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Hydrolysis of Ammonia-pretreated Sugar Cane Bagasse with Cellulase, β-Glucosidase, and Hemicellulase Preparations

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Abstract Sugar cane bagasse consists of hemicellulose (24%) and cellulose (38%), and bioconversion of both fractions to ethanol should be considered for a viable process. We have evaluated the hydrolysis of pretreated bagasse with combinations of cellulase, β-glucosidase, and hemicellulase. Ground bagasse was pretreated either by the AFEX process ($2NH_3$: 1 biomass, 100 °C, 30 min) or with NH_4OH (0.5 g NH_4OH of a 28% [*v*/*v*] per gram dry biomass; 160 °C, 60 min), and composition analysis showed that the glucan and xylan fractions remained largely intact. The enzyme activities of four commercial xylanase preparations and supernatants of four laboratory-grown fungi were determined and evaluated for their ability to boost xylan hydrolysis when added to cellulase and β-glucosidase (10 filter paper units [FPU]: 20 cellobiase units [CBU]/g glucan). At 1% glucan loading, the commercial enzyme preparations (added at 10% or 50% levels of total protein in the enzyme preparations) boosted xylan and glucan hydrolysis in both pretreated bagasse samples. Xylanase addition at 10% protein level also improved hydrolysis of xylan and glucan fractions up to 10% glucan loading (28% solids loading). Significant xylanase activity in enzyme cocktails appears to be required for improving hydrolysis of both glucan and xylan fractions of ammonia pretreated sugar cane bagasse.

Keywords Ammonia pretreatment · Sugar cane bagasse · Hydrolysis · Cellulase · Hemicellulase

Introduction

With the rapid increase in oil prices over the past decade together with the uncertainty about reliable future supplies, alternative sources of liquid fuels have recently received extensive

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attention. Increased ethanol demand as an attractive liquid biofuel has resulted in extensive fermentation facilities being constructed worldwide. Whereas ethanol production from food crops will expand over the next decade, a stage will be reached where insufficient crop substrates will be available to satisfy liquid fuel demands. Lignocellulose biomass, a low cost substrate, is available in much larger amounts than food crops and can be hydrolyzed to sugars. Therefore, biomass can serve as a useful substrate to produce a wide range of commodity chemicals in addition to ethanol [1]. However, the heterogeneous nature of lignocellulose results in a biomass that is recalcitrant to enzymatic hydrolysis and where the component fermentable sugars released are more expensive than those obtained from food crops. This situation has started to change as more efficient and cost-effective enzymes have become available [2, 3].

Sugar cane bagasse is a lignocellulose biomass produced in large amounts as a byproduct from sugar extraction in volumes that will continue with increased worldwide sugar production. Considerable amounts of bagasse are currently used as a combustible energy source, for paper pulp production, or animal feed. However, a relatively high carbohydrate (hemicellulose and cellulose) and medium lignin content make bagasse an attractive substrate for ethanol production, but bioconversion of both carbohydrate fractions to ethanol must be considered for a viable process. Pretreatment of bagasse is necessary to reduce its recalcitrance to enzymatic hydrolysis. Previous studies have reported the pretreatment of sugar cane bagasse with either physical or chemical methods such as acid [4], steam [5], or alkali [6, 7]. Dilute sulfuric acid has been widely used to pretreat sugar cane bagasse [4, 8] and results in the hemicellulose fraction being released as pentoses such as xylose and arabinose. Without detoxification, the subsequent fermentation of the hydrolysate can be seriously inhibited. Alkali pretreatment leaves the hemicellulose fraction relatively intact. The ammonia freeze explosion (AFEX) process is a particularly attractive pretreatment for sugar cane bagasse [7] as ammonia can be potentially recycled whereas only some hemicellulose is removed and the formation of sugar degradation products are minimized [9, 10]. Furthermore, this process enables both the cellulose and hemicellulose fractions to be hydrolyzed enzymatically.

We have evaluated the degree of hydrolysis of sugar cane bagasse pretreated by the AFEX process or with NH_4OH with combinations of cellulase, β-glucosidase, and hemicellulase enzymes. Significant xylanase activity in enzyme cocktails appears to be required for greater hydrolysis of both glucan and xylan fractions of ammonia pretreated sugar cane bagasse.

Materials and Methods

Pretreatment of Sugar Cane Bagasse

Bagasse from sugar cane (*Saccharum officinarum*) was obtained from the Raceland Raw Sugar Corp. sugar mill, Raceland, LA, USA and ground to a particle size less than 12 mm. Ground sugar cane bagasse was submitted to MBI International, Lansing, MI, USA for pretreatment using the AFEX process [7]. Briefly, approximately 1 kg of the bagasse was treated in a 1-gal reactor at 100 °C for 30 min with a 2:1 ammonia loading to biomass and 40% moisture level. After pretreatment, bagasse was removed from the reactor, dried to remove ammonia, and stored in sealed plastic bags at 4 °C. Ground sugar cane bagasse (2.5 kg) was also pretreated by adding a 28% stock solution of NH_4OH to achieve a final concentration

of 0.02 g NH_3/g water in a final water mass of 20 kg. The slurry was placed in a pressure reactor and heated at 160 °C for 60 min. The solid mass was removed from the slurry by filtration through muslin cloth and washed with 40 kg of water. Less than 1% of the carbohydrate in the bagasse, detected as monosaccharides, was found in the pretreatment effluents.

Analysis of the Composition of Sugar Cane Bagasse and Pretreated Bagasse

The carbohydrate composition and lignin were determined using the NREL laboratory analytical procedures (http://www1.energy.gov/biomass/analytical_procedures.html). The moisture content of the bagasse samples was determined using a moisture analyzer (Computrac MAX 1000, Arizona Instrument Corporation, Tempe, AZ, USA).

Enzyme Preparations

Commercial Preparations

A commercial preparation produced by *Trichoderma reesei* (Spezyme CP) and supplied by Genencor (Rochester, NY, USA) was used as the cellulase enzyme in this study. This preparation was supplemented with a β-glucosidase preparation (Novozym 188) produced by *Aspergillus niger* and supplied by Sigma (C6105). Various commercial xylanase preparations were kindly provided by various North American suppliers, and their cellulolytic and hemicellulolytic activities were evaluated (Table 1). BioCat xylanase was in powder form and was suspended in 100 mM sodium acetate buffer (pH 5) at a concentration of 10 mg/ml before use.

Laboratory-produced Enzyme Preparations

Thermomyces lanuginosus strains SSBP and ATCC 34626 and *Aspergillus carneus* ABO 372 were obtained from the culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. The fungi were maintained on malt extract agar plates. With a sterilized needle, samples were inoculated into 1 ml YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) and cultivated for 2 days respectively at the optimum temperature for the fungus (*T. lanuginosus*, 50 °C; *A. carneus*, 30 °C) with shaking. The contents of each inoculant were decanted into 200 ml growth medium (0.67% yeast nitrogen base with amino acids, 0.2% L-asparagine monohydrate, 0.5% KH_2PO_4), containing 4 g of AFEX pretreated sugar cane bagasse or 4 g beechwood xylan (Sigma) as carbon source and cultivated for 5 days at the optimum temperature. Fungal growth was clearly visible in the flasks after 4 days. The culture fluid was filtered through four layers of muslin cloth and then centrifuged at 5,000×*g* for 10 min to remove fungal debris. After centrifugation, 0.1% sodium azide was added to the supernatant to prevent microbial growth and concentrated by filtration through an Amicon concentration apparatus using a 10-kDa membrane filter.

Enzyme and Protein Assays

The filter paper activity of the enzyme samples was determined at 50 °C according to standardized NREL filter paper assay [11]. Carboxymethylcellulase (CMCase; endoglucanase) and Avicelase (exoglucanase) activity was determined by measuring the release of reducing

Table 1 Specific activities (mean of triplicate determinations±standard deviation) of cellulolytic and hemicellulolytic enzymes of selected preparations.

Enzyme and source	Protein concentration mg/ml	Filter paper activity U/mg	Endoglucanase (CMCase) U/mg	Exoglucanase (Avicelase) U/mg	β-glucosidase U/mg	β-xylanase U/mg	β-xylosidase U/mg	α-arabinofuranosidase U/mg
Cellulase (Spezyme CP) *T. reesei*	51.81±5.66	1.40	21.8±2.1	0.09±0.02	1.82±0.08	15±2	0.56±0.02	0.38±0.04
β-glucosidase (Novozym 188) *A. niger*	38.01±1.2	0	1.0±0.2	0.35±0.05	14.75±0.45	10±1	0.22±0	0.09±0.1
Xylanase (Multifect) *T. reesei*	7.63±0.39	0.04	6.3±0.6	0.46±0.07	3.30±0.10	209±10	4.90±0.58	3.21±0.40
Xylanase (PowerPulp TX200A) *Trichoderma*	3.34±0.07	0.42	5.6±2.0	0.31±0.09	1.07±0.13	369±10	1.27±0.01	0.98±0.04
Xylanase (FibreZyme LBL) unknown	28.81±1.00	1.27	97.1±7.6	0.38±0.05	3.09±0.21	69±1	0.94±0.03	0.68±0.03
Xylanase (BioCat; 10 mg/ml)								
Trichoderma longibrachiatum	0.51±0.01	1.43	78.6±10.5	9.50±0.79	4.65±0.26	1235±57	5.87±0.31	5.07±0.45
A. carneus ABO372[a]	0.23	ND	43.3±11.0	0.20±0.03	2.64±0.21	156±5	0.50±0.02	0.19±0.01
T. lanuginosus SSBP[b]	0.47	ND	4.8±1.7	0.05±0.02	0.46±0.02	2498±34	0.09±0.01	0.11±0.01
T.lanuginosus ATCC 34626[b]	0.32	ND	5.6±1.6	0.03±0.01	0.91±0.06	2764±137	0.12±0.01	0.08±0.01
T. lanuginosus ATCC 34626[c]	0.14	ND	19.3±5.5	0.16±0.03	0.66±0.03	8238±64	0.19±0.03	0

ND: not determined

[a] Fungus grown in medium containing AFEX-pretreated bagasse at 30 °C.

[b] Fungus grown in medium containing AFEX-pretreated bagasse at 50 °C.

[c] Fungus grown in medium containing beechwood xylan at 50 °C.

sugars from 3% carboxymethylcellulose (Sigma) and 3% Avicel (FMC Biopolymer pH-102), respectively, at 50 °C and pH 5.0 (100 mM acetate buffer) for 10 min [12]. β-Xylanase activity was determined by following the release of reducing sugars from a 1% birchwood xylan (Sigma) solution at 50 °C for 5 min [13]. One unit of activity was defined as the amount of enzyme that released either 1 μmol of glucose or xylose as reducing sugar equivalents per min.

β-Glucosidase, β-xylosidase, and α-arabinofuranosidase activities were determined by following the release of 4-nitrophenol from 4-nitrophenyl-β-D-glucopyranoside, 4-nitrophenyl-β-D-xylopyranoside, and 4-nitrophenyl-α-L-arabinopyranoside (Sigma), respectively, for 15 min at 50 °C and pH 4.0 (50 mM Na acetate buffer). The reaction was stopped by the addition of 1 M Na_2CO_3, and the absorbance was read at 410 nm from a 4-nitrophenol standard curve. One unit of activity was defined as the amount of enzyme that released 1 μmol of 4-nitrophenol per min.

The protein concentration was determined using the Coomassie Brilliant Blue dye-binding method (BioRad; [14]).

Batch Hydrolysis of Pretreated Sugar Cane Bagasse and Avicel

Enzymatic saccharification experiments of pretreated samples were performed in triplicate in 20 ml glass scintillation vials at 50 °C and 100 rpm for 72 h as described in the NREL (LAP-009) procedure. Briefly, the reaction mixture contained 0.1 g glucan (cellulose dry weight; except with the substrate loading experiment), 0.5 ml 1 M sodium citrate buffer (pH 4.8), 40 μl tetracycline (10 mg/ml), 30 μl cycloheximide (10 mg/ml), Spezyme CP and Novozym 188 in a ratio of 1:2/g glucan, and distilled water to give a final volume of 10 ml. Adjustments were also made for the addition of various activities of commercial and laboratory-produced xylanases. The moisture content in the AFEX-pretreated bagasse (24.53%) and NH_4OH-pretreated bagasse (78.42%) was included in the calculation of the total volume. Avicel (FMC Biopolymer pH-102; 4.54% moisture) was also included as a control. Substrate blanks excluded the enzyme activities whereas enzyme blanks excluded the substrates, and the degree of hydrolysis was used to correct data. Samples were withdrawn initially and after 72 h, and centrifuged at 10,000 rpm in Eppendorf tubes to remove the biomass. Subsequently, the liquid portion was filtered through a 0.45 μm (pore size) Whatman GD/X filter and the liquid was subjected to HPLC analysis. HPLC analysis was conducted using an isocratic system using a refractive index detector (Spectra System, RI 150, Thermo Electron, Milan, Italy). Sugars were separated on an Aminex-HPX-87P column (BioRad) at a flow rate of the mobile phase (deionized water) of 0.6 ml/min and at 85 °C for 30 min.

Calculations

The percent hydrolysis of the glucan fraction of sugar cane bagasse was calculated by adding the glucose concentration to double the cellobiose concentration. This value was multiplied by 0.9 to correct for hydration (each glucose molecule had one molecule of water added during hydrolysis) and divided by the grams of glucan.

Statistical Analysis

A two-way analysis of variance (ANOVA) was performed to determine if any significant differences ($p<0.05$) occurred between factors. Bonferroni and bootstrap multiple comparisons were preformed with STATISTICA version 7.0 (Stat-Soft, Tulsa, OK, USA).

Results and Discussion

Analysis of sugar cane bagasse revealed a composition (dry biomass) of 25.0% lignin, 38.4% glucan, 24.1% xylan, and 1.9% arabinan. After pretreatment with the AFEX process or NH_4OH, the composition consisted of 22.6 and 21.1% lignin, 41.7 and 56.6% glucan, 20.4 and 24.0% xylan, respectively, and 1.2% arabinan for both AFEX and dilute ammonia treatment. Both pretreatment processes resulted in an increase in the glucan concentration and left the hemicellulose fraction of bagasse largely intact. Alkali pretreatments are reported to only partially hydrolyze the hemicellulose fraction to oligomers [9, 15]. In this study, it is apparent that solubilization might also depend on the substrate type as the xylan concentration of the pretreated bagasse was only slightly less than that of the raw sugar cane bagasse.

The cellulolytic and hemicellulolytic activities of six commercial enzyme preparations are shown in Table 1. Filter paper activity could be detected only in some samples and the highest activities were obtained in the BioCat xylanase, Spezyme CP, and FibreZyme LBL. The samples showed a considerable range in CMCase (an indicator of endo-β-1,4-glucanase activity) and Avicelase (an indicator of exoglucanase activity). BioCat xylanase preparation showed the highest specific activity of the various cellulolytic enzymes. Novozym 188 had the highest β-glucosidase activity. The β-xylanase specific activities of the six commercial samples ranged between 10 and 1,235 U/mg. Especially notable was the low β-xylanase activities of Novozym 188 and Spezyme CP, two enzyme preparations commonly used in the hydrolysis of lignocellulose. The hemicellulolytic enzyme profiles of a number of preparations were evaluated to establish which might be appropriate for addition to a cellulase/β-glucosidase enzyme mixture. The BioCat, PowerPulp TX200A, and Multifect preparations were found to have the highest xylanase activity. Furthermore, BioCat xylanase, Multifect xylanase, and PowerPulp TX200A revealed specific β-xylosidase activity >1 and BioCat xylanase and Multifect xylanase were found to have the highest α-arabinofuranosidase activity. Four other commercial xylanase preparations were analyzed but their hemicellulolytic enzyme profiles were deemed not to be appropriate for further evaluation (data not shown). The results suggest that these xylanase preparations might be suitable to boost hydrolysis of sugar cane bagasse to a mixture of hexoses and pentoses when added to a cellulase/β-glucosidase enzyme mixture.

The cellulolytic and hemicellulolytic enzyme profiles of a number of selected fungi were also evaluated (Table 1). The selection of the fungi was based on their properties determined in previous research. *A. carneus* ABO374 was isolated from soil in the Southern Cape region of South Africa and found to efficiently release reducing sugars from wheat straw. The *T. lanuginosus* strains SSBP and ATCC 34626 were shown to be very efficient producers of xylanases, and the β-xylanase activity could be maintained up to 60 °C [16, 17]. The *A. carneus* strain cultivated on AFEX-pretreated sugar cane bagasse produced the highest specific activities of cellulolytic enzymes and β-glucosidase of the fungal strains tested. *T. lanuginosus* strain SSBP produced lower cellulolytic specific activities when compared with ATCC 34626 when cultivated under similar conditions. Most activities of cellulolytic enzymes were higher with strain ATCC 34626 when it was grown on beechwood xylan than on AFEX-pretreated bagasse, suggesting that pure xylan is a better substrate for enzyme production. The highest specific β-xylanase activity was produced by strain ATCC 34626 when cultivated on beechwood xylan, whereas lower activity was found when the *T. lanuginosus* strains were grown on AFEX-pretreated bagasse, and much lower β-xylanase activity was produced by *A. carneus* (Table 1). These results revealed values that were higher than those found in the commercial samples. *A. carneus* produced

the highest activity of β-xylosidase and α-arabinofuranosidase whereas the *T. lanuginosus* strains produced much lower activities of these auxiliary enzymes, as has been reported previously [16].

Spezyme CP is a commercial cellulolytic enzyme preparation produced by *Trichoderma reesei* (Table 1) and has been widely used in the hydrolysis of cellulose-rich biomass. However, this preparation lacks adequate β-glucosidase activity [9] to achieve complete hydrolysis to glucose, resulting in cellobiose accumulation. Therefore, Spezyme CP is usually supplemented with β-glucosidase to promote hydrolysis. In the literature, the ratios of cellulase to β-glucosidase have ranged widely and have depended upon the nature of the lignocellulose material to be hydrolyzed. For example, ratios of 1 filter paper unit (FPU):2 cellobiase units (CBU) [18] and 1 FPU:3.2 CBU [3] have been used to hydrolyze various softwood substrates and barley straw, respectively, whereas Martin et al. [4] used a ratio of 1 FPU:5.4 CBU and the NREL procedure (LAP-009) recommended a ratio of approximately 1 FPU:1 pNPGU to hydrolyze steam pretreated sugar cane bagasse. A ratio of 1 FPU:2 CBU was selected for the hydrolysis experiments in this study, and the ratio was based on the reported commercial enzyme activities of 60 FPU/ml for Spezyme CP and 282 CBU/ml for Novozym 188. Based on the data in Table 1, our calculated ratio was 1 FPU of Spezyme CP:3.3 β-glucosidase units of Novozym 188.

Figure 1 shows the effect of three concentrations of Spezyme CP/Novozym 188 (in an activity ratio of 1:2) on the hydrolysis of NH_4OH-pretreated and AFEX-pretreated bagasse and Avicel (1% glucan loading). As the enzyme level increased, greater amounts of glucose and xylose were released from the bagasse samples. No cellobiose was detected in any of the samples suggesting that the β-glucosidase activity was not limiting in any of the enzyme mixtures. Arabinose was only released in low concentrations from the AFEX-pretreated bagasse. At a 10 FPU:20 CBU/g glucan ratio, the greatest amount of glucose was released from Avicel followed by the AFEX-pretreated bagasse. A significant increase ($p<0.05$) in the release of glucose and xylose from NH_4OH-pretreated and AFEX-pretreated bagasse was observed when the enzyme amount was raised from 10:20 to 30:60. Greater amounts of enzyme activity (60:120) failed to significantly ($p>0.05$) increase the release of glucose and xylose. Only a marginal increase in the amount of glucose ($p>0.05$) released from Avicel was observed with greater enzyme activity, which indicated that the 10:20 enzyme mixture was close to the saturation level for hydrolysis. At the lowest enzyme level, only 28% and 33% of the glucan in respective NH_4OH-pretreated and AFEX-pretreated bagasse samples were hydrolyzed to glucose, whereas 66% of the Avicel glucan was hydrolyzed, suggesting that some glucan was inaccessible in the bagasse samples to enzyme hydrolysis. At the highest enzyme level (60 FPU/g glucan:120 CBU/g glucan), 68% and 78% of the glucan was hydrolyzed in the respective bagasse samples and Avicel samples. Increasing the level of enzyme appeared to have a much lower impact on the hydrolysis of Avicel than on bagasse. This might be because of a synergistic effect of the other enzymes present in the Spezyme CP/Novozym 188 acting on the cellulose and hemicellulose components in bagasse.

Four xylanase preparations with suitable activity profiles were evaluated for their ability to boost the hydrolysis by Spezyme CP and Novozym 188 (10 FPU:20 CBU/g glucan) of NH_4OH-pretreated and AFEX-pretreated bagasse and Avicel (Fig. 2). As these enzyme preparations contain multiple cellulolytic and hemicellulolytic activities (Table 1), the protein concentration was used as the basis to supplement the Spezyme CP/Novozym mixture instead of using the activity of a single enzyme such as xylanase. All enzyme preparations added at 10% and 50% protein levels resulted in increases in the release of glucose (Fig. 2a, c, e, g) and xylose (Fig. 2b, d, f, h). However, in most instances, significant

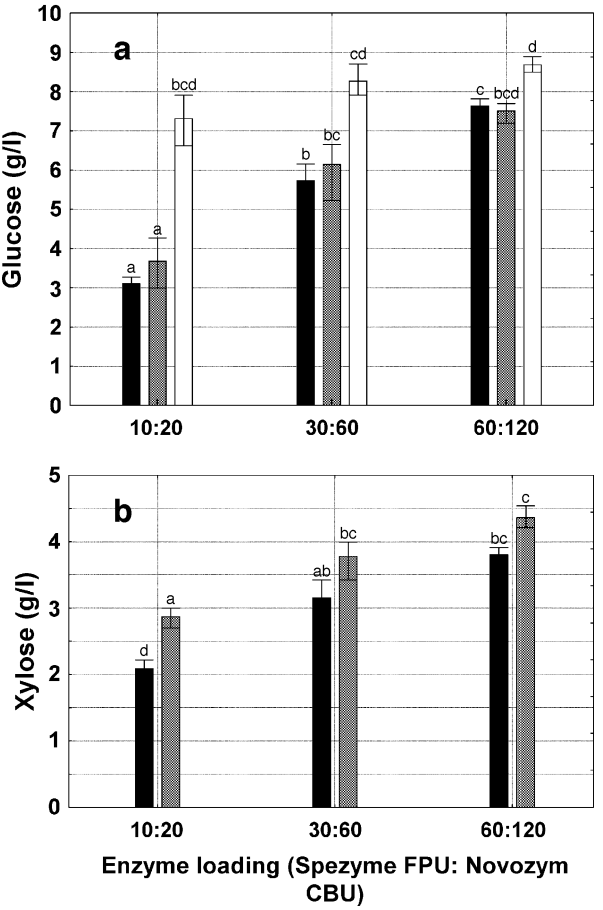


Fig. 1 Release of glucose (**a**) and xylose (**b**) from ammonium hydroxide-pretreated (*black bars*) and AFEX-pretreated (*gray bars*) sugar cane bagasse and Avicel (*white bars*) (1% glucan loading) by Spezyme CP and Novozym 188 (1:2 activity ratio/g glucan). The total protein concentration of Spezyme CP/Novozym 188 mixture was 1.146 (10:20 ratio), 3.438 (30:60 ratio), and 6.876 (60:120 ratio) mg/g glucan. *Vertical lines* indicate 95% bootstrap confidence intervals and means of *bars with identical letters* are not significantly different ($p>0.05$)

differences ($p>0.05$) were not revealed, and only a significant increase in xylose release was observed when Multifect xylanase was added at the 10% protein level to AFEX-pretreated bagasse. Little, none, or trace amounts of cellobiose were detected in these hydrolysates, indicating that β-glucosidase was not limiting under the conditions used. Low concentrations of arabinose were detected in some hydrolysates but no relationship could be found between the levels of the various enzyme preparations applied (data not shown). In all instances, higher levels of glucose and xylose were released from the AFEX-pretreated bagasse than the NH_4OH-pretreated bagasse, and in many cases, these differences were significant ($p<0.05$). The degree of hydrolysis of the glucan of the NH_4OH-pretreated bagasse increased from 26% to 43%, 44%, and 55% when Multifect, FibreZyme LBL, and BioCat xylanases were added, respectively, at the 50% protein level. However, the degree of hydrolysis of the AFEX-pretreated bagasse was greater and increased from approximately 42% to 61%, 54%, 55%, and 73% when Multifect, PowerPulp, FibreZyme LBL, and BioCat xylanases were added at the 50% protein levels. It is interesting to note that the addition of Multifect and PowerPulp xylanase but not FibreZyme LBL or BioCat xylanase to the enzyme mixture increased the release of glucose from Avicel, but bore no relationship to the levels of cellulolytic enzyme activities (Table 1). PowerPulp and Multifect xylanase added at the 50% protein level increased the hydrolysis of Avicel from 65% to 80% and 76%, respectively. Table 1 shows

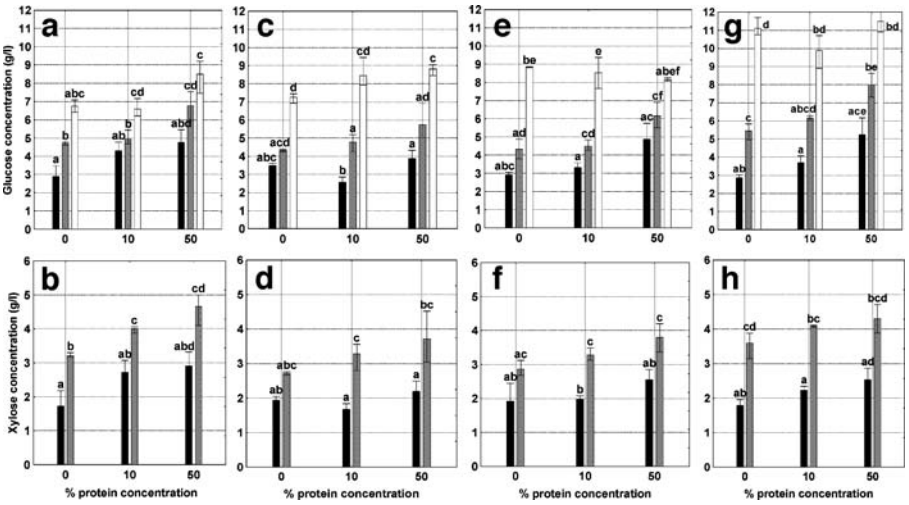


Fig. 2 Release of glucose (**a**, **c**, **e**, g) and xylose (**b**, **d**, **f**, **h**) from ammonium hydroxide-pretreated (*black bars*) and AFEX-pretreated (*checked bars*) sugar cane bagasse and Avicel (*open bars*) (1% glucan loading) by Spezyme CP (10 FPU/g glucan) and Novozym 188 (20 CBU/g glucan). Xylanase from Multifect (**a**, **b**), PowerPulp (**c**, **d**), FibreZyme LBL (**e**, **f**) and BioCat (**g**, **h**) added at 10% and 50% protein to the total protein concentration of Spezyme CP/Novozym 188 mixture (1.146 mg protein/g glucan). *Vertical lines* indicate 95% bootstrap confidence intervals and means of *bars with identical letters* are not significantly different ($p>0.05$)

that both xylanase preparations had relatively low cellulolytic enzyme activities and, therefore, these results point to the possibility that the xylanase might act synergistically in the hydrolysis of cellulose by cellulase.

The impact of laboratory-produced enzymes, added at a 10% protein level on hydrolysis by Spezyme CP and Novozym 188 (10 FPU:20 CBU) is shown in Fig. 3. Enzymes produced by *A. carneus* failed to boost the hydrolysis of NH_4OH-pretreated and AFEX-pretreated bagasse. The enzyme preparations produced by *T. lanuginosus* strains all produced an increase of xylose release from NH_4OH-pretreated and AFEX-pretreated bagasse, but little or no increase in glucose release was observed. *T. lanuginosus* ATCC 34626, grown on beechwood xylan, was the most effective of the various laboratory-produced enzyme preparations in increasing the amount of xylose released, and this could be related to the high specific xylanase activity (Table 1). No clear relation between enzyme preparation of the laboratory-grown fungi and the release of glucose from Avicel was observed. The failure of *A. carneus* to boost xylose release from NH_4OH-pretreated and AFEX-pretreated bagasse might be because of the low xylanase activity (Table 1). The percent hydrolysis of the glucan fraction of NH_4OH-pretreated and AFEX-pretreated bagasse increased from 29% to 40% and from 49% to 54%, respectively, by the addition of an enzyme preparation from *T. lanuginosus* ATCC 34626, grown on beechwood xylan. As shown in the studies with the commercial xylanase preparations, greater amounts of sugar were released from the AFEX-pretreated bagasse than from the NH_4OH-pretreated bagasse when the laboratory-produced enzymes were added to Spezyme CP and Novozym 188, suggesting that the AFEX-pretreated bagasse is more easily hydrolyzed.

As the Multifect xylanase was shown to be one of the most effective xylanase preparations in boosting the hydrolysis of bagasse, the effect on glucan substrate loading on the hydrolysis of NH_4OH-pretreated and AFEX-pretreated bagasse by Spezyme CP and Novozym 188 with and without Multifect xylanase was evaluated (Fig. 4). As a result of

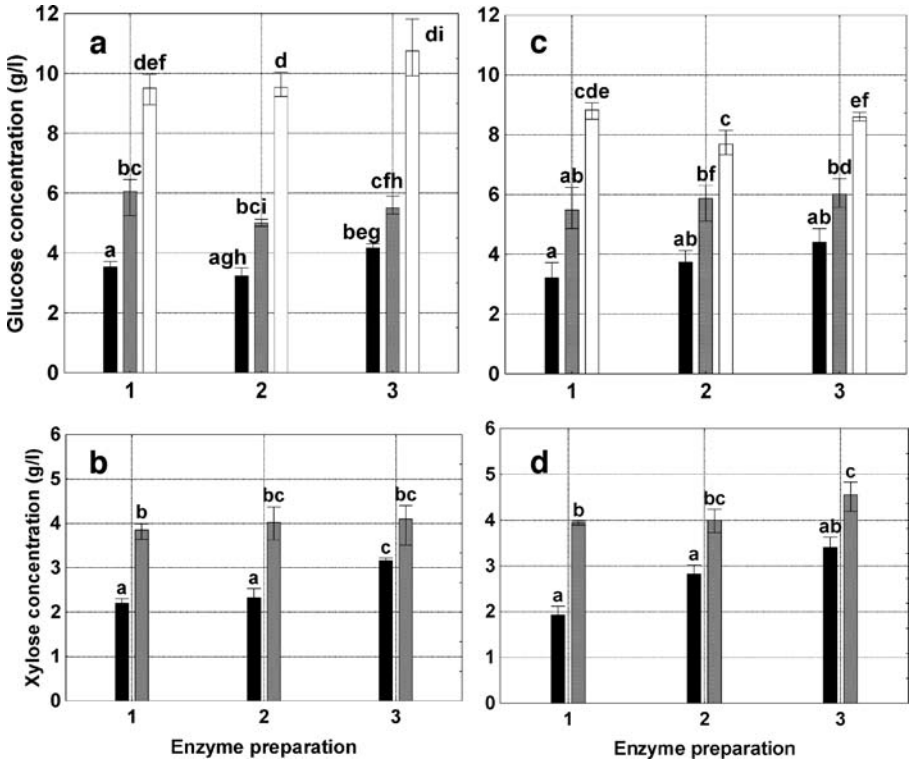
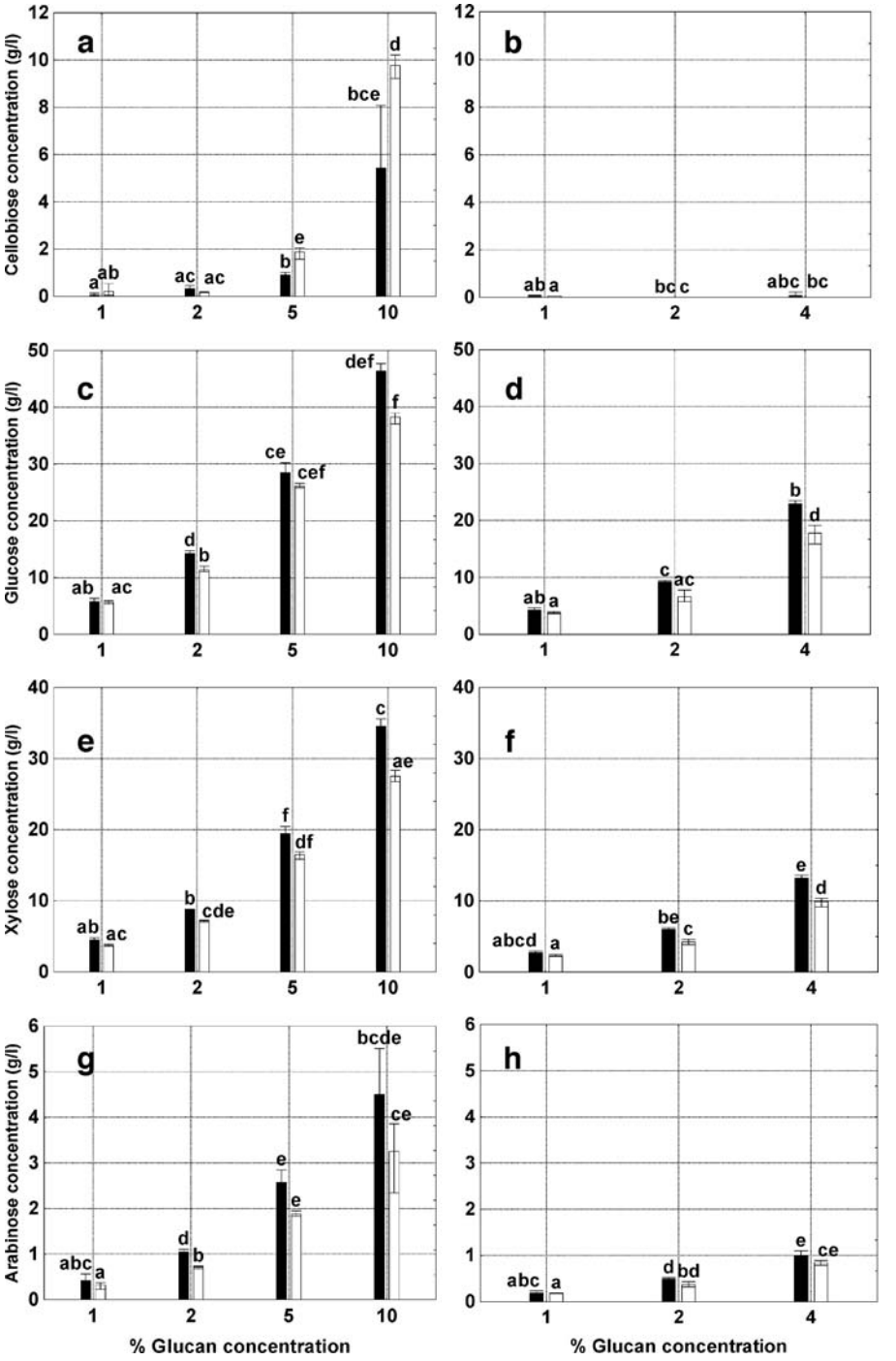


Fig. 3 Release of glucose (**a**, **c**) and xylose (**b**, **d**) from ammonium hydroxide-pretreated (*solid bars*) and AFEX-pretreated (*gray bars*) sugar cane bagasse and Avicel (*open bars*) (1% glucan) when hydrolyzed with Spezyme CP (10 FPU/g glucan) and Novozym 188 (20 CBU/glucan) without (*a1*, *c1*) or with the addition of crude enzyme preparations (10 % protein added) of *A. carneus* ABO372 (*a2*, *b2*), *T. lanuginosus* SSBP (*a3*, *b3*), *T. lanuginosus* ATCC 34626 (AFEX-pretreated bagasse as growth substrate; *c2*, *d2*), or *T. lanuginosus* ATCC 34626 (beechwood xylan as growth substrate; *c3*, *d3*). The total protein concentration of Spezyme CP/Novozym 188 mixture was 1.146 mg/g glucan. *Vertical lines* indicate 95% bootstrap confidence intervals and means of *bars with identical letters* are not significantly different ($p>0.05$)

the high moisture content of the NH_4OH-pretreated bagasse, a maximum glucan substrate loading of only 4% could be tested, whereas the AFEX-pretreated bagasse could be tested up to 10% glucan substrate loading. At all substrate loadings, there were greater amounts of xylose, arabinose, and glucose released when Multifect xylanase was present, although the differences in sugars accumulated in hydrolysates with and without Multifect xylanase were only significant ($p<0.05$) in some instances (Fig. 4). Notable is the significantly ($p<0.05$) higher amount of xylose released from AFEX-pretreated and NH_4OH-pretreated bagasse at 10% and 4% glucan loading, respectively (Fig. 4e, f). However, it was apparent that greater amounts of both glucose and xylose were released from the AFEX-pretreated bagasse than the NH_4OH-pretreated bagasse at an equivalent glucan loading. Whereas cellobiose

Fig. 4 Release of cellobiose (**a**, **b**), glucose (**c**, **d**), xylose (**e**, **f**), and arabinose (**g**, **h**) from various concentrations (% glucan) of AFEX-pretreated (**a**, **c**, **e**, **g**) and ammonium hydroxide-pretreated (**b**, **d**, **f**, **h**) sugar cane bagasse by Spezyme CP (10 FPU/g glucan) and Novozym (20 CBU/g glucan) with (*solid bars*) and without (*open bars*) the addition of Multifect xylanase (10% protein;). The total protein concentration of Spezyme CP/Novozym 188 mixture was 1.146 mg/g glucan. *Vertical lines* indicate 95% bootstrap confidence intervals and means of *bars with identical letters* are not significantly different ($p>0.05$)


a
b
c
d
e
f
g
h
Cellobiose concentration (g/l)
Glucose concentration (g/l)
Xylose concentration (g/l)
Arabinose concentration (g/l)
% Glucan concentration
% Glucan concentration

accumulation was negligible at the various substrate loading of NH_4OH-pretreated bagasse (Fig. 2b), a significant amount of cellobiose was accumulated as the substrate loading of AFEX-pretreated bagasse was increased and the accumulation was significantly exacerbated ($p<0.05$) by absence of the Multifect xylanase (Fig. 2a). This suggests that additional β-glucosidase activity might be necessary at higher loadings of AFEX-pretreated bagasse. β-Glucosidase improves cellulose hydrolysis by reducing end-product inhibition of cellulolytic enzymes because of the accumulation of cellobiose. Evidently, at high substrate loading, the inhibition of β-glucosidase by glucose accumulation may have occurred [19], and glucan hydrolysis could be improved by further optimization of the ratio and levels of cellulase: β-glucosidase or by using a β-glucosidase preparation more resistant to glucose inhibition. A simultaneous saccharification and fermentation to ethanol system may also reduce end-product inhibition and not require additional β-glucosidase [9]. It is surprising to note that the percent hydrolysis of the glucan fraction of bagasse appeared to be unaffected by the load increase (data not shown).

A regression analysis of the relationship between xylanase activity added and sugar release found that increasing units of enzyme activity had significant impact on the release of glucose and of xylose (Fig. 5). The relationship between xylanase activity was significant ($p<0.01$) with commercial xylanases added to AFEX-pretreated ($p=0.0087$; Fig. 5g) and NH_4OH-pretreated ($p=0.0048$; Fig. 5h) bagasse, as well with laboratory-produced xylanases added to AFEX-pretreated ($p=0.0057$; Fig. 5i) and NH_4OH-pretreated ($p<0.0001$; Fig. 5j) bagasse. These results suggest that addition of xylanase boosts xylose release, and this was especially significant with the laboratory-produced xylanase on the NH_4OH-pretreated bagasse. When the release of glucose was analyzed, the addition of commercial xylanase had a highly significant effect on AFEX-pretreated ($p<0.0001$; Fig. 5a) and NH_4OH-pretreated ($p<0.0001$; Fig. 5b) bagasse. The effect of the laboratory-produced xylanase on glucose release was only significant on the NH_4OH-pretreated ($p<0.0021$; Fig. 5d) but not the AFEX-pretreated ($p=0.2137$; Fig. 5c) bagasse. These results point to the xylanase in the commercial preparations, in conjunction with possibly other cellulolytic enzymes acting in synergy to hydrolyze the bagasse. The laboratory-produced xylanase preparations mostly lacked cellulolytic activity (Table 1). The release of glucose from Avicel was only significant ($p=0.0004$) with the commercial xylanase preparations (Fig. 5e), whereas no relationship was observed between xylanase activity and glucose release when laboratory-produced xylanase preparations were added (Fig. 5f). This observation again points to the cellulolytic activities in the commercial xylanase preparations playing an important role in cellulose hydrolysis but the xylanase itself does not appear to have any specific activity on the cellulose and on glucose release. A similar observation was made by Berlin et al. [18] from their studies on the hydrolysis of softwood substrates. However, more in depth investigations with purified cellulolytic and hemicellulolytic enzymes could help to confirm these observations.

Conclusions

In conclusion, enzyme preparations have been assayed for their activities and certain preparations have been identified as being most suitable for use in hydrolysis experiments. A lack of detailed knowledge about the nature of the substrate after pretreatment (porosity, size, insolubility, and crystallinity of the macroscopic particles) required us to follow an empirical approach in this study. Furthermore, the role of each enzyme in the crude enzyme preparations is difficult to assess but fundamental studies have pointed to each enzyme as having a complementary role in the sequential hydrolysis of lignocellulose [9]. The boost in

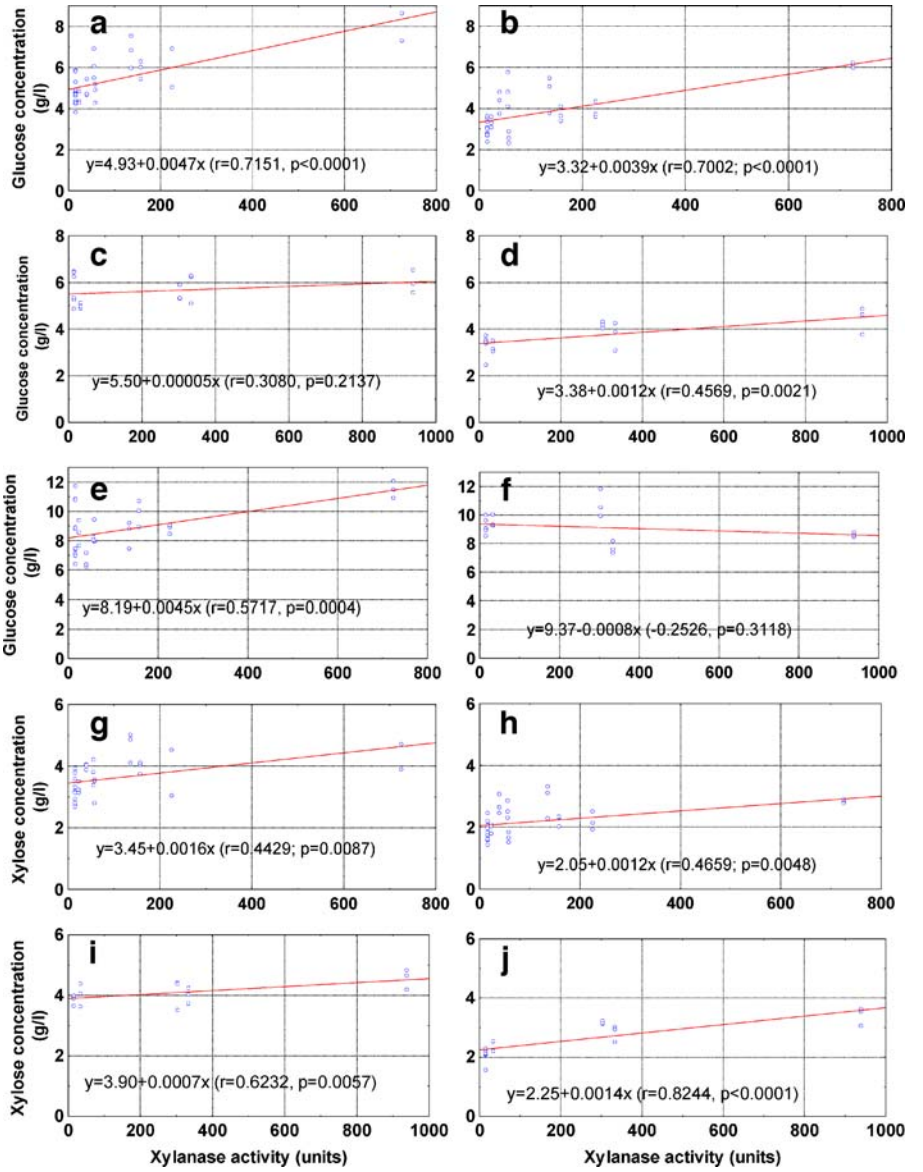


Fig. 5 Regression analysis of glucose (**a**–**f**) and xylose (**g**–**j**) release from AFEX-pretreated bagasse (**a**, **c**, **g**, **i**), NH_4OH-pretreated bagasse (**b**, **d**, **h**, **j**), and Avicel (**e**, **f**) hydrolyzed with Spezyme CP (10 FPU/g glucan) and Novozym 188 (20 CBU/g glucan) and supplemented with xylanase (10% protein addition) from either commercial (**a**, **b**, **e**, **g**, **h**) or laboratory-grown sources (**c**, **d**, **f**, **i**, **j**). The total protein concentration of Spezyme CP/Novozym 188 mixture was 1.146 mg/g glucan

hydrolysis of the xylan fraction but also the glucan fraction of pretreated bagasse by the addition of 10% protein level of a xylanase preparation to the Spezyme CP/Novozym 188 mixture could be interpreted in a number of ways. Previous studies have pointed to the removal of hemicellulose by xylanases increasing the accessibility of cellulose to cellulases [18]. The separation of the lignin fraction from the hemicellulose could also be important in

the overall hydrolysis as lignin acts as a competitive absorbent for cellulase and β-glucosidase [9] and the auxiliary enzymes present in the preparations used in this study might play a significant role. Future work should tailor the levels of xylanases to be added to cellulase/β-glucosidase enzyme mixtures to achieve substantial hydrolysis of AFEX-pretreated and NH_4OH-treated sugar cane bagasse. The development of an optimal cellulase/β-glucosidase/xylanase enzyme mixture is necessary to achieve efficient and economic bagasse hydrolysis.

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References

1. Lynd, L. R., Wyman, C. E., & Gerngross, T. U. (1999). *Biotechnology Progress, 15*, 777–793.
2. Knauf, M., & Moniruzzaman, M. (2004). *International Sugar Journal, 106*, 147–150.
3. Rosgaard, L., Pedersen, S., Cherry, J. R., Harris, P., & Meyer, A. S. (2006). *Biotechnology Progress, 22*, 493–498.
4. Martin, C., Galbe, M., Wahlbom, C. F., Hahn-Hägerdal, B., & Jönsson, L. J. (2002). *Enzyme and Microbial Technology, 31*, 274–282.
5. Kaar, W. E., Gutierrez, C. V., & Kinoshita, C. M. (1998). *Biomass and Bioenergy, 14*, 277–287.
6. Fox, D. J., Gray, P. P., Dunn, N. W., & Marsden, W. L. (1987). *Journal of Chemical Technology and Biotechnology, 40*, 117–132.
7. Holtzapple, M. T., Jun, J.-H., Ashok, G., Patibandla, S. L., & Dale, B. E. (1991). *Applied Biochemistry and Biotechnology, 28/29*, 59–74.
8. Van Zyl, C., Prior, B. A., & du Preez, J. C. (1988). *Applied Biochemistry and Biotechnology, 17*, 357–369.
9. Lynd, L. R., Weimer, P. J., van Zyl, W. H., & Pretorius, I. S. (2002). *Microbiology and Molecular Biology Reviews, 66*, 506–577.
10. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y. Holtzapple, M., et al. (2005). *Bioresource Technology, 96*, 673–686.
11. Adney, B., & Baker, J. (1996). NREL laboratory analytical procedure. In *Laboratory Analytical Procedure No. 006*. Golden, CO: National Renewable Energy Laboratory.
12. Wood, T. M., & Bhat, K. M. (1988). *Methods in Enzymology, 160*, 87–112.
13. Bailey, M. J., Biely, P., & Poutanen, K. (1992). *Journal of Biotechnology, 23*, 257–270.
14. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
15. Dale, B. E., & Moriera, M. J. (1982). *Biotechnology and Bioengineering Symposium, 12*, 31–43.
16. Singh, S., Pillay, B., Dilsook, V., & Prior, B. A. (2000). *Journal of Applied Microbiology, 88*, 975–982.
17. Singh, S., Pillay, B., & Prior, B. A. (2000). *Enzyme and Microbial Technology, 26*, 502–508.
18. Berlin, A., Gilkes, N., Kilburn, D., Bura, R., Matkov, A., Skomatovsky, A., et al. (2005). *Enzyme and Microbial Technology, 37*, 175–184.
19. Dekker, R. F. H. (1986). *Biotechnology and Bioengineering, 28*, 1438–1442.