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Evaluation of Cellulase Preparations for Hydrolysis of Hardwood Substrates

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Abstract

Seven cellulase preparations from *Penicillium* and *Trichoderma* spp. were evaluated for their ability to hydrolyze the cellulose fraction of hardwoods (yellow poplar and red maple) pretreated by organosolv extraction, as well as model cellulosic substrates such as filter paper. There was no significant correlation among hydrolytic performance on pretreated hardwood, based on glucose release, and filter paper activity. However, performance on pretreated hardwood showed significant correlations to the levels of endogenous β-glucosidase and xylanase activities in the cellulase preparation. Accordingly, differences in performance were reduced or eliminated following supplementation with a crude β-glucosidase preparation containing both activities. These results complement a previous investigation using softwoods pretreated by either organosolv extraction or steam explosion. Cellulase preparations that performed best on hardwood also showed superior performance on the softwood substrates.

Index Entries: Cellulase; xylanase; hemicellulose; lignocellulose; bioconversion.

Introduction

Concerns about diminishing resources, national energy security, and the excessive production of greenhouse gases continue to motivate the search for alternatives to petroleum. Lignocellulosic biomass contains large

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amounts of polymeric carbohydrates that represent an attractive source of sugars to produce alternative fuels and other chemical commodities. Potential feedstocks include agricultural residues such as corn stover, "purpose-grown" energy crops such as hybrid poplar, and hard- or softwood wastes from the forest industry.

One of the bioconversion schemes currently under active investigation involves enzymatic hydrolysis of the carbohydrate fraction, included largely of cellulose and hemicellulose, to produce glucose and other simple sugars for fermentation to fuel-grade ethanol. However, this approach is problematic because cellulose is inherently resistant to enzyme attack and because both cellulose and hemicellulose are protected by the surrounding matrix of lignin. Consequently, lignocellulosic biomass requires pretreatment to disrupt cellulose and lignin in order to improve enzyme accessibility.

Typically, pretreatment produces an enriched cellulose fraction containing residual hemicellulose and lignin, although the composition of pretreated material varies considerably, according to the type of feedstock, the pretreatment technology employed, and the process parameters that affect pretreatment severity. Various pretreatment technologies are now being optimized in attempts to produce appropriate substrates for hydrolysis at realistic cost *(1)*. Concurrently, enzyme manufacturers are investigating ways to reduce production costs and improve the specific activities of the enzyme complexes required for the hydrolysis of pretreated feedstocks *(2)*.

Efficient cellulose hydrolysis requires the concerted action of several endo- and exoglucanases *(3)*. These cellulases are prime targets in attempts to improve enzyme activity. A further strategy involves optimization of so-called "accessory enzymes" that hydrolyze the complex array of glycosidic bonds in hemicellulose. Efficient hemicellulose hydrolysis is important, not only for recovery of sugars from residual hemicellulose, but also because hemicellulose appears to hinder the access of cellulases to cellulose fibers. In some feedstocks, similar considerations may apply to residual pectin.

In previous research, we examined the hydrolysis of several softwood substrates by a panel of seven cellulase preparations in order to evaluate their hydrolytic performance *(4)*. Substrates were pretreated by SO_2-catalyzed steam explosion or ethanol organosolv extraction. We demonstrated that evaluation of enzyme performance using the target substrate is essential because the ability to hydrolyze model cellulosic substrates, such as filter paper, provides a poor estimate of activity on pretreated softwood. We also presented indirect evidence that the activity of a cellulase preparation is related to the endogenous levels of two activities: β-glucosidase (cellobiase) and xylanase. In this article, we present complementary data for two hardwood substrates prepared by organosolv pretreatment. This is relevant because hardwoods and softwoods contain different types of hemicellulose and lignin *(5,6)*, factors that may influence enzyme performance.

The flexibility to process a broad range of lignocellulosics would benefit commercial bioconversion process economics, so versatile cellulase preparations offer significant advantages.

Materials and Methods

Substrate and Pretreatment

Representative samples of yellow poplar (*Liriodendron tulipifera*), and red maple (*Acer rubra*), were collected in Eastern Canada. Samples were chipped to approx 2 × 2 × 0.5 cm after removal of bark, screened for uniformity, and equilibrated at 4°C in sealed plastic bags to approx 9% (w/w) moisture content before pretreatment.

Organosolv pretreatment of poplar and maple was carried out in a 1 L-stainless steel pressure reactor (Parr Instrument Co., Moline, IL) using 50% (w/w) ethanol, adjusted to pH 2.4 with 10% (v/v) sulfuric acid, at 195°C and approx 3.2 MPa (460 psi). The solvent:wood ratio was 7:1 (w:w). The pretreatment time was 40 min for both substrates. The time required to reach the target cooking temperature was approx 53 min, in all cases. After cooking, the reactor was quenched in ice until the inside temperature was ≤55°C and the spent liquor removed by decantation. The solids were homogenized for 5 min in 70% (v/v) ethanol at 70°C (solids: ethanol approx 9:1) in a British disintegrator (TMI, Montreal, Canada), then washed three times with 1 L of warm 70% ethanol and rinsed extensively with water. Pretreated solids were then separated by filtration and stored in sealed plastic bags at 4°C.

Chemical Analysis of Untreated and Pretreated Hardwoods

The carbohydrate composition and lignin content of untreated and pretreated hardwood samples was determined using a modified Klason lignin method derived from the TAPPI standard method T222 om-88, as previously described *(7)*. Monosaccharides were analyzed by HPLC with fucose as internal standard according to the procedure described elsewhere *(7)*.

Cellulase Preparations

Three commercial *Trichoderma reesei* cellulase preparations and four laboratory preparations produced by mutant strains of *Penicillium* spp. and *Trichoderma* spp. (*see* Table 2) were evaluated. In some assays, cellulase preparations were supplemented with Novozym 188 (Novozymes), a commercial β-glucosidase preparation from *Aspergillus niger* containing 340 cellobiose units (CBU)/mL, as described later.

Batch Hydrolysis of Pretreated Hardwood and Data Analysis

Hydrolysis experiments were performed in triplicate in 100-mL flasks, at 50°C and shaken at 250 rpm. The reaction mixture contained

0.1*M* acetate buffer, pH 5.0, 5% (w/v) substrate and 10 filter paper units (FPU) of cellulase activity per gram dry substrate, in a total volume of 10 mL. In experiments involving supplementation with Novozym 188, the FPU:CBU ratio was 1 : 2. Samples were taken at 1, 3, 6, and 12 h. Glucose concentrations were determined using the glucose oxidase-peroxidase method *(8)*.

Two indices, specific conversion (SC) and mean specific rate (MSR) were calculated to compare the various hydrolysis progress curves obtained for pretreated hardwoods, as described in the Results and Discussion.

Statistical Analysis

Statistical analyses were performed using Origin 6.0 software (Microcal Software, Inc.). Analyses of variance were performed using Origin's one-way ANOVA test.

Enzyme Assays

Enzymes activities on model substrates were performed as previously described *(4)*, according to the recommendations of the International Union of Pure and Applied Chemistry *(8,9)*. Xylanase activity was determined by monitoring the release of reducing sugars from birchwood xylan (Sigma) by the Somogyi-Nelson method *(10)*, as previously described *(4)*. β-glucanase activity was measured using the xylanase assay procedure, with barley β-glucan (Sigma) replacing xylan. Pectinase and mannanase activities were measured using polygalacturonic acid and galactomannan, respectively, as previously described *(11,12)*. The protein concentration in enzyme preparations was determined by the Lowry method *(13)*, following precipitation with trichloroacetic acid.

Results and Discussion

Carbohydrate and Lignin Composition of Pretreated Hardwoods

The compositions of untreated and pretreated hardwood samples (% dry weight) are shown in Table 1. Untreated poplar and maple contained approx 45% and 42% cellulose (assuming all glucose represents cellulose) respectively. The percentage of cellulose content increased to approx 80% and 72%, respectively, as a result of pretreatment, reflecting partial extraction of lignin and hemicellulose during organosolv extraction. The xylan content of both hardwood samples decreased following organosolv extraction whereas the mannan content increased, suggesting preferential extraction of xylan. Typical hardwood hemicelluloses have a high-xylan content and low-mannan content, relative to softwoods. However, the xylan and mannan contents of organosolv pretreated hardwoods (Table 1) and softwoods *(4)* show no particular trend, demonstrating that the hemicellulosic sugar content of pretreated lignocellulosic substrates does not necessarily reflect that of the untreated feedstock.

Table 1
Carbohydrate and Lignin Composition of Untreated and Pretreated Hardwoods (% From Dry Weight)

	Arabinan	Galactan	Glucan	Xylan	Mannan	Klason lignin	Acid-soluble lignin
Untreated yellow poplar	0.34 ± 0.06	0.40 ± 0.03	44.65 ± 0.99	17.06 ± 0.32	1.82 ± 0.07	21.14 ± 0.19	2.74 ± 0.06
Organosolv-pretreated yellow poplar	0	0.02 ± 0.00	79.57 ± 2.04	7.05 ± 0.17	2.52 ± 0.08	5.35 ± 0.02	1.32 ± 0.32
Untreated red maple	0.29 ± 0.02	0.60 ± 0.03	41.86 ± 0.49	6.22 ± 0.11	1.76 ± 0.10	28.13 ± 0.19	2.06 ± 0.09
Organosolv-pretreated red maple	0	0	72.15 ± 0.28	2.83 ± 0.03	2.01 ± 0.03	17.50 ± 0.32	0.95 ± 0.02

Activities of Cellulase Preparations on Model Carbohydrate Substrates

The protein content of the seven cellulase preparations, and their specific hydrolytic activities against a panel of model cellulosic and hemicellulosic substrates and related glycans, are shown in Table 2. The preparations showed similar specific filter paper activity (0.7–1.0 FPU/mg), CMCase activity (14.1–24.4 U/mg), and Avicelase activity (1.6–2.5 U/mg). However, the preparations demonstrated significant differences in their levels of specific β-glucosidase activity (0.15–1.16 U/mg) and in their levels of xylanase, mannanase, and pectinase activities. All preparations contained similar levels of β-glucanase activity.

Analysis of Hydrolysis of Pretreated Hardwood Substrates

Two indices, MSR and SC, were used to evaluate the hydrolysis of cellulose by the various preparations for the two pretreated hardwood substrates, as previously described for softwood *(4)*. The MSR index (g glucose/L/h/mg) estimates the average rate of cellulose hydrolysis during the first 12 h of hydrolysis, normalized for total protein. The method of calculation, involving curve triangulation is illustrated in Fig. 1A. To validate this method, all hydrolytic progress curves were fitted to an arbitrary hyperbolic function ($G = [k_1 t]/[k_2 + t]$; where G = glucose concentration (g/L), t = time (h), and k_1 and k_2 are constants). Regression analysis showed that this function produced a good fit to all hardwood hydrolysis data described below ($\chi^2 \leq 0.015$; $r^2 = 0.99$). Analysis of variance (Fig. 1B) was then used to demonstrate that MSR values calculated by curve triangulation did not differ significantly from values calculated using the first derivative of the fitted curves (F = 0.02–0.16; p = 0.67–0.95). The SC index (%/mg) describes the % of total cellulose in the sample hydrolyzed to glucose in the 12 h incubation period, normalized for total protein.

Activities of Cellulase Preparations on Pretreated Hardwood Substrates

Data for the hydrolysis of pretreated poplar and maple by the seven different cellulase preparations are shown in Figs. 2A and 3A, respectively. Analysis of these data (Table 3) shows that the rate (MSR) and extent (SC) of cellulose hydrolysis by MSUBC1 (*Penicillium* sp. cellulase preparation, *see* Table 2) were significantly greater than seen for the other preparations tested, as previously reported for a panel of pretreated softwood substrates *(4)*. Also, as previously reported for softwood substrates, the MSR and SC indices for all the cellulase preparations show a poor correlation with activities determined using filter paper ($r_{MSR} = 0.428$, $p = 0.127$; $r_{SC} = 0.508$, $p = 0.06$), CMC ($r_{MSR} = -0.203$, $p = 0.486$; $r_{SC} = -0.124$, $p = 0.674$) or Avicel ($r_{MSR} = -0.462$, $p = 0.097$; $r_{SC} = -0.517$, $p = 0.059$). This result emphasizes the previous conclusion *(4)* that filter paper activity does not provide a reliable prediction

Table 2
Enzyme Activities in Cellulase Preparations

Cellulase preparation and source	Protein concentration[a]	Cellulase activities (U/mg protein)			Other enzyme activities (U/mg protein)				
		FPA	CMC	Avicelase	β-Glucosidase	β-Glucanase	Xylanase	Pectinase	Mannanase
Laboratory preparations									
MSUBC1 (*Penicillium* spp.)	838	0.9	16.9	2	1.16	17	39.1	0.64	0.3
MSUBC2 (*Trichoderma* spp.)	556	0.7	19.2	2.4	0.16	14.6	9.3	0.25	0.26
MSUBC3 (*Trichoderma* spp.)	771	0.8	17.7	1.9	0.15	15.6	3.8	1.56	0.18
MSUBC4 (*Trichoderma* spp.)	460	0.8	24.4	2.5	0.2	12.2	6.1	1.07	0.43
Commercial preparations									
TR1 (*Trichoderma* spp.)	129	0.9	14.1	2.2	0.19	16.8	3.5	0.07	0.11
TR2 (*Trichoderma* spp.)	130	1	20.7	1.6	0.66	19.2	11.8	0.05	0.06
TR3 (*Trichoderma* spp.)	149	0.9	21.6	2.5	0.28	14.2	13.1	0.03	0.03

[a]Protein concentration, — mg/g, except TR1 and TR2 (mg/mL)

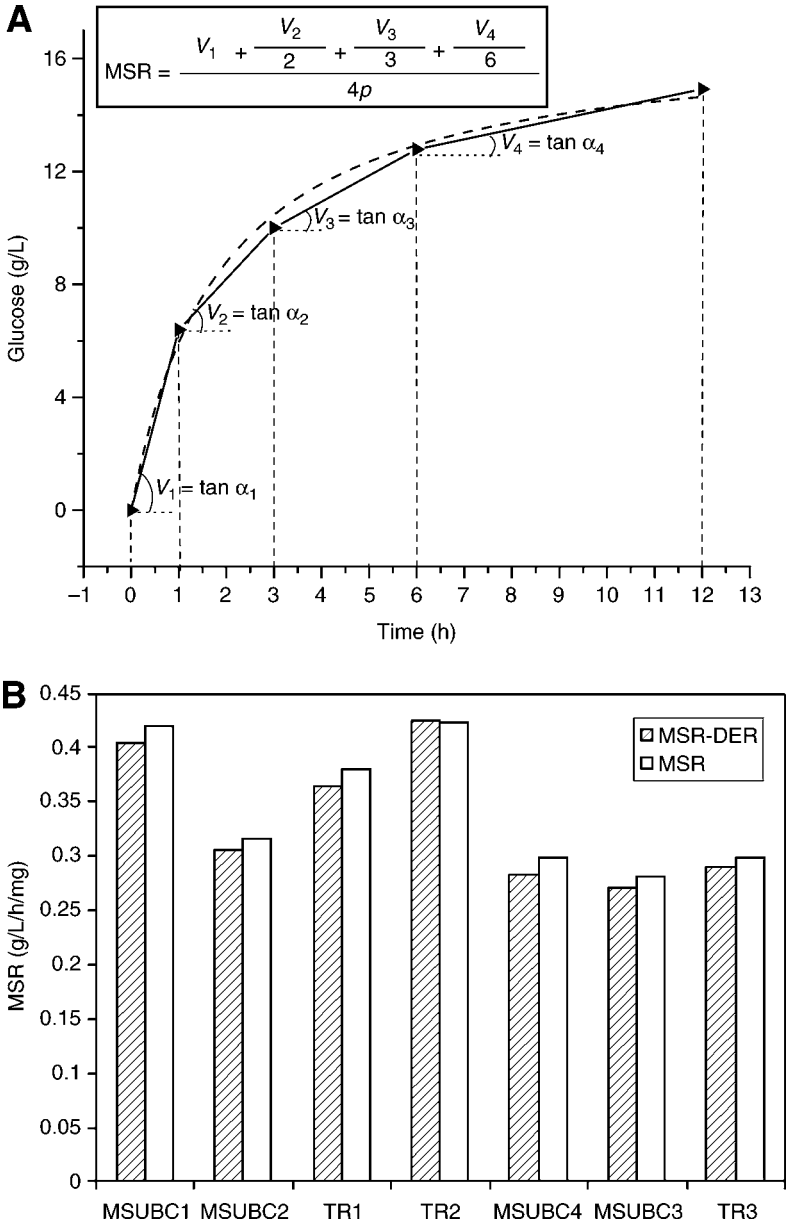

Fig. 1. Calculation of the MSR of hydrolysis by curve triangulation. **(A)** Hydrolysis of organosolv-pretreated poplar by the MSUBC1 cellulase with exogenous β-glucosidase supplementation is illustrated as an example. *p*—total protein loaded (mg) and **(B)** one-way ANOVA. MSR-DER calculated from the first derivative of the fitting function; MSR-calculated by curve triangulation.

of the ability of a cellulase preparation to hydrolyze cellulose into glucose in complex lignocellulosic substrates, despite suggestions to the contrary *(14)*.

However, linear regression analysis revealed a significant correlation ($r \geq 0.80$, $p < 0.0001$) between the efficiency of cellulose hydrolysis (MSR

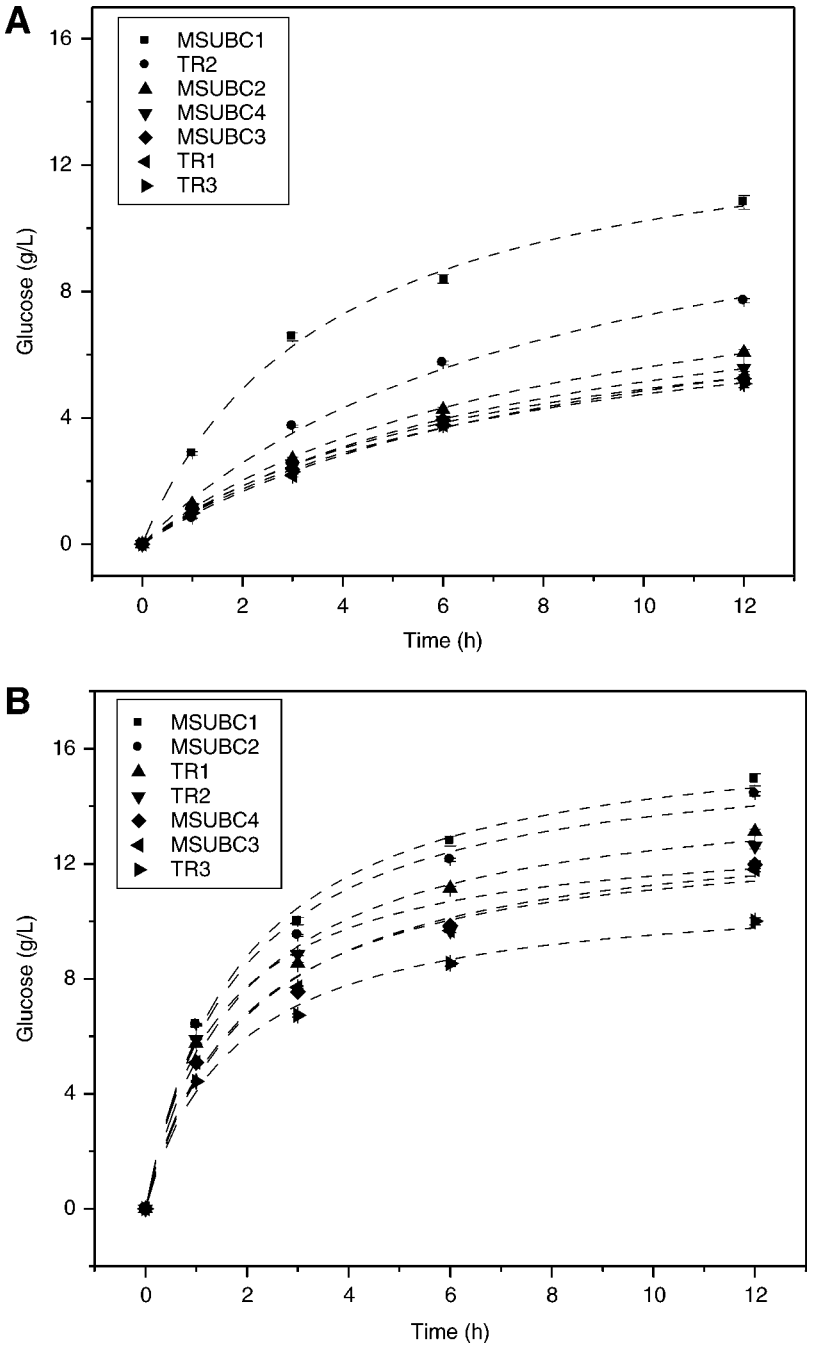

Fig. 2. Hydrolysis of organosolv-pretreated poplar by cellulase preparations. **(A)** Without β-glucosidase supplementation and **(B)** with β-glucosidase supplementation (FPU:CBU 1:2). Dashed lines are fitted curves.

or SC) in pretreated hardwood and the levels of endogenous β-glucosidase and xylanase activities (Figs. 4A, 5A, 6A, and 7A). This result provides indirect evidence for the hypothesis that differences in the endogenous levels of

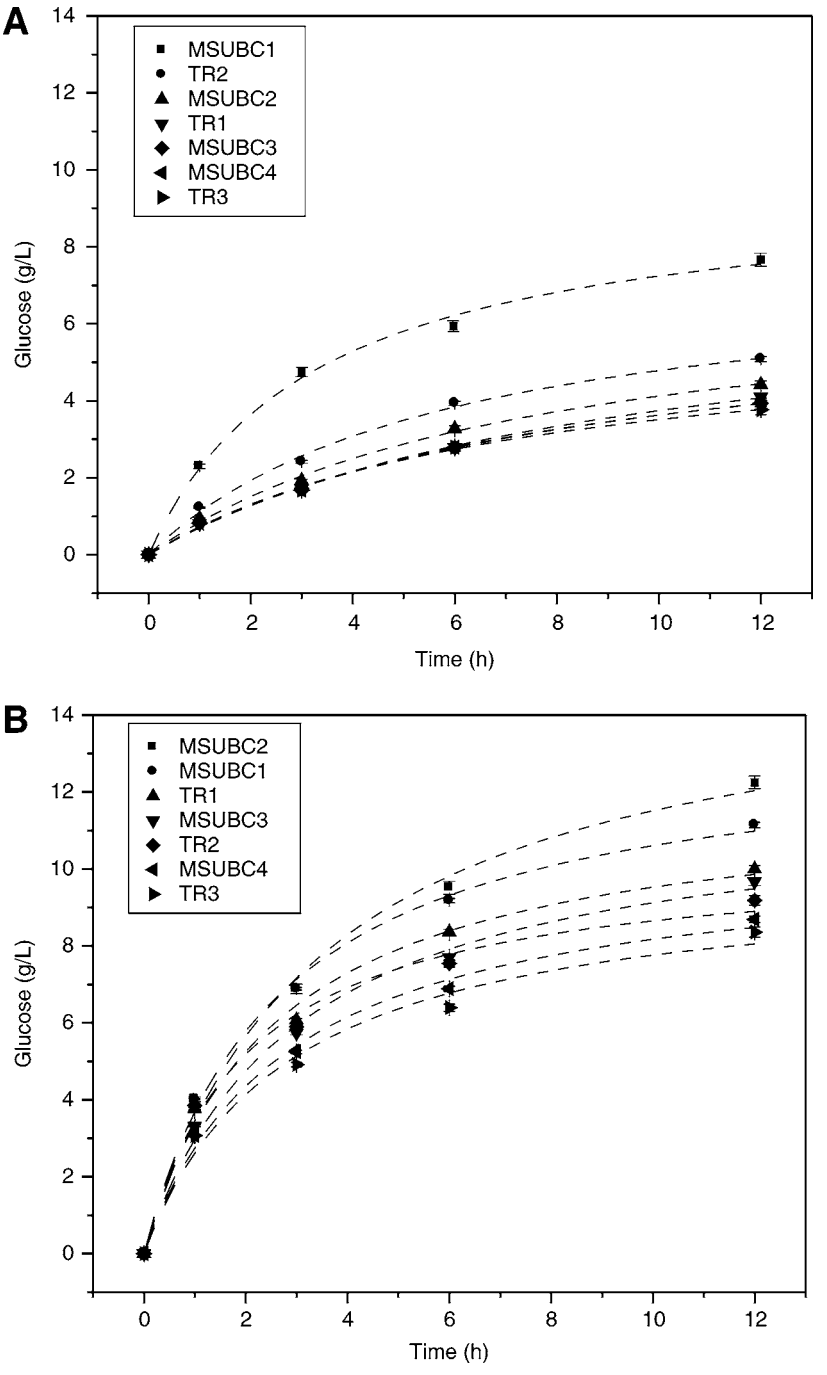

Fig. 3. Hydrolysis of organosolv-pretreated maple by cellulase preparations. **(A)** Without β-glucosidase supplementation and **(B)** with β-glucosidase supplementation (FPU:CBU—1:2). Dashed lines are fitted curves.

these two activities are at least partially responsible for the differences in cellulase performance seen on hardwood substrates, as also reported for softwoods *(4)*. This evidence is supported by the demonstration (Figs. 2B and 3B)

Table 3
Mean Specific Rates (MSR) and SCs for Hydrolysis of Hardwood Samples by Cellulase Preparations With and Without Exogenous β-Glucosidase (β-G) Supplementation

Enzyme	Protein load per assay (mg)	Organosolv-pretreated yellow poplar		Organosolv-pretreated yellow poplar with exogenous β-G		Organosolv-pretreated red maple		Organosolv-pretreated red maple with exogenous β-G	
		MSR[a]	SC[b]	MSR[a]	SC[b]	MSR[a]	SC[b]	MSR[a]	SC[b]
MSUBC1	5.66	0.25	4.32 (24.48)	0.42	5.96 (33.75)	0.19	3.4 (19.26)	0.29	4.22 (30.74)
MSUBC2	7.28	0.1	1.89 (13.72)	0.32	4.48 (32.63)	0.07	1.52 (11.07)	0.23	4.56 (25)
MSUBC3	6.57	0.09	1.81 (11.87)	0.28	4.06 (26.7)	0.07	1.5 (9.84)	0.21	4.76 (22.95)
MSUBC4	6.22	0.1	2.03 (12.61)	0.3	4.35 (27.07)	0.07	1.79 (9.84)	0.2	3.49 (21.72)
TR1	5.48	0.11	2.17 (11.87)	0.38	5.41 (29.67)	0.08	1.65 (10.25)	0.27	3.68 (24.18)
TR2	4.82	0.17	3.62 (17.43)	0.42	5.92 (28.56)	0.13	2.64 (12.71)	0.3	4.92 (27.87)
TR3	5.39	0.11	2.13 (11.5)	0.3	4.20 (22.62)	0.08	1.75 (9.43)	0.22	3.88 (20.9)

[a]Average rate of cellulose hydrolysis from 0 to12 h/mg protein (g glucose/L/h/mg).
[b]Percentage of total cellulose converted to glucose in 12 h/mg protein (%/mg); in parentheses: cellulose conversion (%) from 0 to 12 h.
[c]β-G (Novozym 188—a β-glucosidase preparation from *Aspergillus niger)*.

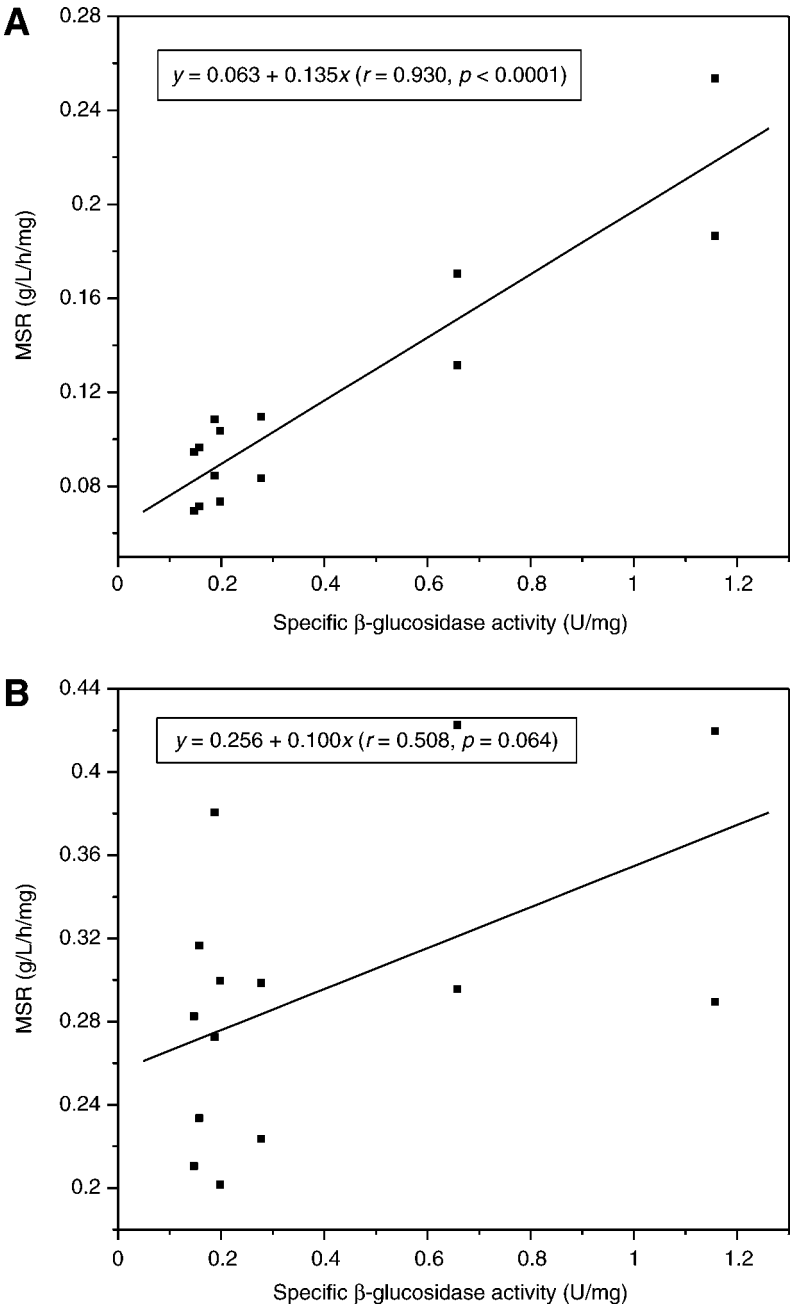

Fig. 4. Regression analyses of MSR for hydrolysis of hardwood samples vs specific β-glucosidase activity without **(A)** and with **(B)** β-glucosidase supplementation (FPU:CBU—1:2). MSR—mean specific rate.

that differences in the efficiencies of cellulose hydrolysis by the various cellulase preparations on hardwood substrates were reduced or eliminated after supplementation with Novozym 188, a commercial β-glucosidase

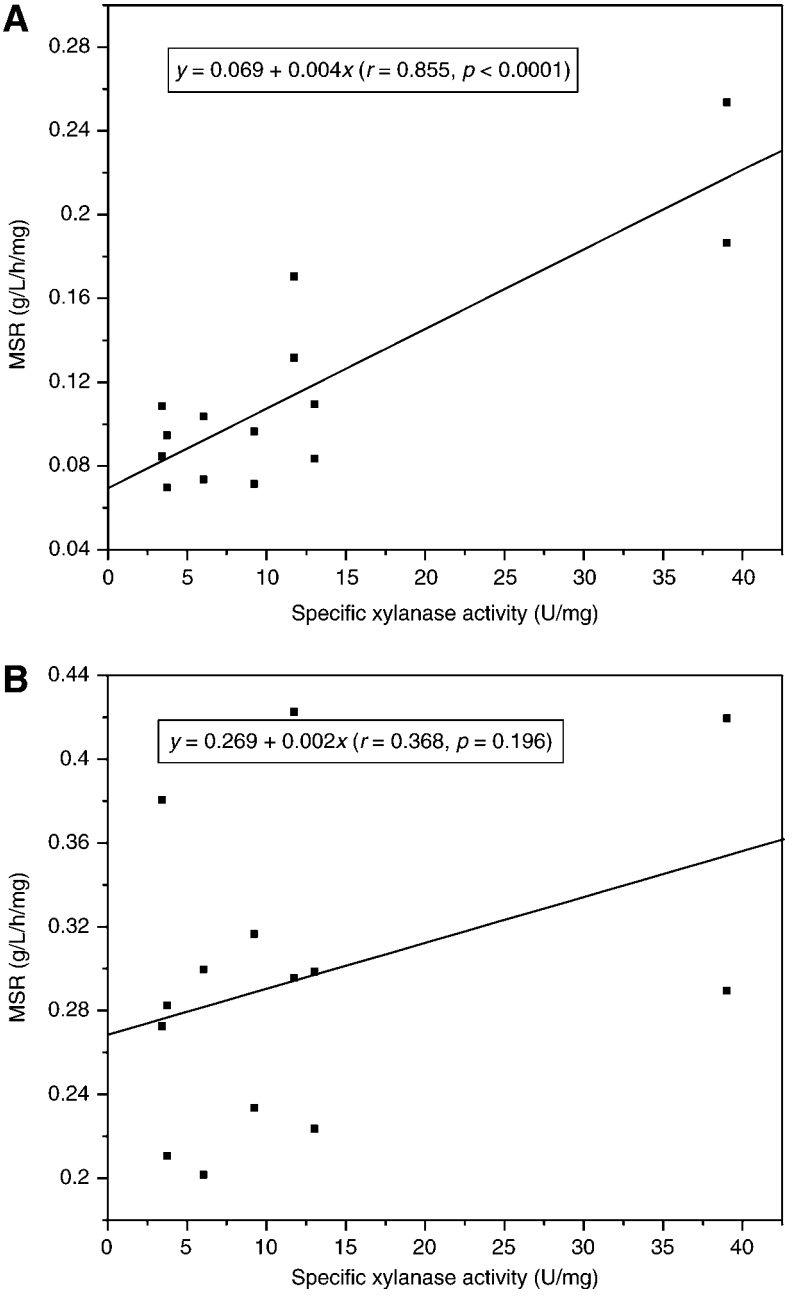

Fig. 5. Regression analyses of MSR for hydrolysis of hardwood samples vs specific xylanase activity without **(A)** and with **(B)** β-glucosidase supplementation (FPU: CBU—1:2). MSR—mean specific rate.

preparation commonly used to improve cellulase performance. Consequently, the correlation between cellulose hydrolysis in pretreated hardwood and level of endogenous β-glucosidase or xylanase activity was markedly reduced following supplementation (Figs. 4B and 6B). It should

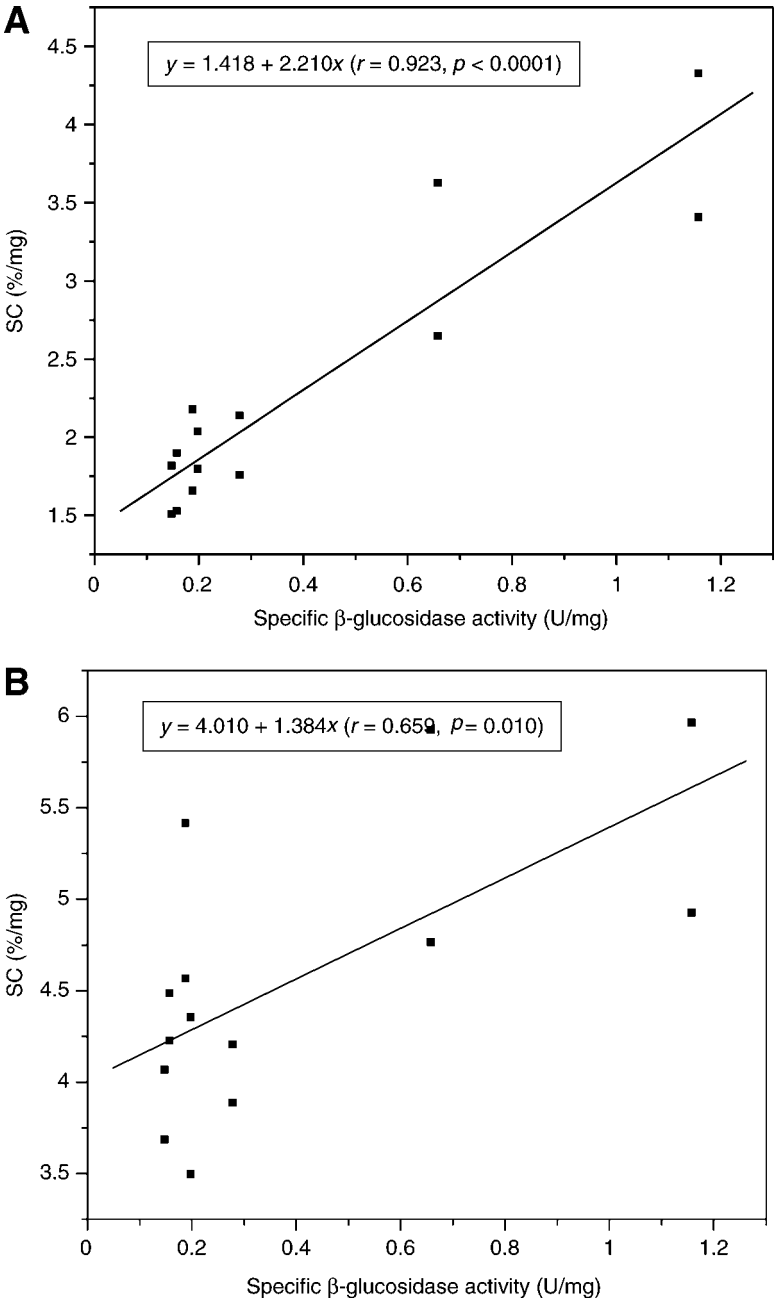

Fig. 6. Regression analyses of SC for hydrolysis of hardwood samples vs specific β-glucosidase activity without **(A)** and with **(B)** β-glucosidase supplementation (FPU: CBU—1:2). SC—specific conversion.

be noted that Novozym 188 contains significant xylanase activity (0.58 U/mg protein, based on hydrolysis of birchwood xylan) *(4)*. Therefore, it appears that deficiencies in the levels of both activities are compensated

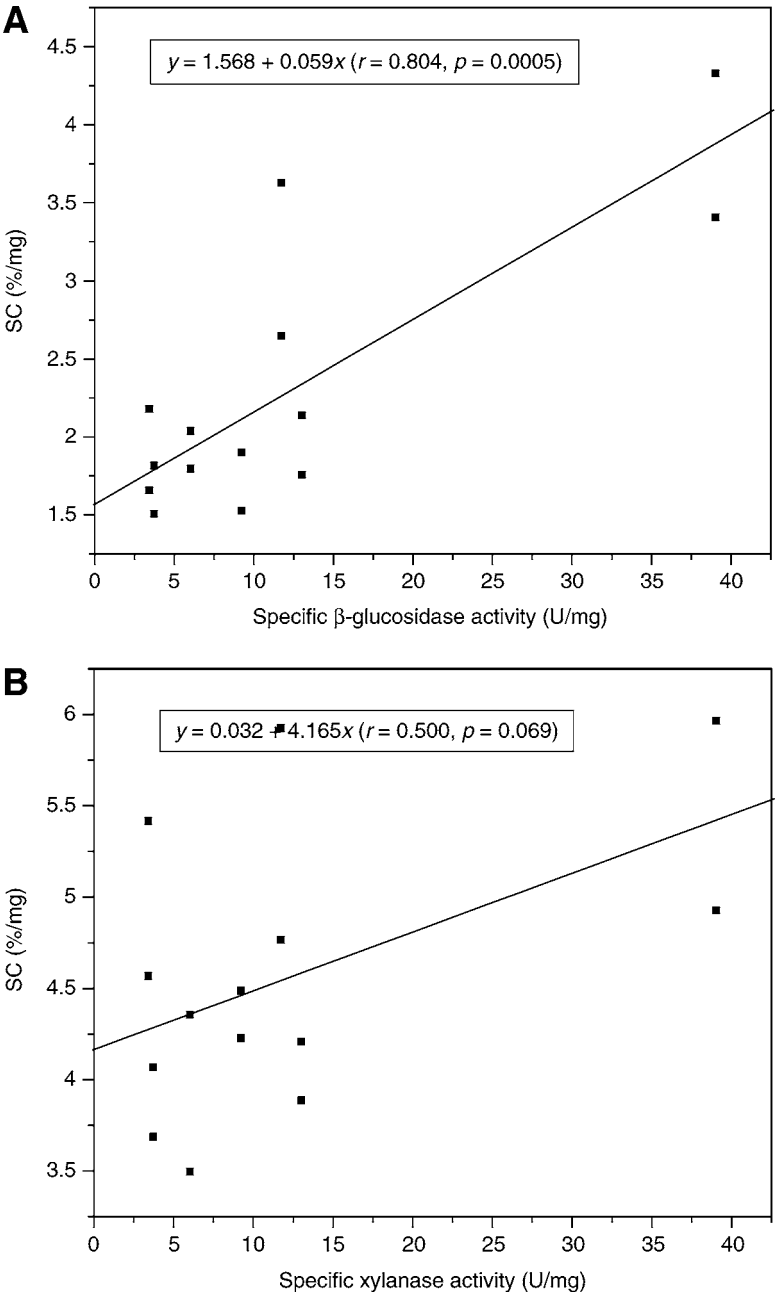

Fig. 7. Regression analyses of SC for hydrolysis of hardwood samples vs specific xylanase activity without **(A)** and with **(B)** β-glucosidase supplementation (FPU: CBU—1:2). SC—specific conversion.

by supplementation with corresponding activities present in the β-glucosidase preparation during the hydrolysis of both softwood and hardwood substrates. Further experiments using defined enzymes are required to determine the relative importance of these two activities.

The role of β-glucosidase in relieving end product inhibition caused by accumulation of cellobiose is well documented *(15)*. Presumably, xylanases improve cellulose hydrolysis by removing hemicellulose on fiber surfaces, thereby increasing the accessibility of cellulose to cellulases. Although the xylan content of pretreated hardwood samples is low (≤ 7.1%; Table 1), it is probable that a fraction of hemicellulose is solubilized during pretreatment and redeposited on fiber surfaces during the late stages of pretreatment, as in kraft pulping *(16)*; consequently, steric hindrance owing to hemicellulose may be significant. Xylanases may also increase cellulose accessibility indirectly by facilitating lignin removal *(16,17)*. In contrast to xylanase, no significant correlation was seen between cellulose hydrolysis in pretreated hardwoods and levels of endogenous mannanase ($r_{MSR} = 0.064$, $p = 0.827$; $r_{SC} = 0.030$, $p = 0.918$), pectinase ($r_{MSR} = –0.227$, $p = 0.435$; $r_{SC} = –0.280$, $p = 0.333$) or β-glucanase activity ($r_{MSR} = 0.536$, $p = 0.046$; $r_{SC} = 0.577$, $p = 0.031$). Mannans are not major components of hardwood hemicelluloses, in contrast to softwoods *(6)*; however, a similar lack of correlation between endogenous mannanase activity and cellulose hydrolysis was also reported for softwood substrates *(4)*. These results suggest that the residual mannan does not significantly restrict access to cellulose in pretreated woody substrates, or that mannan hydrolysis is limited by other factors. It is noted that significant differences in the rate and extent of cellulose hydrolysis by the various cellulase preparations remain after supplementation, and that supplementation of cellulase preparations with Novozym 188 did not improve the correlation between cellulase hydrolysis in hardwood substrates and hydrolysis of filter paper ($r_{MSR} = 0.465$, $p = 0.094$; $r_{SC} = 0.373$, $p = 0.189$), CMCase ($r_{MSR} = –0.290$, $p = 0.315$; $r_{SC} = –0.260$, $p = 0.370$) or Avicelase ($r_{MSR} = –0.420$, $p = 0.135$; $r_{SC} = –0.571$, $p = 0.033$) indicating that the various preparations are distinguished by additional differences in enzyme properties.

These results are relevant to current attempts to reduce the cost of cellulase preparations for bioconversion of lignocellulosic substrates. First, they demonstrate that discovery of novel enzyme complexes, coupled with mutagenesis, is a viable empirical method to significantly improve cellulase activity on lignocellulosic substrates. The MSUBC strains used to provide cellulase preparations in this study were derived by reiterative strain selection and random mutagenesis *(18)*. Screening for improved enzyme complexes should involve the target substrate, because activity on filter paper, or other model cellulosic substrates, provides a poor indication of the ability to hydrolyze cellulose in lignocellulose. Secondly, they support the concept that further improvements in performance can be achieved by supplementation of cellulase preparations with accessory enzymes, such as xylanases, that facilitate the removal of noncellulosic components.

The results presented here for hardwoods, and previously for softwoods *(4)*, provide no indication that the improvements in cellulose hydrolysis produced by both these approaches are restricted to particular

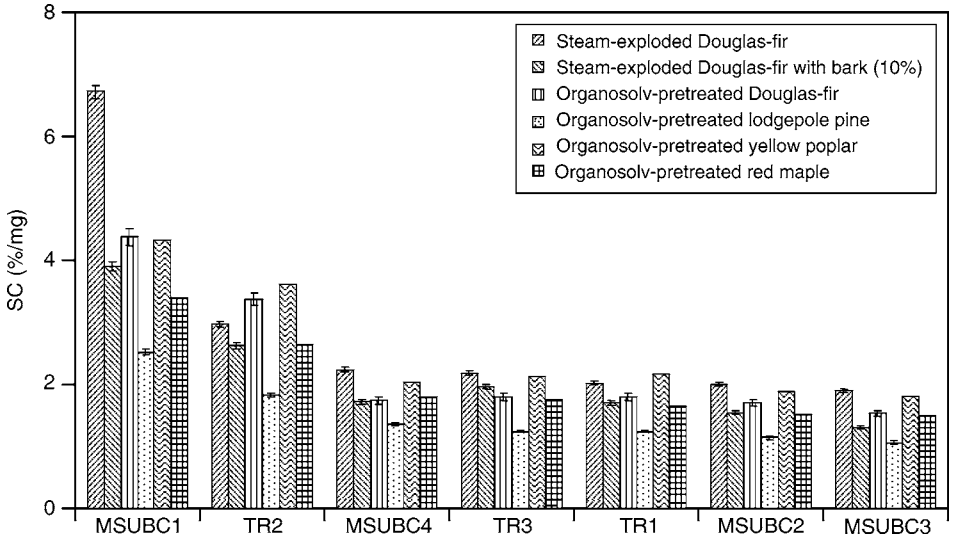

Fig. 8. Comparison of enzyme performance (SC) on a range of substrates produced by SO_2-catalyzed steam explosion or organosolv pretreatment of hardwoods and softwoods.

classes of substrates or pretreatment methodologies: MSUBC1 and TR2 show superior performance on all substrates examined so far (Fig. 8), although further experiments using a broader range of substrates are required to substantiate any conclusion. Robust enzyme preparations (i.e., those that perform well on a broad range of substrates) should simplify enzyme production and biomass conversion processes and reduce costs, and the flexibility to process a range of feedstocks would mitigate potential problems with feedstock supply that may arise from reliance on one feedstock. Nevertheless, it is reasonable to expect that incremental increases in hydrolytic performance could be achieved by systematically fine tuning the composition of enzyme complexes for particular substrates; for example, by addition of further enzymes to existing cellulase preparations to produce specific "cocktails." If all relevant enzymes are assumed to have approximately equal production costs (and stabilities), a simplistic model suggests that this strategy is cost effective only when the improvement, per unit weight of protein added, exceeds that achieved by simply increasing the loading of unsupplemented cellulase preparation. Similarly, it is still unclear whether use of enzyme supplementation to improve the performance of cellulase preparations such as TR2 offers any economic advantage over use of unsupplemented preparations like MSUBC1 (Table 3).

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