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Synergism in Binary Mixtures of *Thermobifida fusca* Cellulases Cel6B, Cel9A, and Cel5A on BMCC and Avicel

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Abstract

In an earlier binding study conducted in our laboratory using *Thermobifida fusca* cellulases Cel6B, Cel9A, and Cel5A (formally *Thermomonospora fusca* E_3, E_4, and E_5), it was observed that binding capacities for these three cellulases were 18–30 times higher on BMCC than on Avicel. These results stimulated an interest in how the difference in accessibility between the two cellulosic substrates would affect synergism observed with cellulase mixtures. To explore the impact of substrate accessibility on the extent of conversion and synergism, three binary *T. fusca* cellulase mixtures were tested over a range of cellulase ratios and total molar cellulase concentrations on Avicel and BMCC. Higher extents of conversion were observed for BMCC due to the higher enzyme to substrate ratio resulting from the higher binding. The processive endoglucanase, Cel9A, had four times the extent of conversion of the endocellulase Cel5A, while the exocellulase Cel6B had three times the extent of conversion of Cel5A. Approximately 500 nmol/g of the Cel9A+Cel6B mixture was needed to obtain 80% conversion, while the Cel6B+Cel5A and Cel9A+Cel5A mixtures required 1500 and 1250 nmol/g, respectively, to obtain 80% conversion. Thus, it appears that the more accessible structure of BMCC, as reflected by its binding capacity, results in relative higher processive activity.

Index Entries: Cellulases; *Thermobifida fusca*; synergism; Avicel; BMCC.

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Introduction

It has been known for some time that a cellulase's accessibility to the cellulose surface is a rate-determining factor in the enzymatic hydrolysis of cellulose *(1–6)*. Cellulases must diffuse into pores large enough to accommodate them, and bind to the cellulose molecule to catalyze the hydrolysis reaction *(2)*. Accessibility is generally defined as the surface area available for cellulase binding and catalysis, and the amount of surface area is a function of the molecular diameter of the cellulase *(1)*. Cellulase binding studies have revealed order of magnitude differences in binding capacities (moles of cellulase per gram of cellulose) between different cellulosic substrates *(7–9)*. These studies have also revealed a factor of 2–5 difference in binding capacity of a given cellulose for several difference cellulases *(7–9)*.

In an earlier binding study conducted in our laboratory using *Thermobifida fusca* cellulases Cel6B (cellulase family 6B), Cel9A, and Cel5A (formally *Thermomonospora fusca* E_3, E_4, and E_5), it was observed that the binding capacities for these three cellulases were 18–30 times higher on bacterial microcrystalline cellulose (BMCC) than on Avicel *(9)*. In addition, there was much less variation observed in the BMCC binding capacities for the three cellulases relative to the Avicel binding capacities. The coefficient of variances of the BMCC binding capacities was 11%, while for Avicel the coefficient of variance was 37%. From these results it was concluded that these three cellulases had similar levels of accessibility to BMCC and varying levels of accessibility to Avicel.

These results stimulated an interest in how the difference in accessibility observed between the two cellulosic substrates would affect the synergism of cellulase mixtures. One might hypothesize that a substrate that provides roughly equal accessibility to the cellulases would give rise to a higher degree of synergistic effect (DSE) than a substrate that exhibits a high degree of variation in accessibility based on the size of the molecule. Implicit in this hypothesis is the assumption that synergism can only occur on a surface that is accessible to all of the reactants participating in the synergistic reaction. To test this hypothesis, binary mixtures of *T. fusca* Cel6B+Cel5A, Cel9A+Cel5A, and Cel6B+Cel9A were tested over a range of cellulase ratios and total molar cellulase concentrations, $[E]_t$, on Avicel and BMCC. The influence of total cellulase concentration and cellulase mole fraction on the extent of conversion and synergism was measured and compared for Avicel and BMCC, respectively.

Materials and Methods

Growth of Streptomyces lividans *Cultures*

A strain of *Streptomyces lividans* containing the *T. fusca* cellulase Cel6B gene (S121) was grown for 48 h at 30°C in tryptic soy broth media (DIFCO Laboratories, Detroit, MI) with 5 µg/mL thiostreptone (Sigma Chemical Co., St. Louis, MO) in two 10 L fermentors. A 20 mL initial culture grown

for 3 d at 30°C was used to start a 200 mL culture, which was grown overnight and used to inoculate a 10 L fermentor. Anti-foam reagent, MAZU (PPG Industries, Gurnee, IL), was used and the pH was maintained at 7.0–7.3 throughout the incubation. After 48 h, the culture was harvested and centrifuged using a Sharples refrigerated centrifuge. Phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO), final concentration 0.1 m*M*, and ammonium sulfate, final concentration 1.2 *M*, were added to the supernatant. The supernatant was filtered using crossflow filtration on a Millipore Pellicon cassette (Millipore Products Division, Bedford, MA) with a 0.45 µm membrane. Strains producing Cel9A (S130) and Cel5A (pGG74) were grown and harvested under the same conditions.

Purification of Cellulases

Purification of Cel6B

All purification procedures were done at 4°C. Cel6B was purified as described in Zhang et al. *(10)* with the following changes. The concentration of ammonium sulfate added to the supernatant was 1.2 *M*. After the supernatant was loaded on the phenyl Sepharose column (Pharmacia Biotech Inc., Piscataway, NJ), 0.8 *M* ammonium sulfate was used in the initial wash of the column, and an affinity column was not used. Fractions from the phenyl Sepharose column were assayed for carboxymethyl-cellulose (CMC) and swollen cellulase activity and only the fractions with high swollen cellulose and very low CMC activity were combined for the Q Sepharose column in order to avoid the CMCase contaminant that Zhang et al. *(10)* found. Ten percent glycerol was added to the combined fractions and to all buffers for the Q Sepharose column (Pharmacia Biotech Inc., Piscataway, NJ). The Q Sepharose column was equilibrated with 0.01 *M* Bis Tris pH 5.2 and Cel6B was eluted with the same buffers as Zhang et al. *(10)* at pH 5.2. A native gel with a CMC overlay was run on the purified Cel6B, and no contaminating CMCase was detectable (data not shown). Cel6B was at least 95% pure based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and a native gel with a CMC overlay.

Purification of Cel9A

Cel9A was purified in the same way as Cel6B. Cel9A was determined to be 95% pure based on SDS-PAGE.

Purification of Cel5A

Cel5A was purified in three steps, a phenyl Sepharose column, a hydroxylapatite column, and a Q Sepharose column as described by Bothwell et al. *(9)* Cel5A was at least 95% pure based on SDS-PAGE.

Quantification of Individual Cellulases

The concentration of individual *T. fusca* cellulases was determined by absorbance readings (Spectronic 1001, Bausch and Lomb) at 280 nm and the extinction coefficients for each cellulase *(14)*.

Reducing Sugar Assay

Dinitrosalacylic acid reagent (DNS) was used to determine the concentration of reducing sugar, as described by Walker et al. *(11)*. Particulate-free supernatant was used to obtain soluble reducing sugar measurements, while a sample including the cellulose particles was used to determine total reducing sugar.

Trinder Assay to Measure Glucose

The Sigma Diagnostic Glucose (Trinder) Assay (Sigma, St. Louis, MO) was modified to measure micromole glucose in reactor samples containing glucose and cellobiose. Instead of reconstituting the dry Trinder reagent in 100 mL of distilled water and using 1 mL reagent with a 5 µL sample to test for glucose, the dry reagent was reconstituted in 90 mL distilled water. Nine-hundred microliters of reagent were added to a 100 µL diluted sample *(12)*.

Thin Layer Chromatography to Detect Oligosaccharides

Thin layer chromatography using 10 µL samples was performed as described in Jung et al. *(13)*.

Substrate and Buffer Preparation for Hydrolysis Experiments

Avicel reactions were run in 2 mL microcentrifuge tubes with O-ringed screw caps (Laboratory Product Sales, Inc., Rochester, NY) using 1 mL working volume. Avicel PH 102 (FMC, Corp., Philadelphia, PA) was mixed with 50 n*M* sodium acetate, pH 5.5, for a final concentration of 4% w/v (40 mg/mL). The buffer contained 0.04 % sodium azide (Sigma Chemical Co., St. Louis MO). Before cellulases were added, the buffer–substrate solutions were pre-incubated in a 50°C incubator (VWR Scientific Inc., Philadelphia, PA) for 1 h.

Bacterial microcrystalline cellulose (Cellulon, Weyerhaeuser, WA) in a moist cake form was weighed out, washed with distilled water, and filtered according to the directions given by Weyerhaeuser. The washed and fully expanded BMCC was then resuspended in distilled water and 0.04 % sodium azide to make a BMCC stock solution that was 7.71 mg/mL. The BMCC reactions were run in 2 mL microcentrifuge tubes with O-ringed screw caps and a 1 mL reaction volume. The final concentration was 0.1 % w/v (1 mg/mL). BMCC in 50 m*M* sodium acetate, pH 5.5, containing 0.04% sodium azide. The buffer–substrate solutions were preincubated at 50°C for 1 h.

Hydrolysis Experiment with Individual Components

For single enzyme reactions on Avicel, the total enzyme concentrations were 0.68–12.92 nmol/mL, which represents 17–323 nmol enzyme per gram cellulose. These concentrations are all below binding saturation for Avicel *(9)*. For single enzyme reactions on BMCC, the total enzyme concentrations were 0.25–9.22 nmol/mL, which represents 250–9220 nmol enzyme per gram cellulose. These concentrations are also all below satura-

tion for BMCC *(9)*. Individual cellulases were tested at higher concentrations than mixtures, since single cellulases have a much lower activity than synergistic mixtures. Microcentrifuge tubes containing the cellulose and cellulase mixtures were placed in a 50°C incubator and rotated end-over-end for 16 h (Tube Rotator, Scientific Equipment Products Division of J.A.G. Industries, Inc., Baltimore, MD). The reactors were then stored at –20°C for reducing sugar assays. Each reaction was run in triplicate *(12)*.

Hydrolysis Experiments with Mixtures

All total molar cellulase concentrations ($[E]_t$) were below half saturation on both Avicel and BMCC. Five different total molar concentrations were used in these experiments ranging from 0.68 to 6.80 nmol/mL and from 0.25 to 2.44 nmol/mL for Avicel and BMC, respectively. Mole fractions ($Cel5A/[E]_t$) of 0.1, 0.2, 0.4, and 0.9 were used for the Cel6B+Cel5A and Cel9A+Cel5A mixtures, and mole fractions ($Cel6B/[E]_t$) of 0.25, 0.50, and 0.75 were used for the Cel6B+Cel9A mixtures. Reactors were incubated at 50°C and rotated end-over-end for 16 h *(12)*.

Quantification of Soluble Reducing Sugar Concentrations

Samples removed from the reactors were centrifuged at 13,600*g* for 3 min. The supernatant was removed and placed into fresh microcentrifuge tubes and centrifuged again at 13,600*g* for 3 min. These particulate-free supernatants were transferred into new microcentrifuge tubes. To obtain particulate-free samples from the BMCC reactors, 500 µL samples were placed in 0.45 µm cellulose acetate filter microcentrifuge tubes (Laboratory Product Sales, Inc., Rochester, NY) and centrifuged for 1 min *(12)*.

Duplicate samples of the supernatant from each reactor were placed in 13 × 100 mm test tubes. The total sample volume was brought to 400 µL by the addition of 50 m*M* sodium acetate. Soluble reducing sugars were measured by adding 1 mL DNS solution to each test tube and boiling the samples for 15 min. After cooling to room temperature, the absorbance of the samples at 600 nm was measured. For single enzymes on BMCC, 400 µL of sample were needed for the DNS assay, so single DNS measurements of soluble and total reducing sugar were made for each reactor.

Results and Discussion

Enzyme Activity

Listed in Table 1 are the specific CMC, FP, and SC activities for the three cellulases. Specific activities were similar to those reported in Irwin et al. *(14)*.

Products of Hydrolysis

The thin layer chromatograph (see Fig. 1) showed glucose and cellobiose were produced in noticeable quantities by Cel9A, Cel5A, and mixtures of Cel9A and Cel5A. Trinder assay indicated that glucose accounted

Table 1
Cellulase Physical Properties and Hydrolytic Characteristics[a]

T. fusca Cellulase	MW (kD)	pH range	Temperature	Catalytic domain family	Mode of attack	CMC activity $\left(\frac{\mu mol}{min \mu mol}\right)$	SC activity $\left(\frac{\mu mol}{min \mu mol}\right)$	FP activity $\left(\frac{\mu mol}{min \mu mol}\right)$
Cel6B	59.6	6.0–9.0	50°C	6	Exo	0.3[b]	3.9	0.13[b]
Cel9A	90.2	6.0–9.0	50°C	9	Exo	647.0	183.0	0.90[b]
Cel5A	46.3	6.0–9.0	50°C	5	Endo	2840.0	90.0	0.83

[a]References *10*, *11*, and *14*. Units of activity are µmol cellobiose/min-µmol enzyme.
[b]Activity calculated used digestion achieved by 0.6 nmol enzyme in 16 h, since 5.2% digestion was not achieved *(14)*.

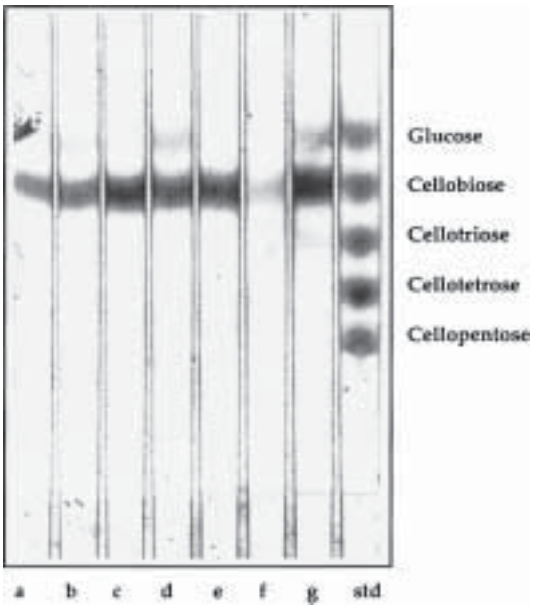

Fig. 1. Thin layer chromatography of products after 16 h of hydrolysis: **(A)** Cel9A on Avicel; **(B)** Cel9A on BMCC; **(C)** Cel6B+Cel9A ($[Cel6B]/[E]_t = 0.50$) on BMCC; **(D)** Cel9A+Cel5A ($[Cel5A]/[E]_t = 0.20$) on BMCC; **(E)** Cel6B+Cel5A ($[Cel5A]/[E]_t = 0.20$) on BMCC; **(F)** Cel5A on BMCC; **(G)** Cel5A on Avicel.

for 28, 18, and 5% of the products of hydrolysis for Cel9A, Cel5A, and Cel6B, respectively. For mixtures, glucose accounted for 6–24% of the absorbance. Thus, the extent of conversion of substrate to reducing sugar was based on the theoretical glucose yield of the substrate, and χ was calculated in units of μmol glucose reducing sugar.

Extents of Conversion for Pure Cellulases

Presented in Figs. 2A and 2B are plots of χ vs $[E]_t/[S]_0$ for Cel6B, Cel9A, and Cel5A on Avicel and BMCC, respectively. $[E]_t/[S]_0$ ranges of 17–323 nmol/g (5–50% of the maximum Avicel binding capacity) and 250–9220 nmol/g (2.5–25% of the maximum BMCC binding capacity) for Avicel and BMCC, respectively. The χs for all experiments were calculated in units of μmol reducing sugar as glucose. For Avicel, Cel5A gave the highest maximum extent of conversion, χ_{max}, of 0.036, or 3.6%, followed by Cel6B then Cel9A. For BMCC, Cel9A yielded the highest χ_{max} of 0.763 or 76.3%, followed by Cel6B and Cel5A. Higher χs were obtained for BMCC partially due to the higher $[E]_t/[S]_0$; however, when comparing χs over the same $[E]_t/[S]_0$, higher values are obtained with BMCC. These differences varied from 2.0 to 20 times higher on BMCC with the biggest difference observed with Cel9A. It is also interesting to note that the processive endocellulase, Cel9A, and the exo-cellulase, Cel6B, yielded the highest extents on BMCC while on Avicel the endocellulase, Cel5A, yielded the highest extents.

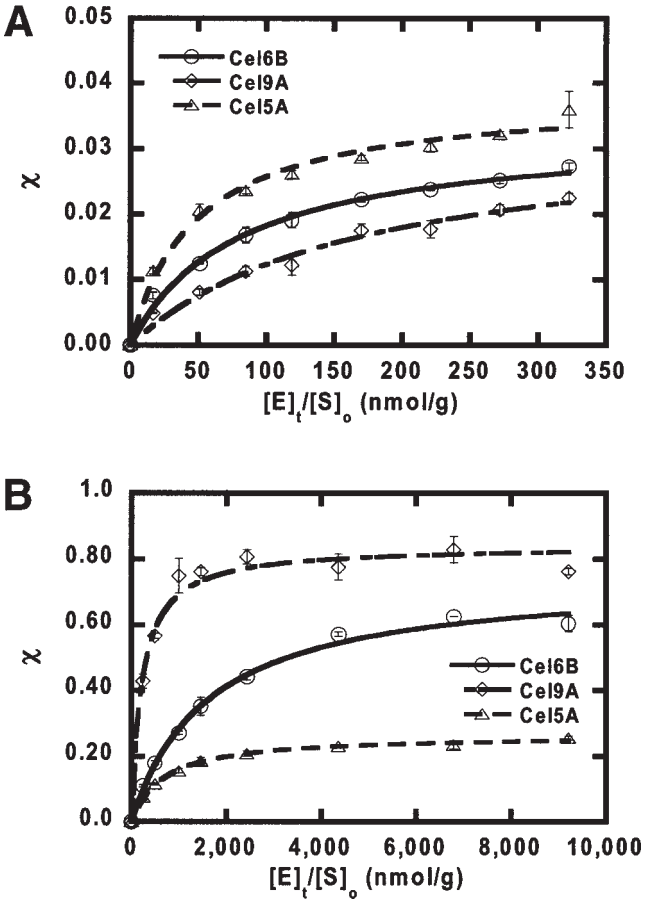

Fig. 2. Extent of conversion (χ) at 16 h hydrolysis vs $[E]_t/[S]_0$ for Cel6b, Cel9A, and Cel5A: **(A)** Avicel; **(B)** BMCC.

Both substrates exhibit a nonlinear response to increasing $[E]_t/[S]_0$ even though binding over this interval was observed by Bothwell et al. *(9)*, and verified in this study, to be linear. This suggests a decrease in enzyme activity with increasing enzyme concentration. Other investigators have noted this loss in enzyme activity with increasing enzyme concentration over the course of hydrolysis *(4–6)*.

Extents of Conversion for Cellulase Mixtures

Presented in Fig. 3 are the χs on Avicel for mixtures of Cel6B+Cel5A. The other two mixtures, Cel9A+Cel5A and Cel6B+Cel9A, exhibited similar nonlinear behavior with increasing $[E]_t/[S]_0$, ranging from 17 to 170 nmol/g for Avicel (5–50% of the maximum Avicel binding capacity). Maximum extent of conversion was 0.044, 0.031, and 0.030 for mixtures of Cel6B+ Cel5A, Cel9A+Cel5A, and Cel6B+Cel9A, respectively. In general, the χs were insensitive to changes in the mole fraction. This is particularly true for

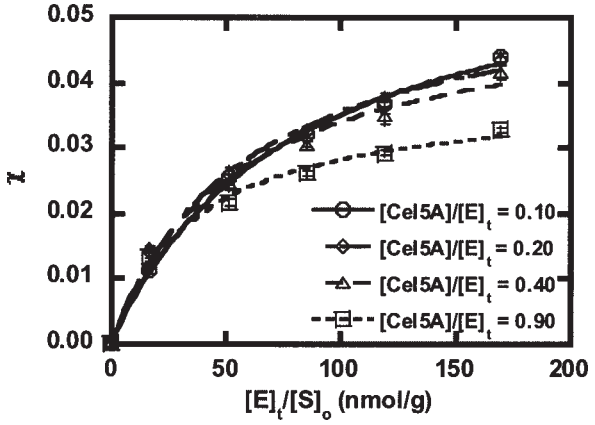

Fig. 3. Extent of Avicel conversion (χ) at 16 h vs $[E]_t/[S]_0$ for different mole faction of Cel6b+Cel5A.

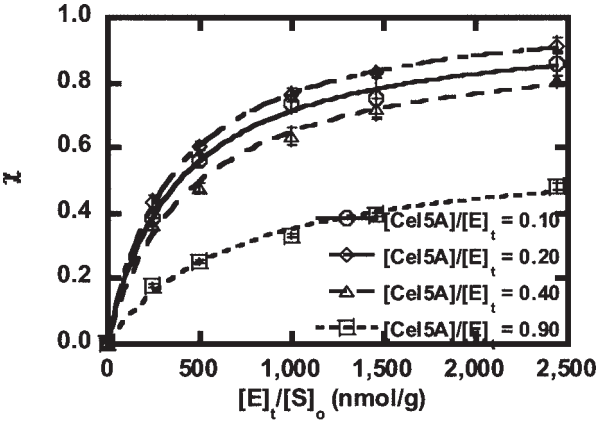

Fig. 4. Extent of BMCC conversion (χ) at 16 h vs $[E]_t/[S]_0$ for different mole faction Cel6B+Cel5A.

the Cel6B+Cel9A mixture (data not shown), where varying Cel6B/$[E]_t$ from 0.25 to 0.75 resulted in no significant change in χ. The χ_{max} observed for the Cel9A+Cel5A mixture for a Cel5A/$[E]_t$ of 0.90 was 0.031, which was 7% greater than the χ_{max} obtained for a Cel5A/$[E]_t$ of 0.10. There was more sensitivity to mole fraction for the Cel6B+Cel5A mixture where a Cel5A mole fraction of 0.90 resulted in a 25% reduction in χ at the higher $[E]_t/[S]_0$ (see Fig. 3).

Figure 4 shows the χs on BMCC for mixtures of Cel6B+Cel5A. The other two mixtures, Cel9A+Cel5A and Cel6B+Cel9A, also exhibited the characteristically nonlinear behavior with increasing $[E]_t/[S]_0$ over the $[E]_t/[S]_0$ range of 250–2440 nmol/g (2.5–25% of the maximum BMCC binding capacity). Maximum extents of 0.91, 0.84, and 0.98 were observed for Cel6B+Cel5A, Cel9A+Cel5A, and Cel6B+Cel9A mixtures, respectively. Overall,

the BMCC extents were more sensitive to cellulase mole fraction. The one exception is the Cel6B+Cel9A mixture, which exhibited the same insensitivity to changes in the mole fraction of Cel6B (Cel6B/$[E]_t$) that was observed with this mixture on Avicel. Both the Cel6B+Cel5A and Cel9A+Cel5A mixtures exhibit a very significant reduction in χ when the Cel5A/$[E]_t$ was 0.90. The χ_{max} observed for the Cel6B+Cel5A mixture was 0.91 at a Cel5A/$[E]_t$ of 0.20. The χ_{max} for the Cel9A+Cel5A mixtures was 0.84 with a Cel5A/$[E]_t$ of either 0.20 or 0.10.

Degree of Synergistic Effect

In several earlier studies *(8,11)*, the degree of synergistic effect (DSE) was defined as follows:

$$DSE = \frac{[RS]_{mix}}{\sum_{i=1}^{n} [RS]_i} \quad (1)$$

where

$[RS]_{mix}$ = soluble reducing sugar production in 16 h observed with a mixture, nmol/mL

$[RS]_i$ = soluble reducing sugar production in 16 h with cellulase component *i* of the mixture, nmol/mL

Reducing sugar concentrations for individual cellulases and cellulase mixtures were determined by first fitting the χ data for each $[E]_t$ for each individual cellulase and for each cellulase mixture at every experimental mole fraction according to the following equation:

$$\chi = \frac{\beta_1 \left(\frac{[E]_t}{[S]_0} \right)}{\beta_2 \left(\frac{[E]_t}{[S]_0} \right)} \quad (2)$$

where β_1 and β_2 are rate constants (nmol/g).

Values of β_1 and β_2 were determined using the nonlinear curve fit package of Kaliedagraph (Synergy Software, Reading, PA) and are presented in Table 2. The resulting curve fits are presented in Figs. 2–4. Equation 2 and the parameter values in Table 2 were used to calculate DSEs for the cellulase mixtures and individual cellulases for the $[E]_t/[S]_0$ of the mixture and the concentration of individual cellulase in the mixture.

The following equation was used to convert χs to [RS]s to obtain $[RS]_{mix}$ and each $[RS]_i$:

$$[RS] = \chi[RS]_0 \quad (3)$$

Table 2
Curve Fit Parameters for Extent of Conversion (χ) vs Cellulase Concentration ($[E]_t/[S]_0$) on Avicel and BMCC

		Avicel			BMCC		
		β_1	β_2 (nmol/g)	R^2	β_1	β_2 (nmol/g)	R^2
CEL6B		0.03285	79.666	0.9930	0.7467	1620.70	0.9939
CEL9A		0.03344	171.550	0.9824	0.8411	213.42	0.9816
CEL5A		0.03807	47.530	0.9863	0.2655	669.10	0.9974
CEL6B+CEL5A	$CEL5A/[E]_t$						
	0.10	0.06328	80.626	0.9979	0.9832	381.60	0.9974
	0.20	0.05672	60.113	0.9915	1.0409	361.61	0.9999
	0.40	0.05244	54.760	0.9886	0.9360	445.66	0.9964
	0.90	0.03843	36.786	0.9950	0.5976	706.38	0.9917
CEL9A+CEL5A	$CEL5A/[E]_t$						
	0.10	0.03881	75.814	0.9779	0.9300	220.94	0.9993
	0.20	0.03851	69.082	0.9846	0.9281	213.91	0.9987
	0.40	0.03721	50.953	0.9881	0.9158	264.61	0.9976
	0.90	0.03686	38.042	0.9978	0.8366	639.70	0.9993
CEL6B+CEL9A	$CEL6B/[E]_t$						
	0.25	0.04309	89.748	0.9725	1.0523	176.26	0.9925
	0.50	0.04429	83.001	0.9724	1.0446	135.64	0.9976
	0.75	0.04171	69.716	0.9846	1.0776	169.47	0.9835

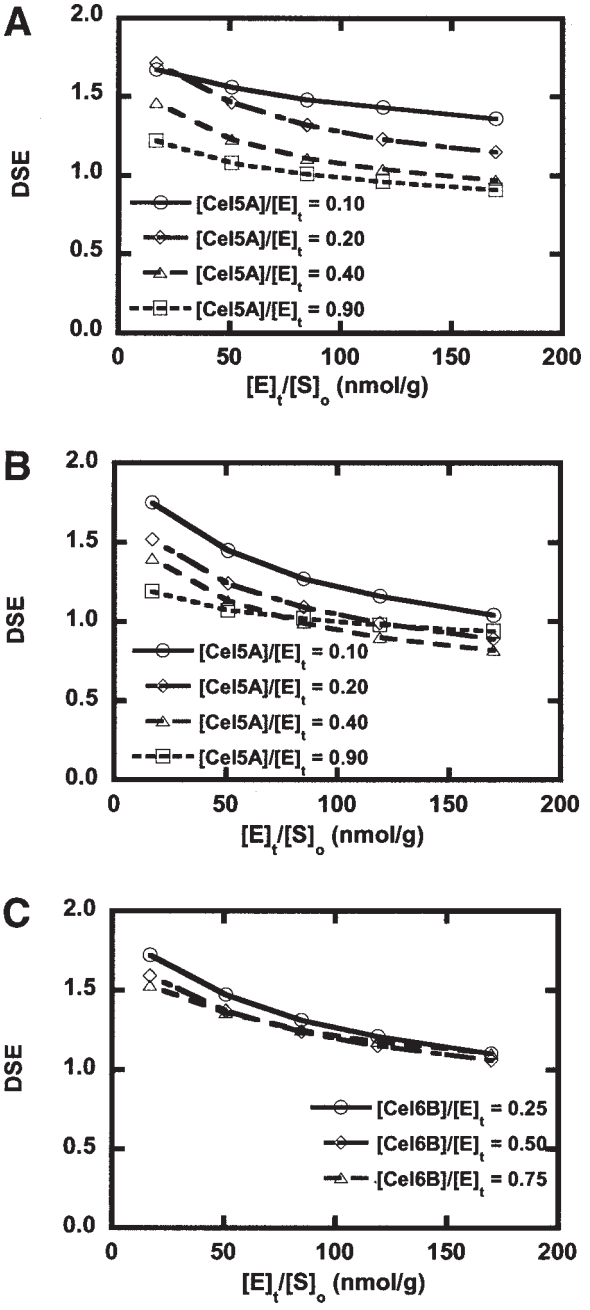

Fig. 5. Degree of synergistic effect (DSE) vs $[E]_t/[S]_0$ on Avicel at different mole faction: **(A)** Cel6B+Cel5A; **(B)** Cel9A+Cel5A; **(C)** Cel6B+Cel9A.

where $[RS]_0$ is the theoretical maximum reducing sugar concentration in μmol glucose/mL: $[RS]_0 = 0.040$ g/mL for Avicel and $[RS]_0 = 0.001$ g/mL for BMCC. Equation 1 was used to calculate DSEs for each mixture at every concentration.

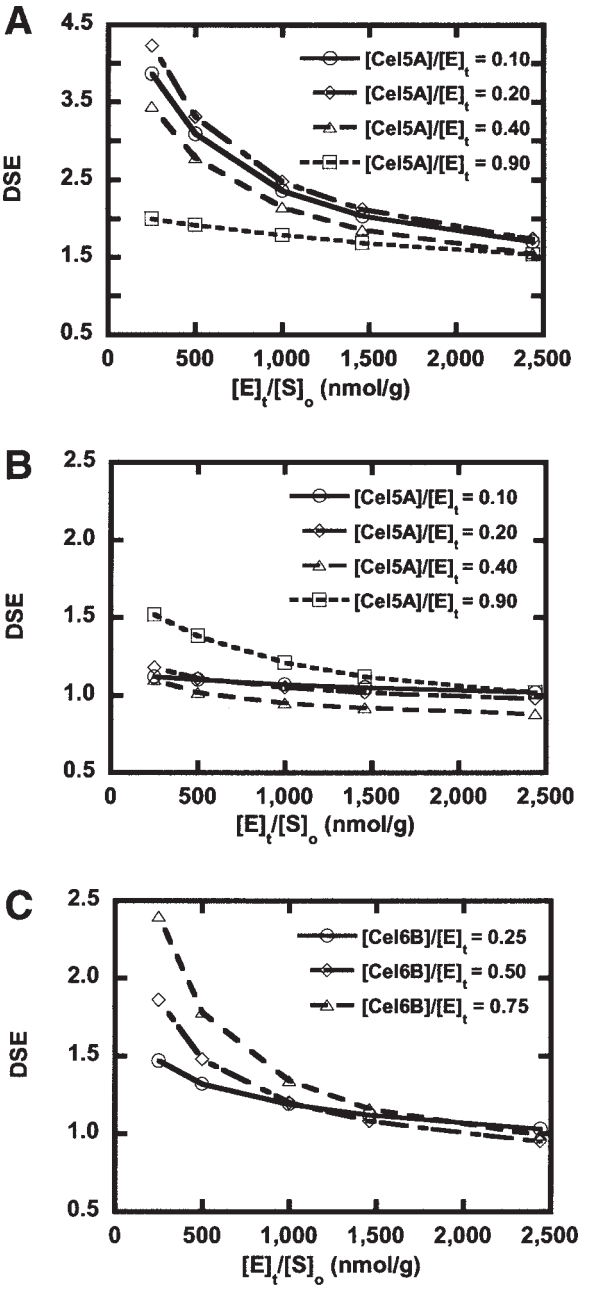

Fig. 6. Degree of synergistic effect (DSE) vs $[E]_t/[S]_0$ on BMCC at different mole faction: **(A)** Cel6B+Cel5A; **(B)** Cel9A+Cel5A; **(C)** Cel6B+Cel9A.

Presented in Figs. 5 and 6 are the DSEs obtained from this method for Avicel and BMCC, respectively. There are two key observations. On both Avicel and BMCC, the maximum DSE occurred at the lowest $[E]_t/[S]_0$ stud-

ied. Second, the greatest sensitivity to cellulase mole fraction was generally observed at the lowest $[E]_t/[S]_0$.

The maximum DSE on Avicel was observed for the Cel6B+Cel5A mixture with a Cel5A/$[E]_t$ of 0.10 and a $[E]_t/[S]_0$ of 17 nmol/g; however, the maximum DSE for the two other mixtures on Avicel are very close to this maximum (see Figs. 5A–5C). For BMCC, the maximum DSE observed was also with the Cel6B+Cel5A mixture at a $[E]_t/[S]_0$ of 250 nmol/g and a Cel5A/$[E]_t$ of 0.20. Maximum DSEs for Cel6B+Cel5A and Cel6B+Cel9A were greater on BMCC than Avicel.

For two of the mixtures, Cel6B+Cel5A and Cel6B+Cel9A, the DSE values were higher for BMCC than for Avicel by a factor of 1.7–2.5. The one exception is the Cel9A+Cel5A mixture, which had a lower DSE for BMCC than Avicel. On BMCC, all three mixtures were sensitive to mole fraction at the lowest $[E]_t/[S]_0$ tested—the lowest $[E]_t/[S]_0$ tested on BMCC is nearly 1.5 times greater than the highest $[E]_t/[S]_0$ tested on Avicel. DSEs for Cel6B+Cel5A and Cel6B+Cel9A are still much more sensitive to mole fraction on BMCC than on Avicel. On BMCC, DSE is insensitive to $[E]_t/[S]_0$ greater than 1000 nmol/g, which corresponds to χs on BMCC greater than 0.60 for Cel6B+Cel5A and Cel9A+Cel5A and greater than 0.80 for Cel6B+Cel9A. On Avicel, all three mixtures are sensitive to the $[E]_t/[S]_0$ in the range tested, 17–170 nmol/g (see Figs. 5 and 6).

Conclusions

It is clear that BMCC is more susceptible to processive enzymes. The processive endocellulase, Cel9A, had four times the extent of conversion of the endocellulase Cel5A, and Cel6B had three times the extent of conversion of Cel5A. This is also reflected in the results obtained with the cellulase mixtures on BMCC. Approximately 500 nmol/g of the Cel9A+Cel6b mixture were needed to obtain 80% conversion, while the mixtures of Cel6b+Cel5A and Cel9A+Cel5A required 1500 and 1250 nmol/g, respectively. Thus, it appears that the more accessible structure of BMCC, as reflected by binding capacity, results in higher processive activity. However, binding capacity alone does not account for the difference in the extent of conversion observed on BMCC. Cel9A, which has the lowest BMCC binding capacity, yielded the highest extent of conversion, while Cel5A that had the highest binding capacity on BMCC had the lowest extent of conversion on BMCC.

It is clear for BMCC that the Cel6B+Cel9A mixture gives the highest extent of conversion that approaches 1.0 (100%). Cel9A is a processive endocellulase that would initially attack the cellulase chain randomly as an endocellulase, then proceed to hydrolyze the length of the chain as an exocellulase.

The DSE results obtained in this study are consistent with earlier work by Woodward et al. *(8)* and Walker et al. *(11)*. Walker et al. *(11)* observed that the DSE for tertiary mixtures of *T. fusca* Cel6B and Cel5A and *T. reesei*

CBHI, hydrolyzing Avicel, generally decreased or remained unchanged at higher concentrations. Woodward et al. *(8)* also observed a decrease in DSE with increasing enzyme concentrations on Avicel. Bothwell et al. *(16)* and Walker et al. *(11)* observed ranges of cellulase fractions in which the DSE was insensitive to the ratio of enzymes. The DSE was sensitive to the endocellulase mole fraction on Avicel for the mixtures Cel6B+Cel5A and Cel9A+Cel5A. On BMCC, the DSEs of all three mixtures were most sensitive at concentrations less than 1000 nmol/g, which is much less than saturation. The decreased sensitivity of DSE to mole fraction at concentrations greater than 1000 nmol/g on BMCC may be due to the high χ obtained for the mixtures at concentrations higher than 1000 nmol/g. The lower DSE of the Cel9A+Cel5A mixture could be because Cel9A has sufficient endocellulase activity on it own.

It is clear that the higher accessibility of BMCC to cellulases is very important and results in higher extents of hydrolysis. Binary mixtures of cellulases are not sufficient to hydrolyze the inaccessible Avicel more than 4%.

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