

Enzymatic Hydrolysis of Steam-Exploded and Ethanol Organosolv–Pretreated Douglas-Fir by Novel and Commercial Fungal Cellulases

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Abstract

Softwood residues are the most abundant feedstock available for bioconversion in many northern countries. However, the high costs for delignification and enzymatic hydrolysis currently deter commercialization of softwood bioconversion processes. This study evaluates the abilities of two novel fungal preparations (MSUBC1 and MSUBC2) and two commercial cellulase preparations (TR1 and TR2) to hydrolyze cellulose in Douglas-fir pretreated by steam explosion or ethanol organosolv process. MSUBC1 showed significantly better performance than the other preparations on both lignocellulosic substrates. In particular, MSUBC1 achieved >76% cellulose conversion for hydrolysis of steam-exploded Douglas-fir (~44% lignin) after 72 h at low enzyme loading (10 filter paper units/g of cellulose) and without β -glucosidase supplementation.

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Introduction

Softwood lignocellulosic residues are considered a potential source of inexpensive and renewable feedstocks for bioconversion in northern countries. However, the physical structure of native lignocellulose is intrinsically resistant to enzyme attack, especially cellulose, which is further protected by the surrounding matrix of lignin, hemicellulose, and pectin (1). Therefore, lignocellulosic biomass must be pretreated to make the cellulose fraction more accessible to enzymes. Various pretreatment processes, including acid or alkali treatment, steam explosion, and aqueous organic solvent approaches, are now undergoing technoeconomic evaluation (2,3). Pretreatment effectiveness is highly dependent on the chemical and physical properties of the feedstock. Agricultural residues, such as corn stover, have low lignin content and are more easily pretreated than softwood residues that contain higher amounts of lignin and have a much more rigid cell wall structure. Presently, the costs for pretreatment and enzymes for cellulose hydrolysis are still the main economic obstacles to commercialization of biomass bioconversion technologies (2,3).

Microorganisms produce a complex array of hydrolytic enzymes during growth on cellulosic substrates. Filamentous fungi, particularly *Trichoderma* species, are the preferred producers for industrial cellulase complexes, because their capacity to produce extracellular protein is greater than that of cellulolytic bacteria. In addition, enzyme profiles of filamentous fungi are dominated by endoglucanases (1,4- β -D-glucan glucanohydrolases) and exoglucanases (1,4- β -D-glucan cellobiohydrolases) that are directly involved in cellulose hydrolysis. Further, fungal cellulases contain a range of accessory enzymes, including some responsible for the hydrolysis of the hemicellulose and pectin matrix. Recent efforts by leading manufacturers of industrial enzymes have reduced the cost of cellulase for hydrolysis of pretreated corn stover by about 10-fold (4). This reduction was achieved by an approximately fivefold reduction in enzyme production costs and a twofold increase in enzyme performance. Nevertheless, it is estimated that a further three to fourfold reduction in cost is needed for full-scale process commercialization. Thus, efforts to further improve enzyme productivity and performance need to continue. Strategies to improve the performance of cellulase complexes typically involve random mutagenesis of fungal strains, genetic engineering of individual 1,4- β -glucanases, and optimization of the ratio of the various 1,4- β -glucanase components, followed by screening for increased specific activity or stability. Generally, screening of cellulase complexes for improved activity uses a standard assay proposed by the International Union of Pure and Applied Chemistry (IUPAC) to measure the hydrolysis of filter paper (5).

In the present study, we compared two commercial cellulase complexes from *Trichoderma* spp. (TR1 and TR2) and two novel complexes obtained from selection and random mutagenesis of *Penicillium* and *Trichoderma* strains in our laboratory (MSUBC1 and MSUBC2), by evaluating their performance in hydrolyzing both steam-exploded and ethanol organosolv-pretreated Douglas-fir. The experiments described are part of a program to develop a cost-effective process for the pretreatment and enzymatic hydrolysis of softwood residues. The purpose of this study is to show that enzymes with similar specific cellulase activities, Carboxymethyl cellulose (CMCase), Avicelase, and filter paper activity, can differ dramatically in their performance on softwood substrates. Furthermore, the study demonstrates that extensive delignification of a softwood substrate, the most costly step of softwood bioconversion, can be avoided by using improved enzyme production strains that produce enzyme mixtures with apparently low susceptibility to lignin inhibition.

Materials and Methods

Softwood Samples

Representative samples of coastal Douglas-fir (*Pseudotsuga menziesii*) sapwood and heartwood were collected in British Columbia. Samples were chipped to approx $2 \times 2 \times 0.5$ cm, screened for uniformity, and equilibrated at room temperature in sealed plastic bags to an appropriate moisture content (10%) prior to pretreatment.

Pretreatment of Softwood

Samples of Douglas-fir were impregnated with anhydrous SO_2 to the extent of 4.5% (w/w) and steam exploded in 50-g batches (dry weight) at 195°C for 4.5 min, as previously described for pretreatment of corn fiber (6). After pretreatment, the liquid fraction was removed by decantation and filtration. Samples were stored in sealed plastic bags at 4°C.

Organosolv pretreatment of Douglas-fir was carried out in a 1-L stainless steel pressure reactor (Parr, Moline, IL) using 50% (w/w) ethanol, adjusted to pH 2.42 (approx 51 mM sulfuric acid), at 195°C and approx 3.2 MPa (460 psi). The solvent:wood ratio was 7:1 (w/w), and the pretreatment time was 40 min. The time required to reach the target cooking temperature was approx 53 min in all runs. After cooking, the reactor was cooled down immediately by immersing it in ice until the interior temperature was $\leq 55^\circ\text{C}$. The brown liquor was removed by decantation. Following manual removal of any recalcitrant chips, the solids were homogenized for 5 min in 70% (v/v) ethanol at 70°C (pulp:ethanol 9:1) in a British disintegrator (TMI, Montreal, Canada). Solids were then washed a further three times with warm 70% ethanol and rinsed extensively with water. After washing, the solid residues were separated by filtration and stored in sealed plastic bags at 4°C.

Analysis of Composition of Pretreated Softwoods

Carbohydrate and lignin contents of pretreated softwood samples were determined using a modified Klason lignin method derived from TAPPI standard method T222 om-88, as previously described (6). Monosaccharides were analyzed by high-performance liquid chromatography (HPLC) with fucose as the internal standard, as previously described (7). All analyses were performed in triplicate.

Cellulase Preparations

Two commercial *Trichoderma reesei* cellulase preparations, TR1 (118.0 filter paper units [FPU]/mL; Novozyme, Franklinton, NC) and TR2 (135.0 FPU/mL; Iogen, Ottawa, Canada), and two preparations, produced in our laboratory by mutant strains of *Trichoderma* sp. MSUBC1 (740.0 FPU/g) and MSUBC2 (382.0 FPU/g), and *Penicillium* sp. were used.

Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted in triplicates in 125-mL flasks on a shaker at 45°C and 150 rpm. Flasks contained 0.1 M acetate buffer, pH 4.8; 2 or 5% (w/w) substrate; and 10 FPU of cellulase complex/g of cellulose, in a total reaction volume of 50 mL. Samples were taken at 3, 6, 12, 24, 48, and 72 h. Glucose concentrations were evaluated by HPLC using fucose (Sigma, St. Louis, MO) as the internal standard. To prevent microbial contamination, tetracycline (Sigma) and cycloheximide (Sigma) were used at concentrations of 40 and 30 µg/mL, respectively. The effectiveness of the enzymatic hydrolysis was determined by evaluating cellulose conversion after 72 h and the initial (0–3 h) rate (grams/[liter][hour]) (see Tables 3 and 4).

Enzyme Activities

All enzyme activities were determined at 50°C, except Avicelase and β-glucosidase (40°C). Filter paper activity was determined as recommended by (5) and is expressed as filter paper units. Carboxymethyl cellulose activity (CMCase) was assayed following the recommendations in ref. 8. Avicelase activity was assayed as described in ref. 5. β-Glucosidase was assayed by monitoring the release of *p*-nitrophenol from *p*-nitrophenol-β-*D*-glucoside (8).

Xylanase activity was measured by using birchwood xylan as substrate, and monitoring the release of reducing sugars by the Somogyi-Nelson method (9). Briefly, 0.15 mL of 50 mM acetate buffer, pH 5.0, plus 0.25 mL of 1% (w/v) xylan solution were preincubated at 50°C for 5 min and then mixed with 0.1 mL of diluted enzyme. After incubating for 10 min at 50°C, 0.5 mL of Somogyi reagent was added and the mixture was boiled for 40 min. After cooling, 0.5 mL of Nelson reagent was added; the total volume was adjusted to 5.0 mL with distilled water. The amount of reducing sugar released was calculated from the absorbance at 610 nm with

reference to a xylose standard curve. Enzymes were diluted so that the absorbance of the final reaction mixture was ≤ 1 absorbance unit. One unit of xylanase activity is defined as the amount of enzyme required to release 1 μmol of reducing sugars (as xylose equivalents)/min. The β -glucanase activity was measured by following the same procedure but with barley β -glucan as substrate replacing xylan. Pectinase activity was measured using polygalacturonic acid as substrate, as previously described (10). Mannanase activity was determined using galactomannan as substrate (11).

The protein concentration of the enzyme preparations was determined by the Lowry method (12) following precipitation with trichloroacetic acid.

Results and Discussion

Effect of Pretreatment on Lignin Removal

Table 1 gives the compositions of Douglas-fir samples pretreated by steam explosion or ethanol organosolv extraction. Glucan content in the organosolv-pretreated sample was significantly higher than that in the sample pretreated by steam explosion. In addition, lignin removal was quite different for the two pretreatment processes. In agreement with our previous observation, less lignin was removed after steam explosion (13). In fact, a substantial increase in lignin content (to approx 46%) was observed for steam-exploded Douglas-fir (Table 1), mainly owing to the removal of most of the hemicellulose fraction (13).

Ethanol organosolv pretreatment also resulted in solubilization of hemicellulose, but in this case it was accompanied by extensive delignification, with lignin content decreasing to about 13%. Samples contained similar amounts (2.5–3.0%) of residual hemicellulose-derived sugars (arabinose, galactose, mannose, and xylose), although there were significant differences in the amounts of the individual sugars. The glucose content of the steam-exploded and organosolv-pretreated samples was approx 47 and 85%, respectively, corresponding to ~42–76% cellulose, assuming that all glucose is derived from cellulose.

Activities of Cellulase Preparations

Table 2 presents the protein contents and hydrolytic activities of the four enzyme preparations on a range of cellulosic, hemicellulosic, and other related substrates. All preparations had similar filter paper activity (0.7–1.0 FPU/mg of protein), CMCase activity (14.1–20.7 U/mg), and Avicelase activity (1.6–2.4 U/mg). There were considerably larger differences in endogenous β -glucosidase activity (0.2–1.2 U/mg), xylanase activity (3.5–39.0 U/mg), mannanase (0.1–0.3 U/mg), and pectinase (0.1–0.6 U/mg) activities. All preparations contained similar levels of β -glucanase activity.

Table 1
Monosaccharide and Lignin Composition of Steam-Exploded and Organosolv-Pretreated Softwoods (% dry wt)^a

	Arabinose	Galactose	Glucose	Xylose	Mannose	Klason lignin	Acid- soluble lignin
Steam-exploded Douglas-fir	0.14 ± 0.00	0.34 ± 0.01	47.39 ± 1.11	0.58 ± 0.00	2.05 ± 0.07	44.35 ± 0.19	1.2 ± 0.03
Organosolv-pretreated Douglas-fir	ND	ND	84.80 ± 2.60	1.12 ± 0.19	1.38 ± 0.28	12.69 ± 0.40	0.33 ± 0.01
Untreated Douglas-fir	0.97 ± 0.00	2.68 ± 0.03	41.28 ± 0.13	3.67 ± 0.08	12.00 ± 0.15	28.19 ± 0.14	0.30 ± 0.01

^aND, not detected.

Table 2
Enzyme Activities in Various Cellulase Preparations

Enzyme	Protein ^a	Cellulase activities (U/mg protein)				Other enzyme activities (U/mg protein)			
		Filter paper activity	CMCase	Avicelase	β -Glucosidase	β -Glucanase	Xylanase	Pectinase	Mannanase
MSUBC1	838.5	0.9	16.9	2.0	1.2	17.0	39.1	0.6	0.3
MSUBC2	556.0	0.7	19.2	2.4	0.2	14.6	9.3	0.3	0.3
TR1	129.3	0.9	14.1	2.2	0.2	16.8	3.5	0.1	0.1
TR2	130.0	1.0	20.7	1.6	0.7	19.2	11.8	0.1	0.1

^aProtein concentration is in milligrams/gram, except TR1 and TR2, which are in milligrams/milliliter.

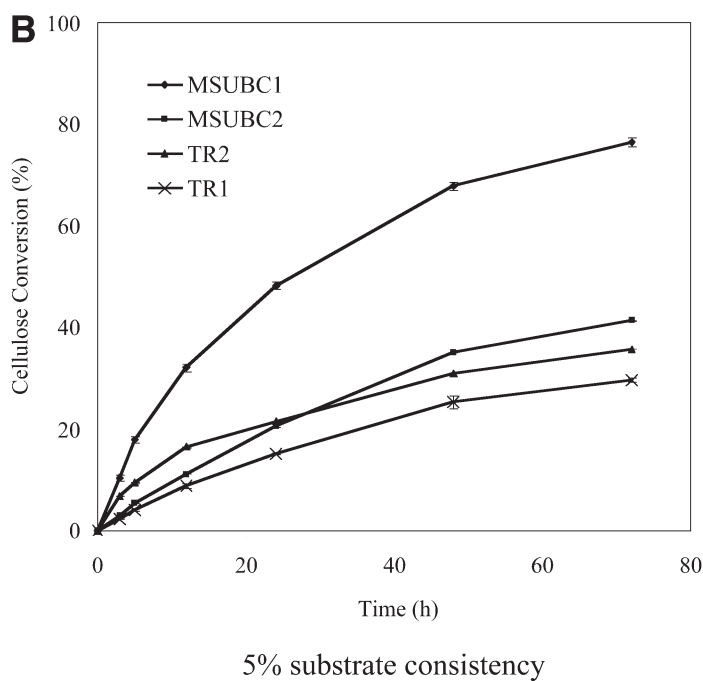
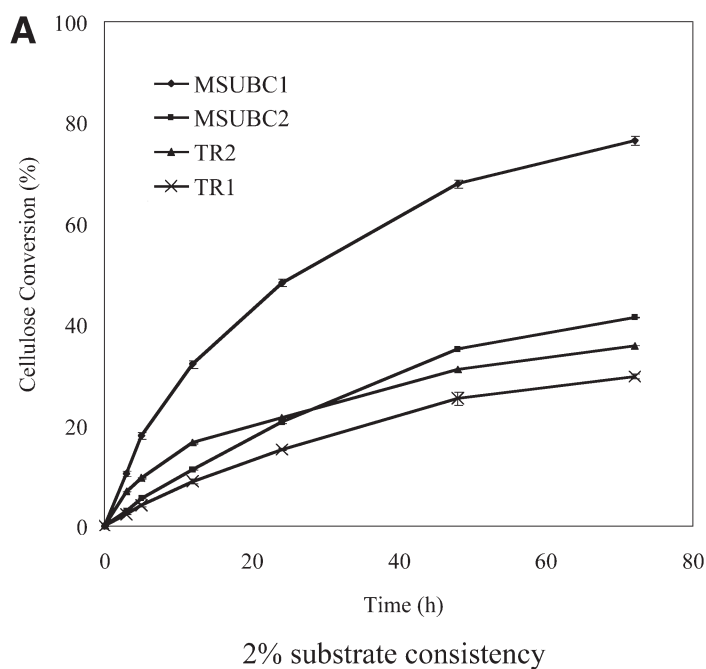


Fig. 1. Hydrolysis of steam-exploded Douglas-fir: **(A)** 2% substrate consistency; **(B)**: 5% substrate consistency.

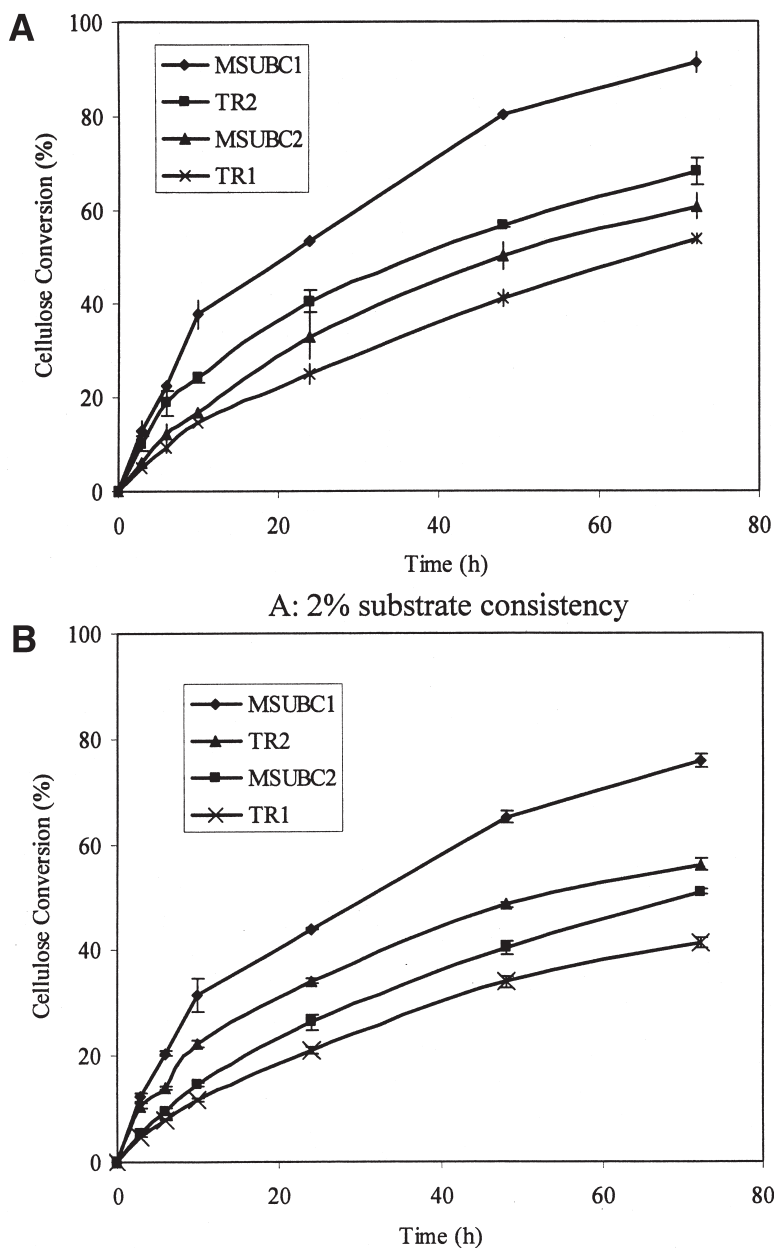


Fig. 2. Hydrolysis of ethanol organosolv-pretreated Douglas-fir: **(A)** 2% substrate consistency; **(B)** 5% substrate consistency.

Hydrolysis of Pretreated Softwood

Data for the hydrolysis of steam-exploded softwood show that MSUBC1 performed significantly better than the other cellulase preparations at both 2 and 5% cellulose consistency (Fig. 1A,B). Superior performance by MSUBC1 was also seen on organosolv-pretreated substrate,

Table 3
Cellulose Conversion and Initial Hydrolysis Rate of Steam-Exploded Douglas-Fir by Various Cellulase Preparations

Cellulase	2% Consistency		5% Consistency	
	% Conversion (%) (72 h)	Initial rate (g/[L · h])	% Conversion (%) (72 h)	Initial rate (g/[L · h])
MSUBC1	76.4 ± 0.9	0.48 ± 0.01	64.7 ± 0.03	1.22 ± 0.04
MSUBC2	41.3 ± 0.05	0.27 ± 0.06	34.0 ± 0.1	0.69 ± 0.00
TR1	29.6 ± 0.5	0.25 ± 0.00	24.0 ± 0.4	0.63 ± 0.03
TR2	35.7 ± 0.02	0.39 ± 0.01	29.2 ± 1.1	0.96 ± 0.03

Table 4
Cellulose Conversion and Initial Hydrolysis Rate of Organosolv-Pretreated Douglas-Fir by Various Cellulase Preparations

Cellulase	2% Consistency		5% Consistency	
	% Conversion (%) (72 h)	Initial rate (g/[L · h])	% Conversion (%) (72 h)	Initial rate (g/[L · h])
MSUBC1	91.4 ± 2.1	0.66 ± 0.07	75.8 ± 1.4	1.57 ± 0.09
MSUBC2	60.6 ± 3.0	0.31 ± 0.01	51.1 ± 0.4	0.71 ± 0.08
TR1	53.7 ± 1.1	0.255 ± 0.02	41.3 ± 0.8	0.61 ± 0.00
TR2	68.0 ± 2.7	0.52 ± 0.09	56.1 ± 0.48	1.37 ± 0.01

although the difference was less pronounced (Fig. 2A,B). Tables 3 and 4 provide more detailed comparison of enzyme performance. The superior performance of MSUBC1 on both softwood substrates evident in Figs. 1 and 2 is reflected by the relatively high values of conversion and initial hydrolytic rates.

Although filter paper activity is commonly used as an index of cellulase performance, it clearly does not provide a reliable indication of the ability of a preparation to hydrolyze complex lignocellulosic substrates. MSUBC1 showed a lower specific filter paper activity (0.88 FPU/mg) than TR2 (1.04 FPU/mg) and approximately the same activity as TR1 and MSUBC2 (Table 2). Similarly, there was no observed correlation between enzyme performance on softwood substrates and CMCase or Avicelase activity. Further examination of enzyme activities shows that MSUBC1 had significantly higher intrinsic β -glucosidase activity than the other three preparations (Table 2). High β -glucosidase activity could be at least partially responsible for the superior performance by MSUBC1 on softwood substrates, presumably by hydrolyzing cellobiose and relieving product inhibition of cellobiohydrolases and endoglucanases (14,15). It was previously shown that supplementation of a commercial cellulase

complex with β -glucosidase beyond relatively low levels did not increase its filter paper activity but did significantly enhance long-term hydrolysis of lignocellulosic substrate (15). Nevertheless, TR1 and TR2, which had similar filter paper activity but a greater than threefold difference in β -glucosidase activity (Table 2), had similar performance on softwood substrates (Figs. 1 and 2, Tables 3 and 4), suggesting that other factors contribute to the efficiency of lignocellulose hydrolysis.

MSUBC1 also showed significantly higher levels of xylanase activity, which may contribute to improved performance by removing residual hemicellulose that impedes cellulase attack. Other possible reasons for the improved activity shown by particular cellulase complexes during long-term incubation with softwoods include better enzyme stability, reduced sensitivity to product inhibition by glucose, and lower affinity for residual lignin. Experiments to evaluate these possibilities are currently in progress.

Conclusion

Fungal cellulase preparations with significantly improved performance on lignocellulosic substrates can be obtained by selection and random mutagenesis. It is anticipated that the use of such preparations will result in substantial cost savings by reducing or eliminating the need to supplement preparations with additional accessory enzymes. It is particularly noteworthy that MSUBC1, unlike the other preparations tested, hydrolyzed steam-exploded Douglas-fir containing approx 44% lignin to >76% cellulose conversion at a relatively low enzyme loading (10 FPU/g of cellulose). It should also be noted that all softwood hydrolysis experiments described herein were performed at 45°C, the optimum for *Trichoderma* cellulase preparations, but that the optimum for MSUBC1, derived from *Penicillium* sp., is 52–55°C (data not shown). Therefore, we can anticipate >80% cellulose conversion of cellulose in steam-exploded Douglas-fir using longer hydrolysis times or higher enzyme loading at optimum temperature. Typically, steam-exploded softwood shows poor hydrolysis characteristics without further treatment to remove lignin, as illustrated in Fig. 1A,B, and the costs of delignification are currently the major drawback to softwood bioconversion schemes based on steam explosion.

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