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Wood adhesives prepared from lucerne fiber fermentation residues of *Ruminococcus albus* and *Clostridium thermocellum*

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Abstract Fermentation residues (consisting of incompletely fermented fiber, adherent bacterial cells, and a glycocalyx material that enhanced bacterial adherence) were obtained by growing the anaerobic cellulolytic bacteria *Ruminococcus albus* 7 or *Clostridium thermocellum* ATCC 27405 on a fibrous fraction derived from lucerne (*Medicago sativa* L.). The dried residue was able to serve as an effective co-adhesive for phenol–formaldehyde (PF) bonding of aspen veneer sheets to one another. Testing of the resulting plywood panels revealed that the adhesive, formulated to contain 30% of its total dry weight as fermentation residue, displayed shear strength and wood failure values under both wet and dry conditions that were comparable with those of industry standards for PF that contained much smaller amounts of fillers or extenders. By contrast, PF adhesives prepared with 30% of dry weight as either unfermented lucerne fiber or conventional fillers or extenders rather than as fermentation residues, displayed poor performance, particularly under wet conditions.

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Introduction

Biomass fermentations to fuels and industrial chemicals are considered by many as an essential centerpiece of sustainable resource utilization in response to reduced supply and increasing costs of fossil carbon sources. It is envisioned that development of a chemical and fuel industry based on cellulosic biomass materials will proceed along the lines of biorefineries, in which biomass will be processed to produce a host of products having different end-uses (Lynd et al. 1999, Kamm and Kamm 2004). The formation and recovery of such "value-added" products are essential for establishing and maintaining the economic viability of these biorefineries.

One type of proposed biorefinery involves "consolidated bioprocessing" (CBP), in which the cellulosic materials are fermented by anaerobic bacteria that produce their own polysaccharide-hydrolyzing enzymes and ferment the resulting oligomers to ethanol (Lynd et al. 1999, 2002). As envisioned, CBP has the advantage of reduced capital costs due to the use of a single reactor for enzyme production, enzymatic hydrolysis of substrate and microbial fermentation to produce ethanol. This feature may compensate for the lower yield and concentration of ethanol obtained, relative to ethanol production by yeast fermentation of soluble sugars (Lynd et al. 2002). Extensive developmental work is necessary to adapt or genetically modify existing organisms to enhance their performance in CBP. Several species, including the thermophilic bacterium *Clostridium thermocellum* and the mesophilic ruminal bacterium *Ruminococcus albus*, have been identified as potential candidates for such development (Lynd et al. 2002).

We have been investigating potential co-products from *R. albus* and *C. thermocellum* fermentations. Both organisms are known to adhere to cellulose during some or all of their growth on cellulose in batch culture. We recently reported that residual solids from the fermentation of pure cellulose by *R. albus* and other cellulolytic ruminococci can serve as adhesives or co-adhesives for bonding wood veneer to produce plywood (Weimer et al.

2003). Here, we extend these observations to include a more practical biomass substrate (lucerne fiber), to characterize the composition of the residue, and to demonstrate that *C. thermocellum* is also useful for the purpose of adhesives production.

Materials and methods

Materials

Lucerne fiber was obtained by a wet-fractionation process that separated expressed juice (containing most proteins, sugars, other soluble materials) from a residual fiber fraction (largely stem material, with some leaf material), as follows. Freshly cut lucerne (*Medicago sativa* L.) was passed, at a rate of ca. 50 kg min^{-1}, through a hammer mill fitted with a 45 kW electric motor operated at ca. 2,500 rpm. The material exiting the hammer mill was squeezed through a multi-cone press (Chase 2000) to ca. 35% dry matter and the retained solids (lucerne fiber) were air-dried indoors on a tarpaulin. Approximately 75% of the original dry matter was retained in the fiber fraction. The dried fiber clumps were passed through a cylinder (ca. 30 cm diam.) fitted with a pair of counter-rotating bars turned by a 2.14 kW electric motor and the resulting fiber was ground through a Wiley mill (1 mm screen size). No additional pretreatment of this fiber fraction was performed prior to its use in the culture media.

GP5778 phenol–formaldehyde (PF) resin was obtained from Georgia–Pacific Resins (Decatur, Ga.). GLU-X wheat flour was from The Robertson Corp. (Brownstown, Ind.). Walnut shell flour (200 mesh) was from Borden Chemical (Columbus, Ohio). Sigmacell 50 microcrystalline cellulose was from Sigma (St. Louis, Mo.).

Cultures

R. albus 7 was revived from glycerol stocks at −80°C and was grown at 39°C under a CO_2 atmosphere in modified Dehority medium (Weimer et al. 2003) that contained Sigmacell 50 microcrystalline cellulose as sole fermentable carbohydrate. *C. thermocellum* ATCC 27405 was grown similarly, except the volatile fatty acid mixture was excluded from the medium and the incubation temperature was 60°C.

Intermediate scale-up was accomplished in glass carboys (4–12 l) containing the same media, but with ground lucerne fiber replacing cellulose. Mass cultures were grown in a modified 380-l fermentor (Fermentation Design; working volume 300 l). The fermentor containing the lucerne fiber and most of the water was sterilized and cooled prior to addition of the other media components as sterile concentrated solutions. The fermentor was then inoculated with ca. 11 l of culture from the carboys. Fermentations were carried out for 50 h under a gentle CO_2 sparge.

Fermentation residues were recovered by pumping the fermentor contents through a Sperry plate-and-frame filter press fitted with 14 cellulose filter sheets (32.0×32.0×0.16 cm, grade 901 paper; BIF America). The filtration process separated the fermentation residue (which contained residual fiber, glycocalyx, adherent bacterial cells) from the fermentation liquid and most of the non-adherent (planktonic) bacterial cells. The filters were hand-scraped to recover the fermentation residue, which was then freeze-dried. These lyophilized fermentation residues (LFR) were ground through a Wiley mill (0.5 mm screen) and used directly for adhesive formulations. A separate sample of lyophilized, unfermented lucerne fiber was prepared by resuspending 75 g of material in 1.5 l of deionized water for 2 h and then filtering through a Nylon screen (50 μm mesh size; Nitex). The residual solids were then lyophilized.

Chemical composition and particle size analysis

Fermentation residues were analyzed for total N using a LECO automated nitrogen analyzer. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid-detergent lignin (ADL) were determined by the methods of Goering and Van Soest (1970), without the addition of α-amylase. Klason lignin was determined by the method of Moore and Johnson (1967), with vacuum filtration through preweighed GF/D glass fiber filters (47 mm diam.; Gelman) to collect the insoluble residues for weighing. Colorimetric analysis of alkali-extractable protein, carbohydrate, and uronic acids were conducted following treatment of ca. 6 mg (weighed to 0.001 mg) of sample with 600 μl of 1 N NaOH for 1 h at 70°C. The samples were then neutralized with 600 μl of 1 N HCl and centrifuged (11,700 *g*, 10 min) to obtain a clear supernatant. Protein was analyzed by Coomassie blue dye binding (Bradford 1976), with lysozyme as standard. Carbohydrate was analyzed by the phenol–sulfuric acid method (Dubois et al. 1956), with D-glucose as standard. Uronic acid was analyzed by the 3-phenylphenol/borate/sulfuric acid method (Blumenkrantz and Asboe-Hanson 1973), with D-galacturonic acid as standard.

Particle size fractions of *C. thermocellum* LFR were obtained by shaking six samples (35 g each) through a series of sieves (United States standard 50, 70, 140, 230, 325 mesh; ASTM E-11 series) in a Roto-Tap sieve shaker for 10 min each.

Adhesive tests

Adhesive formulations, described in Table 1, were used to prepare duplicate three-ply aspen panels. Aspen veneer (178×178×3 mm) was conditioned to equilibrium at 27°C and 30% relative humidity; and then 7 g of adhesive formulation per glueline was hand-applied evenly with a spatula. The veneer was placed together with the grain of the wood in the middle ply oriented perpendicular to that

of the two outer plies. The plies were pressed at 1.14 MPa and 180°C for 5 min, yielding a three-ply panel 9.5 mm thick. These panels were then conditioned at 27°C and 26% relative humidity for 7 days and then cut into lap-shear specimens (82.5×25.4 mm). Six such specimens from each panel were tested for shear strength (National Institute of Standards and Technology 1995), using an Instron universal testing machine at a loading rate of 1 cm min^{-1}. Six other specimens were subjected to a standard vacuum-pressure-soak (VPS) treatment (National Institute of Standards and Technology 1995). A vacuum of 85 kPa was drawn on the specimens while immersed in water and held for 30 min prior to measurement of shear strength as above. Wood failure percentages were determined on the dry shear samples after testing and on wet shear specimens after testing and subsequent air-drying, using procedure D-5266-99 (American Society for Testing and Materials 1999).

To test the adhesive properties of LFR and unfermented lucerne fiber alone (without PF), specimens (50.8×50.8×9.5 mm) were prepared by mixing 17 g of water with 17 g of *R. albus* LFR or unfermented lucerne fiber and pressing as described above.

Statistical analysis

Analysis of variance was conducted using PROC ANOVA in the SAS statistical software package (ver. 7.0), with mean separations determined by Duncan's multiple range test at α=0.01 (SAS Institute 1998).

Results

After freeze-drying and grinding, the fermentation residues were free-flowing powders having an olive-green color. The bulk densities of lyophilized residues (0.150 ±0.008 g cm^{-3} for *R. albus* and 0.181±0.007 g cm^{-3} for *C. thermocellum*) were much lower than that of the unfermented lucerne fiber (0.402±0.007 g cm^{-3}) ground by the same milling procedure (0.5 mm Wiley mill). This latter material, upon rehydration and lyophilization, displayed a bulk density of 0.243±0.012 g cm^{-3}.

Table 1 Composition of adhesive formulations. Percentages in parentheses indicate the %wt of lucerne or fermentation residue in the formulation. *GLU-X/WSF* Mixture of equal weights of commercial GLU-X and walnut shell flour additives, *LFR* lyophilized residue from lucerne fiber fermentation by *R. albus* or *C. thermocellum*, *PF* PF GP5778 (42% solids, pH 11.3), *UFA* unfermented lucerne fiber

Formulation	Component (g)				
	PF	GLU-X/WSF	LFR	UFA	H_2O
PF + GLU-X/WSF	69.0	12.7	0	0	18.3
PF + LFR (30%)	69.0	0	12.7	0	18.3
PF + LFR (45%)	37.0	0	12.7	0	50.3
PF + UFA (30%)	69.0	0	0	12.7	18.3

Washing and lyophilization of the unfermented lucerne fiber removed additional soluble components (particularly nitrogenous compounds and inorganics), resulting in an increase in fiber content (NDF, ADF, lignin; Table 2). Fermentation further increased the relative amount of unfermentable residue components (lignin, ash), which in turn elevated the amount of residual fiber which contains these components. Protein content (determined in alkaline extracts) was slightly (*R. albus*) to considerably (*C. thermocellum*) lower in the fermentation residues than in the unfermented lucerne fiber, probably due to release of lucerne cell wall proteins into solution during the sterilization of the culture media and during the fermentation as polysaccharides were degraded. Similarly, alkali-extractable carbohydrates were lower in the fermentation residues, presumably due to dissolution of sugars, pectins, and low molecular weight heteropolysaccharides during medium sterilization and to degradation of hemicelluloses during fermentation.

LFR from *R. albus* or *C. thermocellum* lucerne fiber fermentation was incorporated into PF resin for the preparation of aspen plywood panels. To evaluate the adhesive performance of these composite resins, shear strength was quantified as a measure of resistance of the panels to breakage under force. Testing in shear of cured panels that contained 30% by weight (dry basis) of LFR from *R. albus* or *C. thermocellum* yielded mean shear strengths of 2.54–2.75 MPa under dry conditions (Fig. 1a). These values were significantly higher than those for panels prepared with PF that contained 30% by weight (dry basis) of unfermented lucerne fiber ground to the same particle size range, or 15% by weight of each of two additives (GLU-X, walnut shell flour) used commercially as co-adhesives or extenders. The shear strength values obtained for the PF/LFR composite resins are comparable with values obtained for PF resins in which the amounts of PF and GLU-X were 92% and 8%, respectively (Weimer et al. 2003), the typical weight ratios used in industrial plywood fabrication. However, PF resins prepared with 45% by weight of either LFR displayed poor shear strengths.

A second, and perhaps better, measurement of adhesive strength is provided by wood failure values, which provide an estimate of the strength of the adhesive relative to that of the wood. High wood failure values are desirable, as they indicate that the adhesive is stronger than the wood itself. Under dry conditions, wood failure values (Fig. 1b) approached 100% for the PF resins prepared with the *R. albus* LFR (at 30% by weight) and were approximately 80% for the *C. thermocellum* LFR (at 30% by weight), near the voluntary industry standard of 85% (National Institute of Standards and Technology 1995). By contrast, PF resins prepared at 30% by weight of unfermented lucerne fiber yielded poor wood failure values, as did resins prepared at 45% by weight of either LFR.

Testing in shear of panels prepared with PF resin that contained LFR at 30% by weight and subsequently exposed to moisture by the VPS method yielded shear strength values of 1.66–2.01 MPa (Fig. 1a). These values

Table 2 Composition of lucerne fiber substrate and residues from lucerne fiber fermentation by *R. albus* and *C. thermocellum*. Values given are the percentage of dry weight of original solid material, expressed as mean value from duplicate analyses ± standard deviation, except for Klason lignin (triplicate determination) and ash (single determination)

Component/ extract	Percentage by weight			
	Unfermented lucerne fiber	Lyophilized unfermented lucerne fiber	Lyophilized *R. albus* residue	Lyophilized *C. thermocellum* residue
Component				
Nitrogen	4.65±0.01	2.96±0.03	7.10±0.05	4.47±0.02
NDF	32.8±1.1	45.7±2.6	57.4±2.4	70.9±1.0
ADF	31.3±0.1	40.9±0.8	41.2±0.6	54.1±0.8
ADL	6.7±0.5	7.5±0.7	18.1±0.1	21.4±1.1
Klason lignin	14.1±0.3	18.1±0.5	25.9±0.5	28.2±0.3
Ash	10.2	7.2	13.8	11.5
Alkaline extract				
Protein	4.7±0.3	5.3±0.2	4.2±0.9	2.8±0.2
Carbohydrate	11.8±0.6	7.5±1.0	7.7±0.9	7.8±0.2
Uronic acid	1.1±0.2	2.2±0.1	0.8±0.1	1.2±0.2

Table 3 Particle size distribution and nitrogen contents of *C. thermocellum* ATCC 27405 LFR (Wiley-milled to pass a 0.5-mm screen) and subsequent adhesive performance of LFR/PF mixtures. The weight percentage of each size fraction was based on the starting material. Total sample recovery was 98.9%. Shear strength and wood failure measurements were performed on aspen panels as described in the Materials and methods, with adhesives formulated to contain 30% LFR. Results are mean values of duplicate panels (each determined with six samples per panel) ±SEM between panels. *NT* Not tested

Sample	Weight percentage	Nitrogen content (% ±SEM)	Shear strength (MPa)		Wood failure (%)	
			Dry	Wet	Dry	Wet
LFR of particle size						
<45 μm	10.85	4.89±0.01	2.51±0.51	1.38±0.62	99±1	100±0
45–75 μm	7.71	4.87±0.03	2.71±0.81	1.60±0.62	85±12	98±2
75–106 μm [a]	15.69	4.82±0.01	2.59±0.51	1.39±0.44	65±22	60±52
106–212 μm [a]	41.01	4.41±0.04	2.71±0.16	1.81±0.27	46±7	35±24
212–300 μm	20.01	3.17±0.03	NT	NT	NT	NT
>300 μm	3.58	2.24±0.02	NT	NT	NT	NT
Unfractionated LFR			2.91±0.26	1.97±0.05	74±4	83±9
Unfractionated UFA			2.19±0.54	1.31±0.21	11±3	20±14
PF+GLU-X/WSF[b]			2.67±0.94	1.52±0.67	100±0	65±41

[a]Slightly more (6% by weight) water was used to prepare the formulation for one of the two panels tested to facilitate spreading of the relatively viscous formulation
[b]PF resin was prepared with 15% (dry weight basis) each of GLU-X and walnut shell flour

are significantly higher than those obtained for adhesives prepared with equivalent concentrations of unfermented lucerne, or with GLU-X and walnut shell flour. Moreover, the wood failure values for the LFR-containing resins are equivalent to those obtained for conventional resins containing GLU-X or walnut shell flour at 8% by weight (Weimer et al. 2003); and these values exceed the voluntary industry standard value of 85% wood failure (National Institute of Standards and Technology 1995).

LFR from the *C. thermocellum* lucerne fiber fermentation, ground through a Wiley mill and 0.5-mm screen, displayed a range of particle sizes, with over three-quarters of the LFR mass having particle sizes in the 75–300 μm range (Table 3). The nitrogen content of the particles increased with decreasing particle size, presumably due to a higher surface-to-volume ratio and thus a greater coverage of microbial cells and glycocalyx per unit mass of LFR. Adhesives prepared from different size fractions of the LFR residue did not differ in shear strength, but wood failure values for the composite resins increased with decreasing LFR particle size (Table 3). Use of smaller particle size LFR also yielded formulations which performed more consistently (i.e., displayed less variation among replicates).

Discussion

Adherence to cellulose appears to be a general strategy among anaerobic cellulolytic bacteria and in at least some species has been reported to be a necessary prerequisite for cellulose degradation (Kudo et al. 1987; Weimer and Odt 1995; Lynd et al. 2002). We previously reported that residues from the fermentation of pure cellulose by *R.*

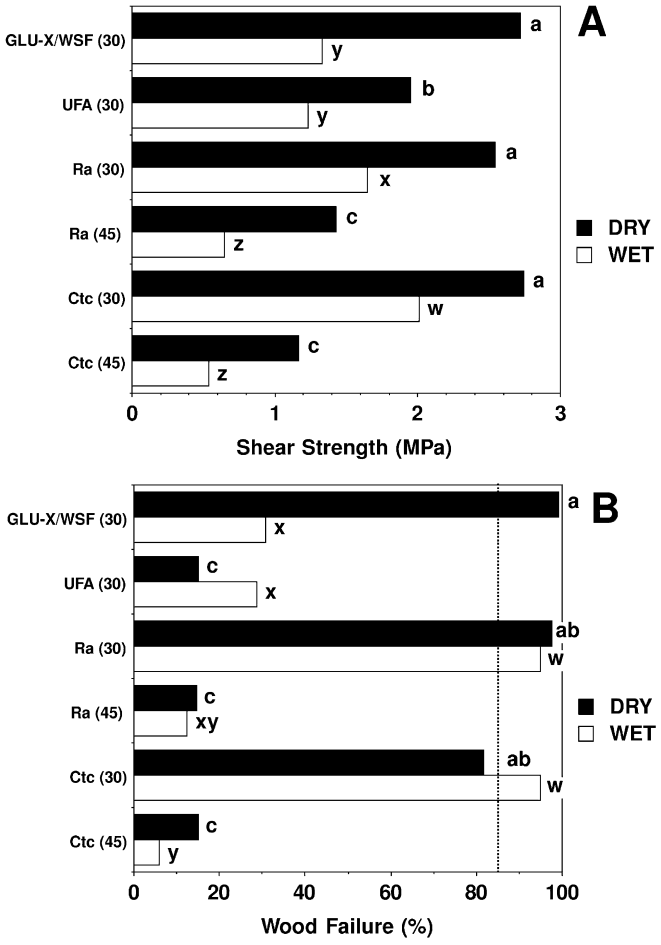

Fig. 1 Shear strength (**A**) and wood failure (**B**) data for plywood adhesives formulated with PF resin and the indicated co-adhesive. *GLU-X/WSF* GLU-X and walnut shell flour, *UFA* unfermented lucerne fiber, *Ra* residue from *R. albus* 7 lucerne fiber fermentation, *Ctc* residue from *C. thermocellum* ATCC 27405 lucerne fiber fermentation. Values in parentheses on the vertical axis labels indicate percentages (dry weight basis) of additive, with the remainder being PF (see Table 1 for formulations). In the case of GLU-X/WSF, each was added at 15% by dry weight for a total of 30%. Different a, b, c letters indicate significant differences ($P<0.01$) in shear strength or wood failure among the panels under dry conditions. Different w, x, y, z letters indicate significant differences ($P<0.01$) in shear strength or wood failure among the panels under wet (VPS) conditions. The vertical line in **B** indicates the industry standard for wood failure (85%)

albus and *R. flavefaciens* display adhesive properties and these properties are improved (particularly for exposure to wet conditions) by blending the residues with PF resins commonly used in plywood manufacture (Weimer et al. 2003). The results presented here extend these observations to include a more authentic biomass substrate (lucerne fiber), along with the bacterium *C. thermocellum*, an organism with reported potential as the basis for consolidated bioprocessing of biomass to produce ethanol (Lynd et al. 1999, 2002). The ability of the fermentation residues to serve as a co-adhesive could potentially allow plywood manufacturers to adjust additive compositions to achieve the desired adhesive performance consistent with the application conditions, open time, close time, and press conditions unique to each manufacturing plant.

The PF-based adhesives formulated with the residues of the *R. albus* and *C. thermocellum* lucerne fiber fermentations displayed similar performance in shear strength and wood failure tests, despite substantial differences in their gross chemical composition. Relative to the *R. albus* residues, the *C. thermocellum* residues contained higher levels of fiber and lower levels of protein. Indeed, the protein levels in the *C. thermocellum* residues were no greater than in the original lucerne fiber, probably as a result of offsetting loss (particularly solubilization) of substrate protein and production of bacterial protein during fermentation. Although protein is considered to be the active ingredient in soy and other biologically derived adhesive materials (Lambuth 2003), it appears that high levels of protein in the residue are not an absolute requirement for adhesive performance and other residue components produced by the bacteria themselves (perhaps the carbohydrate components of the glycocalyces) are important in facilitating adhesion. In this regard, it is interesting that the residues of lucerne fiber fermentation from both organisms contained similar levels of alkaline-extractable carbohydrate and that the monosaccharide compositions of residues obtained from the two species during fermentation of pure cellulose were very similar (data not shown).

Grinding the LFR to smaller particle sizes enhanced the adhesive properties of the LFR/PF formulations. This result is consistent with known properties of adhesive extenders, such as GLU-X and walnut shell flour, which are ground to a fine flour prior to mixing with PF. Additional benefits may result if particle size reduction of the alfalfa fiber is performed prior to bioconversion, as the fermentation of the more finely ground particles should yield particles having a higher ratio of fermentation products (glycocalyx plus cells) to residual substrate. Moreover, a more finely ground substrate would likely be fermented at a greater rate and with a shorter lag time by the bacteria, as shown for pure celluloses (Weimer et al. 1990). Performance improvements may also result from a better understanding of the chemical interactions among wood components, PF, and residue components, particularly in the highly alkaline environment of the PF resin mixture.

Fermentations of cellulosic biomass that yield both ethanol and marketable co-products can potentially improve the overall economics of biomass conversion. The lucerne fiber fermentation residues described here display interesting properties as a co-adhesive, but the high nitrogen content and bulk fiber properties of the residue also allow consideration of other potential uses (e.g., as an animal feed or a fertilizer/soil conditioner). Consolidated bioprocessing schemes in which biomass is fermented by microoorganisms that produce their own polysaccharide-hydrolyzing enzymes and then ferment the hydrolytic products to ethanol have several process advantages over systems in which hydrolytic enzymes must be produced in a separate reactor (e.g., simultaneous

saccharification and fermentation) schemes (Lynd et al. 1999, 2002). In most bioconversion schemes, it is desirable to achieve as complete a conversion of substrate as possible, as the residue is not considered to hold great value and may even require expensive disposal. Consequently, various pretreatments have been proposed to improve total conversion of substrate to simultaneously increase product (viz., ethanol) yield and reduce the fermentation residue (for a recent review, see Dien et al. 2004). Productive use of the fermentation residue thus has the potential to fundamentally alter the bioconversion strategy, in that producing a residue having value equal to or exceeding that of the ethanol co-product obviates the need to strive for maximum substrate conversion. In this particular case, costly pretreatments aimed at improving total substrate conversion may be unnecessary for, or even counterproductive to, overall process economics.

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