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# Hydrolysis of Cellulose Using Ternary Mixtures of Purified Cellulases

JOHN O. BAKER,* CHRISTINE I. EHRMAN, WILLIAM S. ADNEY, STEVEN R. THOMAS, AND MICHAEL E. HIMMEL

*Biotechnology Center for Fuels and Chemicals, National Renewable Energy Laboratory, Golden, CO, 80401*

## ABSTRACT

The saccharification of microcrystalline cellulose by reconstituted ternary mixtures of purified cellulases (one endoglucanase and two cellobiohydrolases) has been studied over the entire range of mixture compositions. Ternary plots are used to compare the performance of five synthetic mixtures drawn from the cellulase systems of *Acidothermus cellulolyticus, Trichoderma reesei, Thermomonospora fusca,* and *Thermotoga neapolitana.* Results reveal that at least one synthetic mixture utilizing enzymes from three different organisms delivers performance competitive with that of a "native" (i.e., co-evolved) ternary system drawn exclusively from *T. reesei.* This heterologous system, consisting of the endoglucanase E1 from *A. cellulolyticus* and the exoglucanases CBHI from *T. ressei* and $E_3$ from *T. fusca,* is forgiving from the system-design point of view, in that it delivers high saccharification rates over a wide range of mixture compositions.

**Index Entries:** Cellulases; *Trichoderma reesei; Aspergillus niger; Acidothermus cellulolyticus; Thermomonospora fusca; Microbispora bispora.*

## INTRODUCTION

The potential of lignocellulose to provide fermentable sugars as a carbon source in the production of fuels and chemical feedstocks is now well appreciated *(1,2)*. It has long been recognized that cellulase activity is not the activity of a single enzyme, but the result of multiple activities working cooperatively, or synergistically, to efficiently solubilize crystalline cellulose *(3,4)*. There are three categories of cellulolytic enzymes

* Author to whom all correspondence and reprint requests should be addressed.

required for this process: endo-1,4-β-D-glucanases or endoglucanases (1,4-β-D-glucan-4-glucano-hydrolases; EC 3.2.1.74), which cleave glycosidic bonds randomly in the interior of the cellulose polymer chain; the exo-1,4-β-glucosidases, which include the 1,4-β-D-glucan glucohydrolases (EC 3.2.1.74), which liberate D-glucose from 1,4β-D-glucans and hydrolyze cellobiose slowly, and the 1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91), which liberate D-cellobiose from 1,4-β-glucans; and β-D-glucosidases, or β-D-glucoside glucohydrolases (EC 3.2.1.91), which relieve end-product inhibition of the endoglucanases and cellobiohydrolases by hydrolyzing the penultimate product, cellobiose, to the less inhibitory final product, glucose.

The development during the past two decades of powerful molecular-biological methods for identifying, transferring and/or modifying, and overexpressing the genetic material that encodes specific proteins, combined with the understanding that cellulase action is a multi-activity process, suggests that cellulase mixtures might be engineered by combining enzymes that originate from various organisms to form effective, and possibly improved, hybrid cellulase systems. Among the potential benefits offered by such heterologous systems are reductions in the production cost that result from fewer enzymes being expressed, higher specific activity of the protein expressed, and the ability to tailor enzyme systems in accordance with the demands of specific processes *(5)*.

In the present study, we have assembled an array of three endoglucanases (one fungal and two bacterial) from three organisms, plus three exoglucanases (two fungal and one bacterial), and have measured soluble-sugar production from ternary mixtures of these enzymes acting on microcrystalline cellulose. Each ternary system studied consisted of one endoglucanase, one exoglucanase (R) specific for the reducing cellulose terminus, and one exoglucanase (NR) specific for the nonreducing cellulose terminus. This paper presents the performance of selected cellulase mixtures using ternary contour diagrams to describe glucose production.

## MATERIALS AND METHODS

### Enzyme Purifications

Only highly purified enzymes were used to construct the cellulase cocktails evaluated in this study. The *T. fusca* enzymes *(6)*, $rE_3$ and $rE_3$ (the prefix "r" denotes a recombinant enzyme), were purified in the laboratory of D. Wilson at Cornell University from cell lysates of *Streptomyces liyidans* TK24 that contained a plasmid carrying the appropriate *T. fusca* gene *(7)*. rEndoglucanase A (rEndoA) from *Microbispora bispora*, expressed from a genomic fragment cloned in *Escherichia coli*, was purified in the laboratory of D. Eveleigh at Rutgers University *(8)*.

Enzymes purified at NREL for this study included *A. cellulolyticus* endoglucanase I, EI, *(9)* expressed from *S. lividans* TK24, plus a truncated form of this enzyme (rEIcd) produced by subjecting the *S. lividans*-expressed enzyme to proteolytic treatment to remove the cellulose-binding domain *(10)*, *T. reesei* endoglucanase EG I, *T. reesei* exoglucanases CBH I and CBH II, and *Aspergillus niger* β-glucosidase. *A. niger* β-glucosidase was purified chromatographically from samples of Novozym 188 Cellobiase (Novo Nordisk, Franklinton, NC), and the *T. reesei* enzymes were prepared from samples of Laminex Cellulase, Lot 13-90091-01, code 6-5960 (Genencor International, Palo Alto, CA) according to the procedures described by Baker et al. *(10,11)*.

The purity of each enzyme preparation used in this study was verified by silver-stained, sodium-dodecylsulfate polyacrylamide gel electrophoresis. In addition, the purity of the *T. reesei* cellulases was verified by Western blot analysis using monoclonal antibodies *(12)*. These preparations were found to have compositions of at least 98% of the protein intended for evaluation.

## Cellulose Digestions

Enzyme digestions of crystalline cellulose were carried out at 50°C in 50 m*M* acetate buffer, pH 5.0, to which 0.004% (w/v) sodium azide had been added to prevent microbial growth. Total cellulase loadings were held constant at 0.36 μ*M* in the 1.0-mL reaction mixtures, which contained as substrate 5% (w/v) microcrystalline cellulose (Sigmacell, Type 20, Product number S-3504, lot 79F-0454, Sigma, St. Louis, MO). The total weight of protein added varied slightly with the molecular weights of the components involved, but in all cases the total cellulase loading (endoglucanase plus exoglucanase) was close to 20 μg protein per mL of digestion mixture, or 0.4 mg cellulase per g cellulose. Sufficient purified *A. niger* β-glucosidase (4.17 μg/mL of digestion mixture, equal to 0.61 units/mL or 12.2 units/mg of cellulose) was added to eliminate the problem of cellobiose inhibition. As described previously *(11)*, the sufficiency of the β-glucosidase loading was established (for the individual enzymes and for representative mixtures) by means of experiments in which reducing sugar output was measured in the presence of various loadings of β-glucosidase, to determine loadings above which further increases in β-glucosidase loading produced no further increases in yield of reducing sugar. The standard loading described above, equal to 83.33 μg β-glucosidase per g cellulose, was approx 10 times the minimum loading required to render the reducing-sugar output independent of the β-glucosidase loading *(11)*.

To reduce the consumption of purified enzyme, a miniaturized digestion apparatus was devised, using 1.5-mL Wheaton autoinjector vials as reaction vessels. The enzyme cocktails (0.3 mL total for each digestion of endoglucanase, exoglucanase, and β-glucosidase) for the various digestion mixtures were first placed in the vials, then substrate was added (as

0.7 mL of a 7.15% stirred slurry) to initiate the reaction. The vials were sealed with aluminum crimp-caps (PTFE-faced silicone septa, Kimble Glass, Vineland, NJ), placed in a custom-built rotator head immersed in a 50°C water bath, and continuously mixed by inversion at 10 rpm. After a standard 120-h digestion period, representative 0.04-mL aliquots of each (well-dispersed) digestion mixture were withdrawn, diluted to 2.0 mL with deionized water, and centrifuged to remove all solid substrate.

### Analysis of Soluble Sugars Released

Sugar concentrations in aliquots harvested from the reaction vials were determined by ion-moderated partition chromatography on a Bio-Rad HPX-87P (lead-form; Hercules, CA) carbohydrate analysis column also equipped with a Bio-Rad deashing precolumn. This column system was installed in a Hewlett-Packard 1090 chromatograph and operated at 85°C with deionized water as mobile phase at a flow rate of 0.6 mL/min. The amount of glucose and cellobiose present in each sample was quantified by comparing the area of the peak against a linear calibration curve.

## RESULTS AND DISCUSSION

### Selection of Enzymes

The seven enzymes selected for this study included four endoglucanases along with examples of exoglucanases specific for either the reducing terminus or the nonreducing termini of the cellulose chain. Five ternary systems were developed using these purified cellulases. Table 1 shows some fundamental characteristics of the cellulases chosen for this study. Although many other cellulase component enzymes have been reported in the literature, the enzymes used in this study are well-characterized and either readily available in high purity as single-gene, recombinant products, or, in the case of the *T. reesei* enzymes, readily purified from commercial preparations. The choice of 50°C, and pH 5.0 as digestion conditions was made to accomodate the less thermostable *T. reesei* enzymes *(13)*, which constitute the reference system for our comparisons. Several other enzymes in this study are significantly more thermostable than the three *T. reesei* enzymes used. The *T. fusca* endoglucanase $E_5$ and the NR cellulose-terminus-specific exoglucanase, $E_3$ from the same organism have, respectively, temperature optima of 55 to 60° and 65°C in the hydrolysis of filter paper (D. Wilson, personal communication). *A. cellulolyticus* EI is even more thermostable, being optimally active against carboxymethylcellulose between 80 and 85°C *(9)*. Should a more thermostable reducing-terminus-specific (i.e., CBH I-like) exoglucanase be made available, either by characterizing a new enzyme or modifying CBH I itself, experiments run at higher temperatures will probably show EI, $E_3$, and $E_3$ to even greater advantage than is the case in the present study.

Table 1
Characteristics of the Exo- and Endoglucanases Used in This Study

| Enzymes Studied | Glycosyl Hydrolase Family[a] | Cellulose Terminal Specificity | Product Stereo-chemistry | Form of Enzyme in Study | Thermal Tolerant |
|---|---|---|---|---|---|
| *T. reesei* CBH I | 7 | reducing | retaining | native | no |
| *T. reesei* CBH II | 6 | non-reducing | inverting | native | no |
| *T. fusca* $E_3$ | 6[b] | non-reducing[b] | inverting[b] | recombinant | moderately |
| *T. reesei* EG I | 7 | none | retaining | native | no |
| *A. cellulolyticus* EI | 5 | none | retaining | recombinant | yes |
| *T. fusca* $E_5$ | 5 | none | retaining | recombinant | moderately |
| *M. bispora* Endo A | 6 | none | inverting | recombinant | yes |

[a] From Bairoch *(14)*.
[b] From D. Wilson (personal communication, 1997).

## Summary of Saccharification Performance

The saccharification of microcrystalline cellulose by reconstituted ternary mixtures of purified cellulases (one endoglucanase and two exoglucanases) has been studied over the entire range of mixture compositions, and the results are shown in Figures 1–5 as ternary contour plots of the percentage of cellulose converted to soluble sugars. The actual compositions used in the experiments are indicated by the nodes (small circles). The contour lines in these figures represent a surface fitted to the experimental results using the program Statistica (Statsoft, Tulsa, OK) with the cubic spline option chosen. Assays measuring the activities of mixtures of the three *T. reesei* enzymes (Fig. 1) and those involving rEIcd and the two *T. reesei* exoglucanases (Fig. 3) were carried out in duplicate; the other experiments relied on single assays. For the assays run in duplicate, the average deviation of the duplicates from the mean was 4.2%.

The results shown in Figs. 1–5 can be compared in at least two ways. The first and simplest comparison is on the basis of maximum sugar yields, as shown in Table 2. The second way of comparing the systems considers the extent to which the system is forgiving in terms of deviations of the composition from the optimum. A useful measure of the forgiving nature of a system is the area on the ternary plot over which the sugar release is at least 85% of the maximum. For all plots shown, this area is fairly closely approximated by the two highest contour zones. In viewing these plots, the reader should bear in mind that the detail of the plots is limited by

Table 2
Maximum Saccharification from Selected Ternary Cellulase Digestions of Sigmacell-20

| Ternary Cellulase System | Ratio Giving Highest Sugar Release (%) | Highest Sugar Release (%) |
|---|---|---|
| CBH I:CBH II:EG I | 60:20:20 | 16.5 |
| CBH I:CBH II:EI | 40:20:40 | 16.2 |
| CBH I:$E_3$:EI | 40:20:40 | 14.1 |
| CBH I:$E_3$:$E_5$ | 60:20:20 | 11.3 |
| CBH I:$E_3$:Endo A | 40:40:20 | 11.6 |

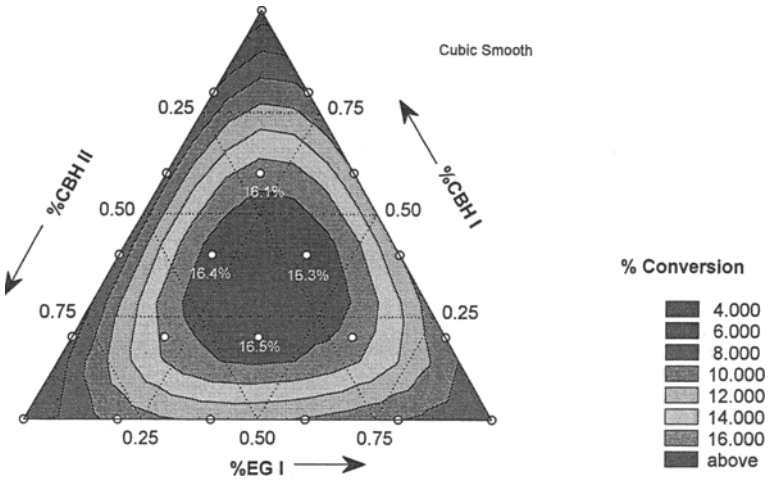


Fig. 1. Ternary plot of the saccharification of Sigmacell-20 using purified *T. reesei* CBH I, *T. reesei* CBH II, and *T. reesei* EG I. The apex indicated by each arrow represents a composition of 100% of the indicated cellulase; data points on the opposite side of the triangle represent assays that have the concentration of that cellulase set at zero.

both the relatively low resolution of the distributions of compositions (the "step" in composition being 20% in all plots), and by the relatively "stiff" surface-fitting routine employed. For instance, the authors have partial ternary diagrams run at higher resolution (data not shown) revealing that the high-yield plateau for the system of EG I, CBH II, and CBH I (Fig. 1) actually extends farther into the 100%-CBH I apex of the plot than is shown by the present plots.

The low-resolution but full-range plots shown here do, however, support some potentially useful observations. Comparison of the performance of five synthetic mixtures drawn from the cellulase systems of *A. cellulolyti-*

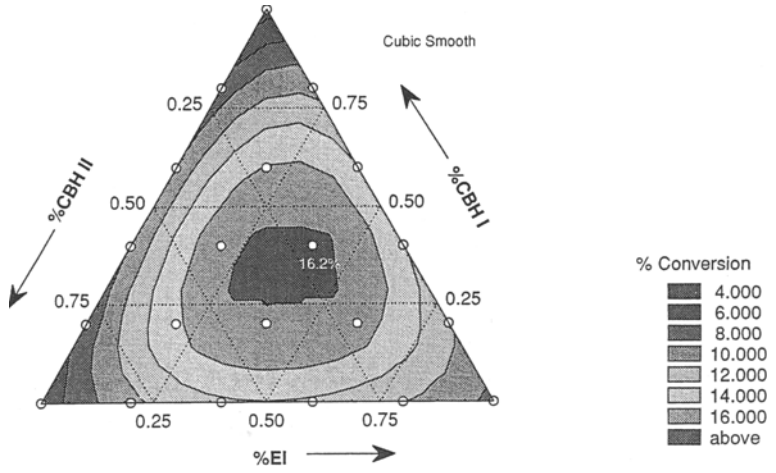


Fig. 2. Ternary plot of the saccharification of Sigmacell-20 using purified *T. reesei* CBH I, *T. reesei* CBH II, and *A. cellulolyticus* EI.

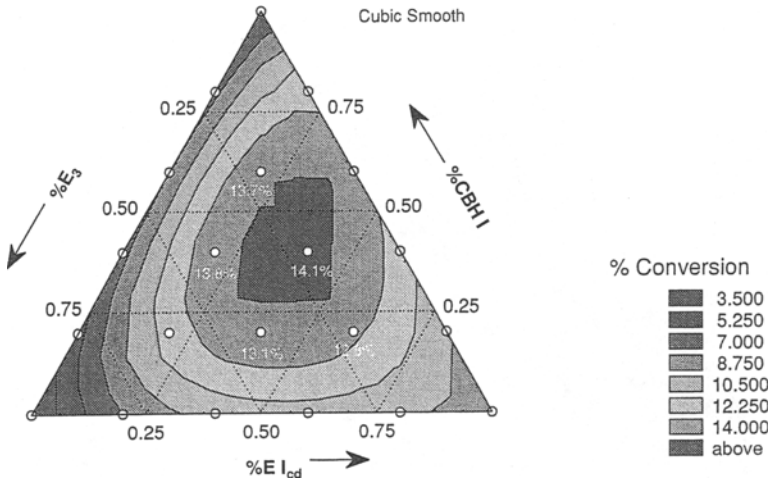


Fig. 3. Ternary plot of the saccharification of Sigmacell-20 using purified *T. reesei* CBH I, *A. cellulolyticus* rEIcd, and *T. fusca* $F_3$.

*cus*, *T. reesei*, *T. fusca*, and *M. bispora*, reveals that the performance of at least one completely heterologous synthetic mixture utilizing enzymes from three different microorganisms is competitive in both senses with the performance of a native (i.e., evolved) ternary system drawn exclusively from *T. reesei*. This heterologous system, which consists of the catalytic domain of endoglucanase EI from *A. cellulolyticus* (EIcd) and the exoglucanases CBH I from *T. reesei* and $E_3$ from *T. fusca*, is a forgiving system from the system-design point of view in that it delivers high saccharification rates over a wide range of mixture compositions. A

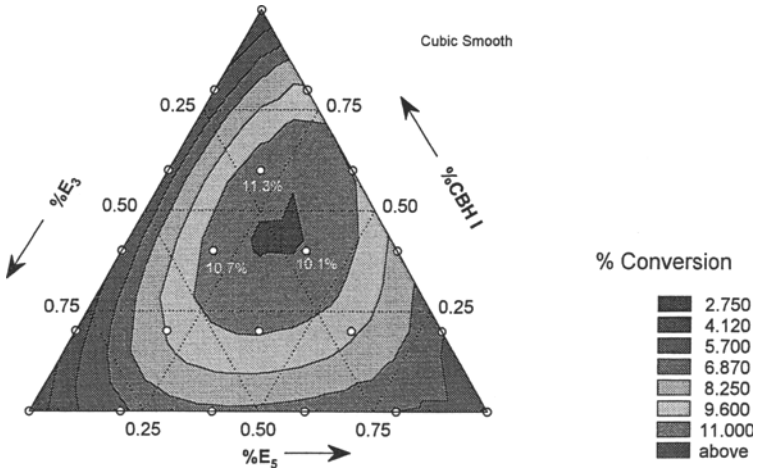


Fig. 4. Ternary plot of the saccharification of Sigmacell-20 using purified *T. reesei* CBH I, *T. fusca* $E_3$, and *T. fusca* $E_3$.

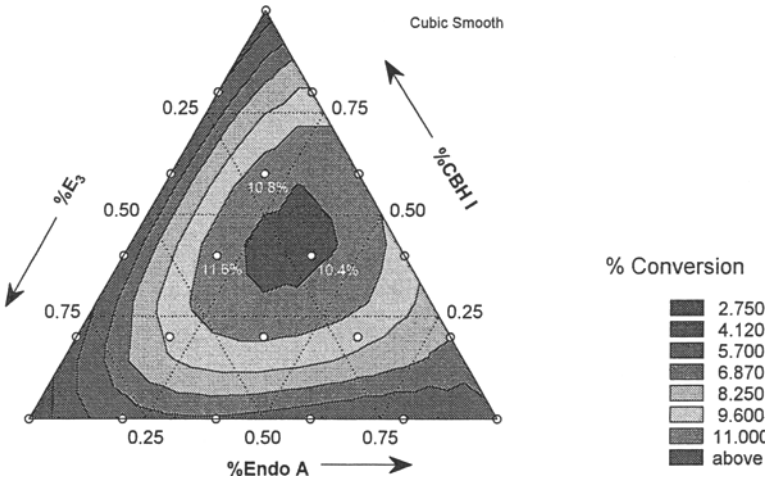


Fig. 5. Ternary plot of the saccharification of Sigmacell-20 using purified *T. reesei* CBH I, *T. fusca* $F_3$, and *M. bispora* EndoA.

two-organism system drawn from *A. cellulolyticus* (EI) and *T. reesei* (CBH I and CBH II) is also competitive with the native coevolved *T. reesei* system. The other completely heterologous system (*M. bispora* endoglucanase-A with *T. fusca* $E_3$ and CBH I, Fig. 5) and the other two-organism system (endoglucanase $E_5$ and exoglucanase $E_3$ from *T. fusca* with *T. reesei* CBH I, Fig. 4) are both less efficient and less forgiving in terms of composition than the systems of Figs. 1–3, but still illustrate that enzymes from different organisms, and indeed from different phylogenetic kingdoms, can work effectively together.

## ACKNOWLEDGMENTS

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