Volumes 107, Number 1 - 3 Spring 2003, ISSN: 0273–2289

Applied Biochemistry and Biotechnology

Biotechnology for Fuels and Chemicals

The Twenty-Fourth Symposium

Editors

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HUMANA PRESS

0273-2289/03/105-108/0689/$20.00

Automated Filter Paper Assay for Determination of Cellulase Activity

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Abstract

Recent developments in molecular breeding and directed evolution have promised great developments in industrial enzymes as demonstrated by exponential improvements in β-lactamase and green fluorescent protein (GFP). Detection of and screening for improved enzymes are relatively easy if the target enzyme is expressible in a suitable high-throughput screening host and a clearly defined and usable screen or selection is available, as with GFP and β-lactamase. Fungal cellulases, however, are difficult to measure and have limited expressibility in heterologous hosts. Furthermore, traditional cellulase assays are tedious and time-consuming. Multiple enzyme components, an insoluble substrate, and generally slow reaction rates have plagued cellulase researchers interested in creating cellulase mixtures with increased activities and/or enhanced biochemical properties. Although the International Union of Pure and Applied Chemists standard measure of cellulase activity, the filter paper assay (FPA), can be reproduced in most laboratories with some effort, this method has long been recognized for its complexity and susceptibility to operator error. Our current automated FPA method is based on a Cyberlabs C400 robotics deck equipped with customized incubation, reagent storage, and plate-reading capabilities that allow rapid evaluation of cellulases acting on cellulose and has a maximum throughput of 84 enzyme samples per day when performing the automated FPA.

Index Entries: Filter paper assay; cellulase; cellulose; *Trichoderma reesei*; filter paper unit.

Introduction

Cellulose, an unbranched β-1,4-linked homopolymer of glucose, is the most abundant renewable fuel resource on Earth, accounting for about half

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of the organic material in the biosphere, and it is the major polysaccharide found in plant biomass. The hydrolysis of cellulose, aided by endocellulase, exocellulase, and β-D-glucosidase catalysis, produces glucose, an easily fermentable monosaccharide. Intense research is currently aimed at the conversion of cellulose to sugars and ethanol via fermentation because this process has great economic potential and is environmentally friendly *(1–3)*. Unfortunately, the main impediment for ethanol production via enzymatic saccharification of cellulose is the low activity of native cellulases *(4,5)*.

Cellulose is insoluble and crystalline; hence, it is largely resistant to enzymatic hydrolysis. In many biomass utilization schemes, the raw material is first treated with dilute acid at moderate temperatures *(6)* to remove hemicellulose and to speed up subsequent cellulose hydrolysis by enzymes. The pretreated biomass can then be subjected to carefully chosen mixtures of endo- and exoglucanases for maximum cost-effectiveness *(7,8)*.

The advent of in vitro molecular evolution techniques has opened up a new area for enzyme improvement. Although these techniques have demonstrated improvements in several enzymes, the axiom "You get what you screen for" remains the dominant truth in the high-throughput screening (HTS) arena *(9,10)*. This certainty, however, is conditional. You must have both an HTS-compatible expression host and a suitable screening method in order to accomplish anything. Typically, the host is *Escherichia coli*, and the screening involves either improvement-dependent growth or colorimetric discrimination between improved and unimproved enzyme. For cellulase enzymes, HTS-compatible host expression has been limited to either bacterial enzymes or fungal endocellulases. Currently, fungal exocellulases have not been demonstrated to be capable of native-like expression in an acceptable HTS host. Additionally, coexpression of an intact whole cellulase system has not been demonstrated outside of native systems. Therefore, detection of improvements to enzyme activity is limited to single enzymes expressed in non-HTS hosts.

Worldwide, screening of whole cellulase preparations is done predominantly using the International Union of pure and Applied Chemists (IUPAC) standard filter paper assay (FPA). This assay, developed by Ghose in 1984 *(11)*, uses the dinitrosalicylic acid (DNS) method to determine reducing sugars released from 50 mg of Whatman #1 filter paper by a complex cellulase mixture. The method requires simple reagents and equipment, but it is plagued by long assay times, exacting dilutions, and many manual manipulations. It is valid only at low levels of hydrolysis and generally requires several iterations to pin down a valid activity measurement. Although Whatman #1 filter paper is fairly ubiquitous and readily available in most laboratories, it requires careful manual manipulation in order to partition it evenly into 1×6 cm strips (~50 mg). Inconsistencies in size, shape, and folding methods can all lead to errors in activity determination. In addition, because of the filter paper's physical properties, it is very difficult to distribute it evenly through automated methods, especially if the scale is reduced to microtiter plate levels. We

examined several cellulose compounds for their hydrolytic similarities to Whatman #1 filter paper, including Solka-Floc®, SigmaCell-20, Avicel, and cotton linters. Although these substrates differ in their physical properties from Whatman #1 filter paper, they have the distinct advantage of being capable of forming pipetable slurries in aqueous solution.

One of the most confounding problems is the determination of the initial dilution needed to bracket 3.6% hydrolysis (The 4% usually stated does not account for the water added during hydrolysis). Because cellulase activity in whole broths is nonlinear in regarding enzyme concentration, this atypical assay dictates dilution of the cellulase preparation to a point where 2.0 mg of reducing sugar equivalents is released in 1 h at 50°C and pH 4.8. This amount of enzyme is defined as one filter paper unit (FPU). Traditionally, a wide dilution range is used and then the assay is repeated to fine-tune the level of confidence. For commercial broths, the dilution required is usually considerable, since the starting titers are often >50 FPU/mL. These high dilutions can result in significant error if not done carefully.

Recent advances in HTS equipment have led us to automate this traditionally manually intensive assay. Although the intent behind the project was to streamline cellulase production determination from fungal strains producing complete cellulase systems, it should be possible to assess the effect of alterations in a single component by augmenting the single enzyme with the other required components. The goals of the present study were to reduce operator involvement and potential operator error in determining the activity of cellulase preparations, to reduce the amount of reagent usage and associated disposal costs, and to allow high throughput of samples through automation of the assay.

Materials and Methods

Reagents

The enzyme used in this study was a commercial broth obtained from Genencor (Palo Alto, CA) formulated for commercial sale and storage. Protein concentration was 108 mg/mL and the traditional FPU value was determined to be 38.6 FPU/mL. The buffer used was 50 m*M* citrate, pH 4.8. The reducing sugar reagent was the standard FPA DNS reagent containing 1416 mL of deionized water, 10.6 g of 3,5-dinitrosalicylic acid, 19.8 g of NaOH, 306 g of Rochelle salts (Na-K tartrate), 7.6 mL of phenol, and 8.3 g of sodium metabisulfite. Glucose (3.0 mg/mL) was used as the reducing sugar stock standard and was diluted to produce a standard curve. All chemicals were ACS reagent grade or better and were obtained from Sigma-Aldrich (St. Louis, MO).

Substrates

The substrates assayed included Whatman #1 filter paper (1 × 6 cm strips for traditional method, 1/4 in. diameter circles for microtiter plate

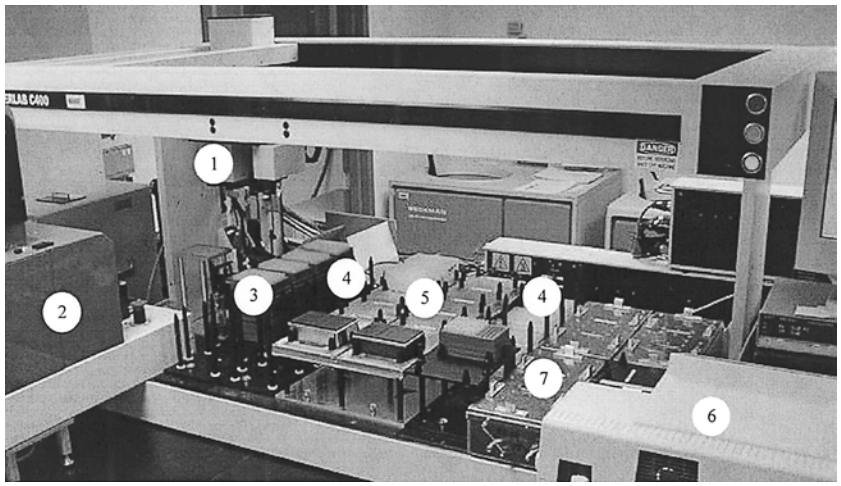

Fig. 1. Layout of Cyberlabs C400 robotics deck. 1, probe head; 2, ALPS plate sealer; 3, tip storage; 4, plate storage; 5, chilled reagent/plate rack; 6, plate reader; 7, incubators.

scale), Solka-Floc (Brown, Berlin, NH), SigmaCell-20 (Sigma-Aldrich), Avicel PH101 (FMC, Philadelphia, PA), and cotton linters (Fluka/Sigma-Aldrich, St. Louis, MO). The cellulose powders were suspended in distilled H_2O at 2.65% (w/v). Glycerol was added to 1.5% (v/v) to prevent the dried pellets from electrostatically jumping out of the wells.

Hardware

The assay was developed on a Cyberlabs C400 XYZ chassis robotics deck (Cyberlabs, Inc., Brookfield, CT) (Fig. 1). The deck consisted of a probe head with single-, 8-, and 96-channel pipettor probes as well as a center-pull gripper hand. The deck of the robot was laid out with racks for pipet tip, assay plate, and dilution plate storage; a 96-channel tip installation station; a 96-channel O-ring greasing station; reagent reservoirs; an ALPS-100 automated plate sealer (Advanced Biotechnologies, Surrey, UK); a barcode reader; a waste chute; a chilled elevated rack for assay setup and enzyme and reagent storage; three independently controlled and electronically heated microtiter plate incubators (Fig. 2A), and a SpectraMax 190 microtiter plate reader (Molecular Devices, Sunnyvale, CA). The system was controlled through CYBOS operating software. The incubators were originally developed at Cyberlabs and modified at National Renewable Energy Laboratory by our in-house machine shop. This modification included the addition of machined aluminum form-fitting inserts designed to enhance heat transfer to the bottom of the assay plates, brass insert guides to assist in placement of the plates on the form-fitting inserts, and brass plate lids designed to passively heat the plates from the top and weigh down the plates to ensure good contact with the inserts and prevent plates from warping at high temperatures (Fig. 2B). The incubators were controlled through independent

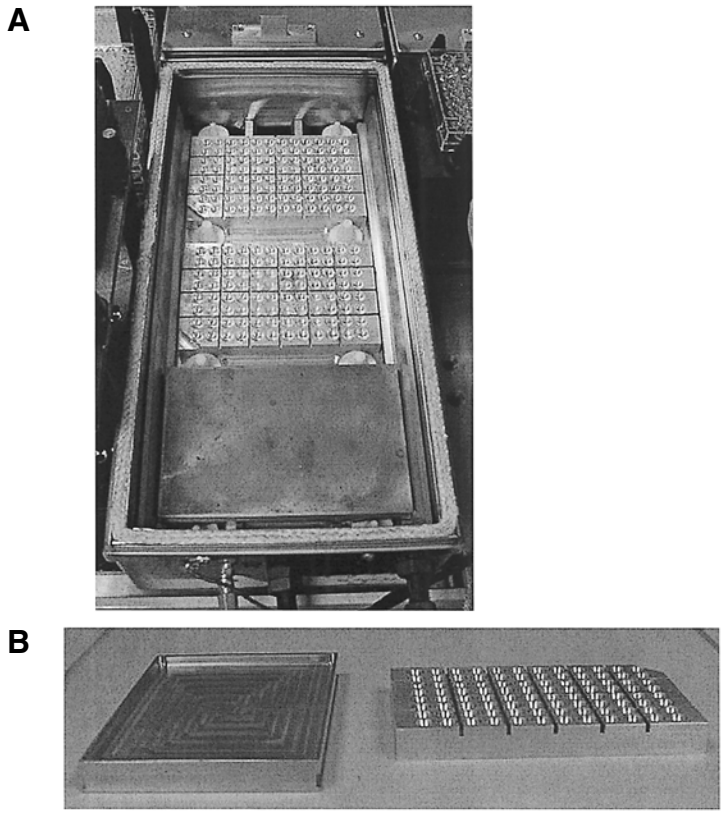

Fig. 2. Custom-modified microtiter plate incubators showing (**A**) brass insert guides, lid, and aluminum form-fitting inserts and (**B**) closeup of brass plate lid and aluminum insert. The gripper arm of the probe head moves the incubator lids and the individual brass plate lids.

Digi-Sense temperature controllers (Cole-Parmer, Vernon Hills, IL) using Type J thermocouples. Output data from the SpectraMax plate reader were exported as ASCII text and analyzed in Excel. The chilled deck was cooled by circulating a of coolant through the deck using a Julabo F240 recirculating cooler (Julabo, Allentown, PA).

Calculations

The standard FPA uses 50 mg of filter paper (FP) in 1.5 mL of assay solution. For the automated assay, the reaction was carried out in a 96-well, polypropylene, round-bottomed microtiter plate with a capacity of 300 µL/well. The volume of reaction mix in each well of the automated assay was set at 0.080 mL:

$$\frac{50 \text{ mg FP}}{1.50 \text{ mL}} \times \frac{0.080 \text{ mL}}{\text{well}} = 2.67 \text{ mg cellulose/well}$$

A standard 1/4-in. office paper punch yielded filter paper disks with an average weight of 2.65 mg, so this amount was used for each substrate. The target concentration for each well was 2.65 mg of cellulose/0.080 mL or 33.1 mg/mL, which compares with 33.3 mg/mL for the standard FPA.

The standard FPA targets 2 mg of reducing sugar released from 50 mg of filter paper, equivalent to 3.6% hydrolysis. Note that this is not given in units of concentration. In the standard FPA, the reaction volume is 1.5 mL and a concentration of 1.333 mg/mL of glucose (glc) equivalents comprises 2.0 mg in 1.5 mL. Because the standard assay looks for the release of a predefined amount of reducing sugar (2 mg) and not the concentration of product, a conversion is needed to adjust the standard 2.0 mg of glucose equivalents released to a concentration equivalent to 3.6% hydrolysis. Owing to the reduction in substrate amount and reaction volume, the required release of sugar to give 3.6% hydrolysis needed to be determined:

$$\frac{2 \text{ mg glc/mL}}{50 \text{ mg cellulose}} \times \frac{1.0 \text{ mg cellulose}}{1.11 \text{ mg glc}} \times 100 = 6.3\% \text{ conversion}$$

$$0.036 \times \frac{2.65 \text{ mg cellulose}}{\text{well}} \times \frac{1.11 \text{ mg glc}}{\text{mg cellulose}} \times \frac{\text{well}}{0.080 \text{ mL}} = 1.325 \text{ mg glc/mL}$$

Because the FPA is based on total product present at a specific time in a nonlinear reaction, a conversion factor is used to approximate a specific activity-type unit. The standard FPA bases this conversion on 0.5 mL of enzyme, even though the total reaction mix is 1.5 mL. In the automated assay, the enzyme and buffer are premixed and added together, resulting in a different volume factor. The two conversion factors are calculated as follows:

Standard:

$$\frac{2.0 \text{ mg glc}}{0.5 \text{ mL}} \times \frac{1 \text{ µmol glc}}{0.18016 \text{ mg glc}} \times \frac{1}{60 \text{ min}} = 0.37 \text{ µmol glc/(min} \cdot \text{mL)}$$

Automated:

$$\frac{1.325 \text{ mg glc}}{\text{mL}} \times \frac{1 \text{ µmol glc}}{0.18016 \text{ mg glc}} \times \frac{1}{60 \text{ min}} = 0.123 \text{ µmol glc/(min} \cdot \text{mL)}$$

Evaporative Losses

Methods were examined to determine the extent of water loss from the plates under various incubation conditions, including uncovering the plates; humidifying the incubator with water-saturated filter pads; and using plastic lids, mineral oil overlays, or preheated brass lids. Each method was tested by filling the plate with 80 or 300 µL of water, weighing, and heating it at 50°C for 1 h. After incubation, the plates were weighed and average water loss per well was determined.

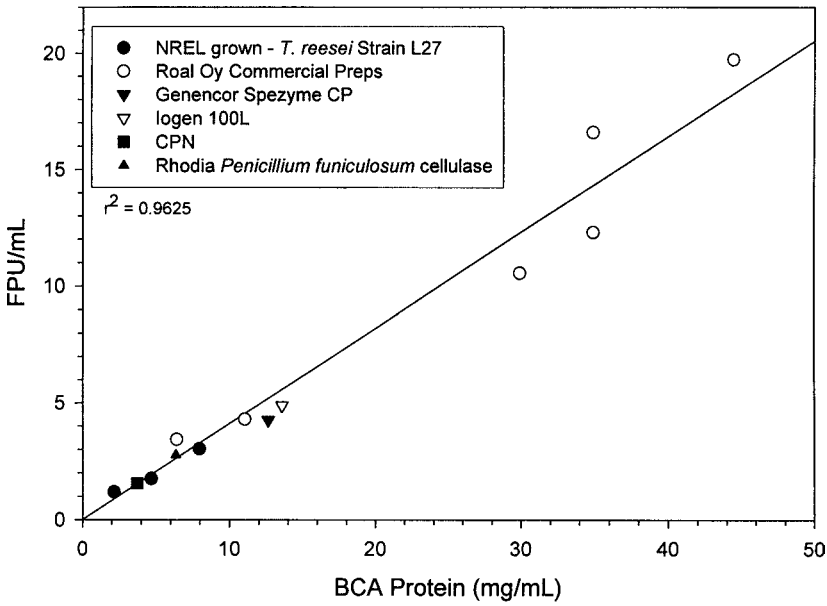

Fig. 3. Commercial and in-house-produced *T. reesei* cellulase preparations assayed for protein content and FPU activity plotted to determine correlation between the two factors. FPUs were determined using the standard FPA, and protein concentration was determined using the Pierce BCA method following desalting chromatography into 20 m*M* acetate, 100 m*M* NaCl, pH 5.0 buffer to remove interfering compounds.

Protein Determination

Cellulase preparations were assayed using the traditional FPA and the FPU value was plotted against total protein as determined using the Pierce micro–bicinchoninic acid (BCA) method (Pierce Endogen, Rockford, IL) standardized using bovine serum albumin (Fig. 3). The protein concentration of the cellulase preparations was measured following desalting of the enzyme sample into 50 m*M* acetate, pH 5.0 buffer using a HiPrep 26/10 desalting column prepacked with Sephadex G-25 with a nominal exclusion limit of 5×10^3 as specified by the manufacturer (Amersham Pharmacia Biotech, Uppsala, Sweden). The samples were desalted to eliminate interfering compounds in the preparations, and to standardize the sample buffers. Only the macromolecular fractions were collected and assayed according to the manufacturer's instructions.

Enzyme Assays

The traditional FPA was carried out according to Ghose *(12)*. The paper was carefully rolled and inserted into the assay tubes in order to minimize irregular and broken fibers that can affect the assay. For the scaled-down automated version, the assays were carried out in 96-well polypropylene microtiter plates containing a 1/4-in.-diameter filter paper disk in each well. The disks were cut with a standard office paper punch and loaded into each

well by hand using forceps. Since this became very tedious very quickly, alternative powdered cellulose substrates were explored. These substrates were also loaded at 2.65 mg/well in a 96-well polypropylene microtiter plate. A 2.65% (w/v) slurry containing 1.5% glycerol was stirred overnight at 4°C and dispensed by hand from a constantly mixing reservoir using a 12-channel pipettor equipped with wide-bore pipet tips. One hundred microliters was dispensed into each well of the assay plate, and the plates were dried for 2 d at room temperature. Initially, the plates were made without glycerol, but after drying, many of the cellulose pellets spontaneously jumped out of the wells, either while in storage on the bench or while being moved around the deck. Glycerol was added in an attempt to alleviate this problem. A concentration of 1.5% was determined to be the minimum needed to prevent the substrate from popping out of the wells on drying. Regardless of the substrate used, each assay was run with duplicate substrate plates and a nonsubstrate-containing control plate. Each assay required two assay plates to be made, and the validation consisted of four assays per run.

The automated assay was carried out as follows. Two substrate plates and a blank plate were set on the deck. The enzyme preparation to be tested was diluted 1:100 or 1:200 and placed on the deck in the first seven rows of each column of the enzyme master plate. A glucose standard of 3.0 mg/mL was placed in row eight of each column. Using an eight-channel pipettor, the robot proceeded to make further dilutions of the enzyme and glucose standard in a 96-well polypropylene master plate. Column 1 contained undiluted enzyme, columns 2–10 contained dilutions of 1:1.11, 1:1.25, 1:1.43, 1:1.67, 1:2, 1:2.5, 1:3.33, 1:5, and 1:10, respectively. Columns 11 and 12 contained only buffer. Dilutions were made with 50 m*M* citrate buffer, pH 4.8. After making the dilutions, the 96-channel pipettor was used to mix the diluted samples by titration and dispense 80 µL to each well of each assay plate: two with substrate and one control plate without substrate. After dispensing, the plates were moved to the 5°C incubator, covered with preheated brass lids, and incubated for 60 min. The plates were then transferred back to the deck and 150 µL of DNS reagent was added to each plate using the 96-channel pipettor. After mixing by titration with the 96-channel pipettor, the plates were incubated at 98°C for 10 min, again with preheated brass lids. Plates were then relocated back to the deck, and 96-well polystyrene dilution plates were set up containing 200 µL of deionized water. A 10-µL aliquot from each assay plate (with and without substrate) was aspirated using the 96-channel pipettor, dispensed into a dilution plate, and mixed by titration (repeated aspiration and dispensing). A Spectramax 190 microtiter plate reader read the dilution plates at a wavelength of 540 nm. The data collected was sent to an ASCII file and imported into Excel. The assay plates, dilution plates, and master plate were all sent to the waste stacks, and the process was started over with the next set of enzymes. This process was repeated for up to six replicates before the robot ran out of tips and plates. Forty-two samples per run (six replicates × seven samples per replicate) were assayed, with two runs being carried out per d.

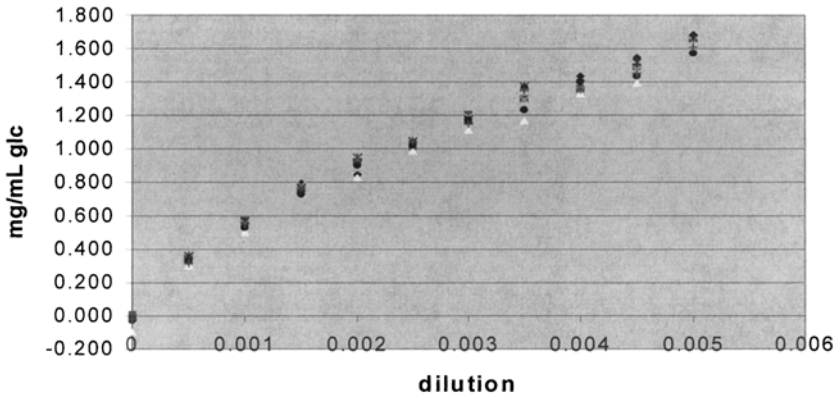

Fig. 4. Tests of evaporation control carried out in the microtiter plate incubators. Ninety-six-well polypropylene microtiter plates were filled with the indicated amount of distilled water, weighed, and incubated at 50°C under the various conditions for 1 h, and weighed again. Average water loss per well was calculated as (initial wt – final wt)/96 wells and converted to a percentage based on initial water volume per well. Each bar represents data from a single 96-well plate (three plates per condition).

Data Analysis

The data exported to Excel contained absorbance data for duplicate substrate assay plates and a no-substrate control plate, each with its own internal glucose standard and blanks. To correct for any potential uneven heating in the three plates, each plate was calculated based on its own internal glucose standard. Background color development was accounted for by subtracting the average of the last two columns (no enzyme) from each column for each plate. The no-substrate control plate was then subtracted from each substrate assay plate. The resulting reducing sugar concentrations were plotted against enzyme dilution, and the dilution yielding 1.325 mg/mL glucose (3.6% hydrolysis) was determined by interpolation of the closest two points. This dilution was then divided by the conversion factor to attain the FPU number.

Results

Evaporation Studies

Tests of evaporation control carried out in the microtiter plate incubators indicated that no lids gave a base loss of approx 45% from 80 µL at 50°C for 1 h (Fig. 4). To minimize this loss, several additional methods were evaluated. Layering mineral oil over the sample was attempted but showed a decrease to only a 25% loss. Placing standard polystyrene microtiter plate lids on the plates or humidifying the chamber by placing water-saturated filter paper pads along the sides reduced the loss to approx 13%. The result of using heated brass lids was a water loss of only 7% during the test conducted at 50°C for 60 min.

Table 1
Summary of FPU Determination
by Automated Assay on Different Cellulosic Substrates

		Automated Measurement	
Substrate	No. of replicates	Average FPU/mL	SD
Whatman #1 FP	28	62.6	17.2
Whatman #1 FP	28	58.2	6.8
SigmaCell-20	28	33.4	3.9
SigmaCell-20	21	37.1	3.2
Avicel PH101	28	27.8	3.4
Solka-Floc	28	34.7	6.2
Cotton linters	21	14.1	1.2
Whatman #1 FP traditional method	—	38.6	—

Automated FPA

Determination of the degree of hydrolysis of Whatman #1 filter paper by a known cellulase was carried out in both normal scale and miniature scale. The standard method, carried out by hand, resulted in an FPU activity of 38.6 FPU/mL in the commercial preparation. Assaying the same cellulase mixture on Whatman #1 filter paper and other cellulose substrates in microtiter plates resulted in a varying range of activities. In the microtiter plate–based assays carried out on the C-400, the commercial cellulase had apparent FPA activities of 60.4 FPU/mL on filter paper, 35.2 FPU/mL on SigmaCell-20, 34.7 FPU/mL on Solka-Floc, 27.8 FPU/mL on Avicel PH101, and 14.1 FPU/mL on cotton linters. These results are summarized in Table 1. Representative curves for each substrate are shown in Fig. 5.

Discussion

Cellulase Assays

Current literature describing the assay of total cellulase activity (or of individual component enzymes) has broadened considerably since the first reports by Mandels et al. *(13)* that reducing sugar release and substrate weight loss could serve as suitable cellulase assay methods. To some extent, and for appropriate substrates, these methods are still considered generally adequate and the basis for numerous product surveys *(14)*. However, considering the focus in biomass biotechnology on cellulase improvement, and the desire to compare cellulase preparations rapidly using smaller qualities of sample, application of laboratory automation to cellulase performance measurement is important. To put the automated assays in proper context, we review next the current state of the art for cellulase assays.

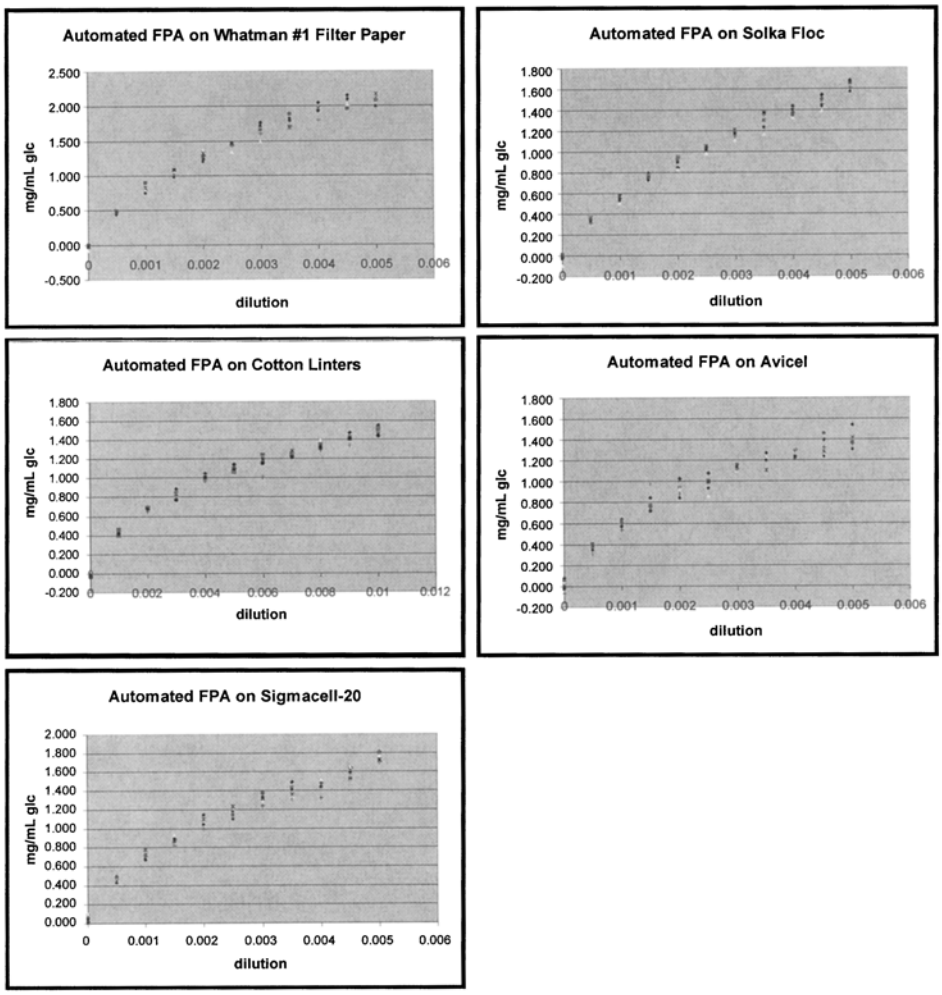

Fig. 5. Microtiter plate–based assays carried out on modified Cyberlab C400 system. Reducing sugar equivalents concentrations (mg/mL of glucose) were determined using the DNS method and plotted against various enzyme dilutions used to generate the reducing sugar equivalents.

IUPAC Methods

As a result of significant effort by an international committee of cellulase researchers and the IUPAC, a procedure was published in 1987 describing the use of filter paper and measurement of reducing sugar by the DNS method of Miller *(15)* in the context of a highly specific assay protocol *(12)*. In fact, the text of this protocol must be followed carefully to achieve comparable results. The rationale developed in this IUPAC method is that to be maximally useful, all assays for cellulase activity must be applied to an identical cellulosic substrate—Whatman #1 filter paper—and that exposure of enzyme preparation to substrate must be

permitted to proceed until 3.6% (w/w) of the cellulose in a 50-mg test coupon; that is, 2 mg, is converted to glucose after a 60-min incubation at 50°C. The concentration (or actually dilution) of enzyme preparation required to effect this is converted, through a somewhat indirect procedure, to the cellulase activity in filter paper units per milliliter. For example, an undiluted cellulase preparation that yields exactly 2 mg of glucose during the IUPAC assay has 0.37 FPU/mL. This fractional unit is the lowest cellulase activity measurable with the IUPAC assay. Note that because the IUPAC FPU assay is nonlinear owing to hydrolysis of an insoluble substrate of variable structural composition, the use of traditional international units of enzyme activities based on initial velocities is invalid. Here, a single incubation time and temperature are used for all samples.

The IUPAC cellulase assay has many significant limitations; it merely serves as the best existing method. The IUPAC commission warns, e.g., that extrapolation of required glucose release from highly dilute or concentrated solutions of enzyme is not permitted. Indeed, the assays used to confirm the release of 2 mg of glucose must be conducted with enzyme dilutions that closely bracket the actual value. The implication is that cellulase solutions too dilute to release 2 mg of glucose must be either concentrated to an appropriate level or pronounced unassayable by the IUPAC method. This latter issue is important for consideration of the assay miniaturization dictated by automation in microtiter plates.

Non-IUPAC Methods

Many cellulase enzyme preparations are simply not concentrated enough to cause the required release of 2 mg of glucose from the 50-mg filter paper sample in 60 min. If these samples cannot be concentrated accurately (which is often the case) traditional FPU cannot be measured. In such cases, however, the IUPAC committee recommends that the reducing sugar release per unit time be accepted as a "provisional" measure of enzyme activity. This is similar to the pseudo–initial rate approach often used in the decade previous to the IUPAC report to measure cellulase activity from a wide variety of substrates. These substrates may include filter paper *(16)*, Avicel *(17)*, dewaxed cotton *(18)*, or phosphoric acid–swollen cellulose *(19)*. Methods based on the use of antibiotic disks *(20)* and turbidity development *(21)* also predated the IUPAC study. More recently, Johnson et al. *(22)* have developed methods to use cellulose solvents to solubilize cellulose treated with purified enzymes and characterize these products using size-exclusion chromatography.

Automated Cellulase Assays

To automate cellulase assays, several requirements are necessary, including the following:

1. Creation of substrate plates.
2. Correct dilution of enzyme stock.
3. Scale-down of assay volume/substrate.

4. Consistent and rapid heating during hydrolysis and color development.
5. Prevention of evaporative losses at low-volume scale.
6. Simultaneous reagent addition/sampling in the assay plates.
7. Automated absorbance reading of samples.
8. Data analysis.

Of these, precise distribution of substrate and prevention of evaporative losses at low volumes are the most important. The target reaction volume of 80 µL required evaporative losses to be minimized and substrate concentration be tightly controlled. Ensuring that these requirements are met in an automated FPA that can assay up to 84 enzyme preparations per day cuts reagent use and disposal 20-fold and requires only ~10% as much researcher time as the traditional method.

Evaporation Studies

Initially, we expected to be able to seal the plates with a polypropylene/foil laminate heat seal using the ALPS sealer integrated into the deck. This plate sealing method worked well, but because of the frequent pipeting to and from the sealed plates, gaining access to the well contents with a pipet tip proved problematic. Even after solving this problem by integrating a custom seal-piercing tool onto the deck, the tips from the 96-channel pipettor would get stuck in the holes during pipeting and the plate would ride up with the pipettor when it retracted. Hold-down pins were then used to help retain the plates on the deck after pipeting, but this made placement of the plates difficult, because the plates tended to flex. Increasing the hand grip enough to hold down the plates resulted in increased difficulty in inserting the plates onto the deck as well. Additionally, it was observed that water tended to condense on the undersurface of the seal during incubation steps. Sometimes these droplets would return to the well (sometimes not), thus increasing the scatter in the sample analysis.

As the solutions to these problems began compounding themselves, it was determined that a simpler method needed to be developed. Mineral oil was overlaid on the surface of the assay mix with the intention of minimizing evaporation. The oil had an apparent affinity for polypropylene and tended to creep up the sides of the plate wells, leaving the middle of the aqueous surface exposed to the air in the chamber. Additional oil was added, but eventually the volume of oil became prohibitive by diminishing the available assay mixture volume. Polystyrene and polypropylene lids were used to cover the plates, and similar condensation problems were noted. In addition, the lids were flimsy and difficult for the C400 gripper arm to handle without distortion. Humidification of the incubators was attempted by inserting thick sections of blotting paper inside the chambers and saturating with water. The results showed an improvement, but the blotting paper tended to fall apart and get in the way during plate insertion and retrieval. There was also a concern that high humidity in the incubators, which are heated with electric heat tape, would cause

problems with the system's electronics. Water also tended to condense on the incubator lids and drip off as the gripper arm moved around the lids. Heated lids, similar to techniques developed for polymerase chain reaction machines, were considered in order to minimize evaporation and condensation. Initial thoughts regarding electrically heated lids with wires attached were quickly discarded in favor of simply maintaining the lids in the hot incubator and allowing their retained heat to control condensation. The prototypes were constructed of lead and had numerous problems; low heat capacity, easy deformation by the gripper, and their weight stressed the gripping system. The final design was based on brass lids, which solved all of the problems associated with lead and allowed us to minimize water loss from the cellulase assay plates, both at 50°C for 60 min, and at 98°C for 15 min.

FPU-to-Protein Correlation

We have determined that at least for fungal cellulase mixtures, FPU activity is closely correlated to total protein content, allowing us to approximate FPU activity quickly and give a good starting point for determining a hard FPU value. Analysis of commercially available cellulase preparations and in-house-produced *Trichoderma reesei* broths demonstrated a wide variance in both protein content and FPU activity. A direct comparison of these two properties yielded an interesting correlation: the number of FPU was directly related to the protein content (Fig. 1). This holds for both *T. reesei*–derived cellulases and at least one non–*T. reesei* commercial preparation. The samples were desalted prior to determination of protein by BCA and FPU activity. Previous work in this area had shown a tendency for stored commercial cellulases to contain a high amount of partial peptides and low molecular weight stabilizing compounds. Several cellulase preparations were examined for filter paper activity before and after desalting and no difference was detected (data not shown). Although the data have some scatter in them, it is likely that such a plot can be used to give a first approximation of the filter paper activity of a cellulase preparation based on the protein content. This will allow fewer and narrower dilutions in determining the FPU value of cellulase samples.

Acknowledgments

This work was funded in part by the Ethanol from Biomass Program of the Biofuels System Division of the US Department of Energy.

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