R. flavefaciens* FD-1

Adhesive <sup>a</sup>	PF (g) <sup>c</sup>	GLU-X (g) <sup>c</sup>	FR <sup>b</sup> (g) <sup>c</sup>	H <sub>2</sub> O (g)	Press time (min)	Number of panels
PF	18.9	3.9	0	0	5	3
Ra7 WFR1	0	0	9.24	0	10	2
Ra7 LFR 1	0	0	11	22	10	2
PF + Ra7 LFR 2 (8)	11.76	0	2	0	10	2
PF + Ra7 LFR 2 (40)	2.94	0	2	4	5	1
PF + Ra7 LFR 2 (73)	1.47	0	4	8	8	1
RfB34b LFR	0	0	11	22	10	2
RfFD-1 LFR	0	0	11	22	10	2
PF + RfB34b LFR (40)	2.94	0	2	4	10	2
PF + RfB34b LFR (73)	1.47	0	4	8	10	2
PF + RfFD-1 LFR (40)	2.94	0	2	4	10	2
PF + RfFD-1 LFR (73)	1.47	0	4	8	10	2

<sup>a</sup> Values in parentheses correspond to percentage by weight of FR

<sup>b</sup> WFR or LFR

<sup>c</sup> Dry weight basis

#### Analysis of adhesive properties

Each three-ply panel was conditioned at 27°C, 30% RH for ~1–2 weeks before cutting into 12 standard lap shear specimens as outlined in PS 1–95 (National Institute of Standards and Technology 1995). Six specimens from each panel were tested for dry shear strength using a universal testing machine at a loading rate of 1 cm min<sup>-1</sup>. The remaining six specimens from each panel were subjected to a standard vacuum-pressure soak treatment (National Institute of Standards and Technology 1995). A vacuum of 85 kPa (25 inches of Hg) was drawn on the specimens while in water and held for 30 min. The vacuum was broken and a pressure of 450–480 kPa (65–70 lb/in<sup>2</sup>) was applied to the specimens while still in water, and held for 30 min. Shear strength was determined on the wet specimens using a universal testing machine at a loading rate of 1 cm min<sup>-1</sup>. Wood failure percentages were determined on the dry shear samples after testing and on wet shear specimens after testing and subsequent air drying using ASTM procedure D-5266-99 (American Society for Testing and Materials 1999).

#### Statistics

Analysis of variance was performed using the ANOVA protocol of the SAS system (SAS Institute 1998). Mean separations were performed using Duncan's multiple range test, at a significance level of  $P < 0.05$ .

## Results

The three *Ruminococcus* strains differed in the extent of cellulose fermentation (amount of residue remaining at the end of the fermentation; Table 1). The residues

(Table 3) differed nearly 10-fold in protein content (0.42–4.88% by weight), but less than 2-fold in the content of alkali-soluble polysaccharide (13.6–23.1%). Parallel treatments of residue subsamples by boiling in neutral detergent solution to remove microbial cells yielded NDF that retained the yellow color of the FR. Treatment of these NDFs with hot TFA released substantial amounts of neutral sugars, the molar composition of which (0.7 glucose:0.18 xylose:0.08 mannose:0.02 galactose) was remarkably similar across bacterial strains. The data suggest that the three strains may synthesize similar exopolysaccharides.

Aspen plywood panels prepared using PF adhesive resin displayed shear strengths of 3.37±0.55 MPa and wood failure values approaching 100% (98.3±2.6%; Fig. 1). High wood failure values are desirable, as they indicate the adhesive material is stronger than the wood itself. Panels prepared using either wet or lyophilized (and subsequently rehydrated) residues from the *R. albus* 7 fermentation, in the absence of PF, had values for both shear strength and wood failure that were similar to one another, but these values were two-fold lower under dry conditions and five-fold lower under wet [vacuum, pressure, soak, dry (VPSD)] conditions, than the values obtained for the PF resin (Fig. 2). Because wet residues provided similar results to lyophilized residues that were rehydrated just before use, the latter were used for subsequent experiments, owing to their ease of handling and their resistance to decomposition during storage.

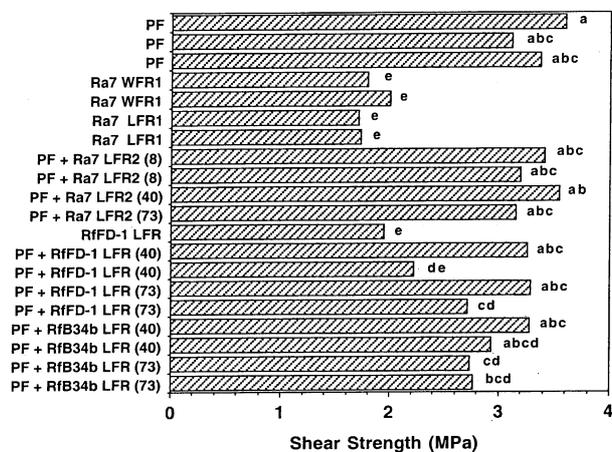
**Table 3** Composition of FR. TFA Trifluoroacetic acid, NDF neutral detergent fiber of residue isolated by the method of Goering and Van Soest (1970)

	Protein (% dry wt)	Alkali-soluble carbohydrate (% dry wt) <sup>a</sup>	Mol% neutral sugar composition of TFA hydrolyzate of NDF <sup>b</sup>				
			Glucose	Galactose	Mannose	Xylose	Arabinose
Ra7 FR1	4.88±0.14	23.1±2.1	NT <sup>c</sup>				
Ra7 LFR 2	3.77±0.13	20.3±0.1	69.2±0.6	2.3±0.3	8.3±0.3	19.4±0.5	0.5±0.2
RfB34b LFR	0.42±0.01	13.6±1.1	72.2±1.9	1.9±0.1	7.4±0.5	18.0±1.3	0.2±0.1
RfFD-1 LFR	1.41±0.01	22.4±6.9	73.1±0.4	2.6±0.5	7.3±0.3	16.8±0.5	0.3±0.1

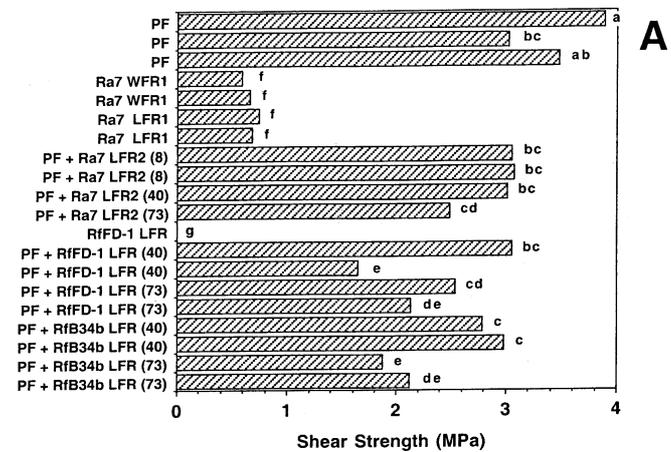
<sup>a</sup> Percentage of residue dry matter converted to phenol/sulfuric acid-reactive carbohydrate after treatment with 1 N NaOH, 70°C, 1 h. Results are mean values of duplicate samples ±SE

<sup>b</sup> See text for details of TFA hydrolysis. Results are mean values of duplicates samples ±SE; rhamnose and fucose were <0.2% for all samples

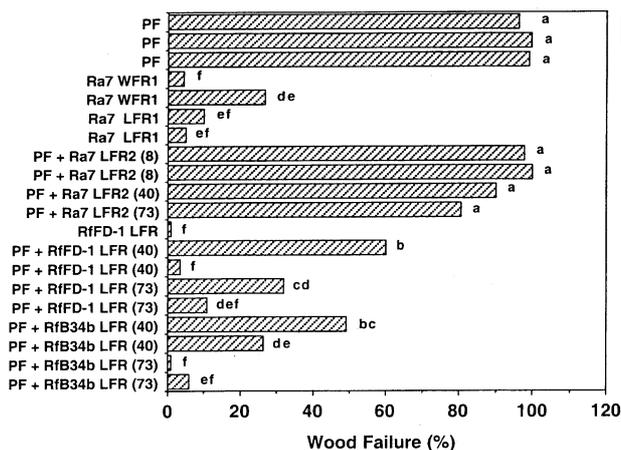
<sup>c</sup> Not tested



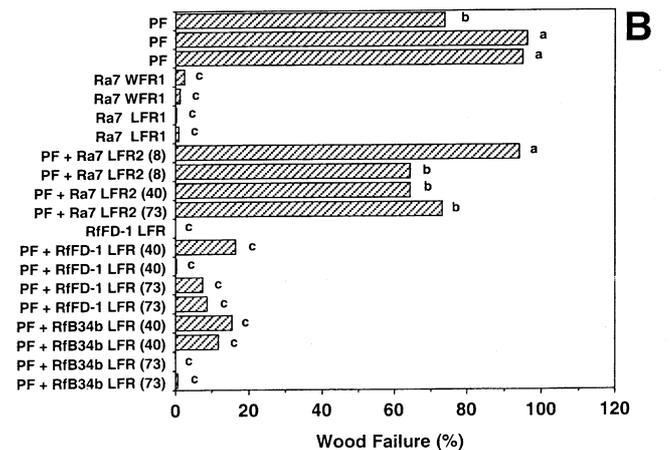
A



A



B



B

**Fig. 1** Shear strength (A) and wood failure percentage (B) for three-ply aspen plywood panels prepared with adhesives described in Table 2, tested under dry conditions. Numbers in parentheses indicate percentage of fermentation residue (FR) by weight in the adhesive formulation. Samples having different lower-case letters within treatments differ ( $P < 0.05$ ). Pooled standard error (SE) for shear strength = 0.58 MPa. Pooled SE for wood failure = 16.9%

**Fig. 2** Shear strength (A) and wood failure percentage (B) for three-ply aspen panels prepared with different adhesives, tested after vacuum/pressure/soak/drying [VPSD] treatment. Numbers in parentheses indicate percentage of FR by weight in the adhesive formulation. Samples having different lower-case letters within treatments differ ( $P < 0.05$ ). Pooled SE for shear strength = 0.46 MPa. Pooled SE for wood failure = 16.4%

Mixing LFR with PF to an LFR content of 8, 40, and 73% (dry basis) of the mixed adhesive resulted in an adhesive that produced shear strengths similar ( $P < 0.05$ ) to that of PF under dry conditions, although a tendency toward decreased performance under wet VPSD conditions was observed with increasing LFR concentration.

Fermentation residues from the two *R. flavefaciens* strains displayed reduced performance relative to those of *R. albus* 7 (Fig. 1). Both *R. flavefaciens* fermentations were less complete than were the *R. albus* fermentations (Table 1). The higher content of residual cellulose and lower content of presumably more adhesive materials (bacterial cells and glycocalyx; Table 3) were probably responsible for the poorer adhesive properties of the *R. flavefaciens* formulations.

Aspen panels prepared with the FR or PF-FR mixtures did not have an odor detectable above the aroma of the wood itself. Visual inspection revealed that the adhesive bondlines of the panels pressed with the FR or PF-FR mixtures were lighter in color than those pressed using PF alone, a desirable feature of plywood used in some interior applications (e.g., for furniture construction).

## Discussion

Direct fermentation of cellulosic biomass to ethanol has several potential advantages over fermentations based on yeast fermentations of sugars derived from corn. Cellulosic biomass is abundant and inexpensive, and is available in the form of agricultural and forestry wastes that have little commercial value. One of the major impediments to developing industrial fermentations of biomass is the lack of valuable co-products. We have shown here that the residue of a *R. albus* fermentation of purified cellulose (composed largely of residual cellulose with smaller amounts of cells and adhesins) do not by themselves meet the shear strength and wood failure standards of conventional phenol-formaldehyde (PF) adhesives for plywood when pressed under conditions typical for PF adhesives. However, they can be added to PF adhesives in large amounts (up to 73% by weight) without significant loss of the adhesive properties of the resulting resin.

The combination of carbohydrate materials and PF adhesives is not new (Loetscher 1934; Feigley 1959; Conner et al. 1986). Addition to PF resins of carbohydrates with large amounts of reducing end groups is known to result in loss of adhesive properties if the carbohydrate exceeds about 10% of the weight of the PF resin (Feigley 1959). By contrast, adhesive properties of PF resins are maintained upon addition of up to 30–50% of the total adhesive weight of sucrose, methyl monosaccharides or sugar alcohols (Conner et al 1986). Our results extend these observations both with respect to the level and source (viz., FR) of the modifier used.

Proteins of biological origin (e.g., blood or soybeans) were commonly used in the adhesives industry prior to the development of formaldehyde-based synthetic chemical

adhesives. Neither these biological materials, nor most carbohydrates, are typically involved in adhesion in nature. However they can display adhesive properties when properly denatured, mixed with other materials, and cured under heat and pressure (Lambuth 1994). The resulting mixed resins show acceptable strength under dry conditions, but often display reduced adhesive strength under wet or humid conditions (Lambuth 1994). During degradation of cellulose, *R. albus* and other ruminal cellulolytic bacteria adhere tenaciously to cellulose fibers immersed in an aqueous environment (rumen fluid or culture media) (Kudo et al. 1987; Weimer and Odt 1995). This adherence is thought to be mediated by several different types of adhesins, including cellulose-binding domains of cellulosome components, pilin-like proteins in fimbriae, and glycocalyx materials synthesized following irreversible adsorption to cellulose fibers (Miron et al. 2001). The intrinsic affinity of the various adhesins for cellulose is likely to contribute, in part, to the strength of the mixed adhesive compared to that of other biologically derived adhesives.

Additional experimentation is necessary to characterize *R. albus* FR more completely; to determine the suitability of the residue from authentic cellulosic biomass fermentations as adhesive components from the standpoints of wet and dry strength, lack of odor, and resistance to moisture and to mold and insect damage; and to determine pressing conditions for optimum adhesive performance. Additional experiments also will reveal if FR derived from different feedstock or having a higher content of cells and adhesins can substitute for some or all of the formaldehyde-based adhesives.

Currently, approximately  $6.4 \times 10^8$  kg phenol (selling price ~US \$0.89 kg<sup>-1</sup>) and  $7.7 \times 10^8$  kg formaldehyde (37% solution, selling price ~ US \$0.48 kg<sup>-1</sup>) are used for production of PF resins in the United States (Chemical Marketing Reporter 2002). The value of the *Ruminococcus* fermentation residue, even if sold at a discount relative to PF, is likely to be greater than that of the ethanol co-product, whose current selling price with government supports is approximately US \$0.40 kg<sup>-1</sup> (Chemical Marketing Reporter 2002). Detailed economic analysis for these adhesive formulations must await data from experiments with residues from authentic biomass substrates that are far less expensive than is purified cellulose, and that offer the prospect of simplifying the culture medium by virtue of their supplying vitamins and minerals that are lacking in pure cellulose.

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