Introduction

Many microorganisms including fungi and bacteria had been found to degrade cellulose and other plant cell wall fibers. In nature, degradation of cellulosic biomass is performed by mixtures of hydrolytic enzymes collectively known as cellulases. The cellulases include endo-acting (endoglucanases, EGs) and exo-acting (cellobiohydrolases, CBH) enzymes, which act in a synergistic manner in biomass-degrading microbes (Dashtban et al., 2009). The cellobiose and cello-dextran products of exoglucanases and CBH are inhibitory to their activity. Thus, efficient cellulose hydrolysis requires the presence of β-glucosidases to cleave the final glycosidic bonds of cellobiose-producing glucose (Maki et al., 2009).

Assays for determining cellulase activity have been classified differently over years of cellulase research. Sharrock (1988) grouped cellulase assays into two basic approaches: (1) determining the activities of individual cellulases (EGs, exoglucanases, and β-glucosidases), and (2) measuring the total saccharifying activity of a crude cellulase system (Sharrock, 1988) whereas Zhang et al. (2006) classified all cellulase activity assays into three main groups: (1) assays in which the accumulation of products after hydrolysis was targeted, (2) assays in which the reduction in substrate quantity was monitored, and (3) assays in which the change in the physical properties of the substrate was measured (Zhang et al., 2006). Due to the complexity of cellulose–cellulase systems and differences between kinetic characteristics of initial hydrolysis reaction and the extended time, cellulase activity assays are either expressed based on the initial hydrolysis rate or using the end-point hydrolysis. The first one is preferred when measuring an individual cellulase activity in a short time; however, the last one is a method of choice for the total enzyme activity within a given time (Wu et al., 2006; Zhang et al., 2006).

Cellulase activity is mainly evaluated using a reducing sugar assay to measure the end products of cellulase hydrolysis activities. Thus, the results of such an assay are typically expressed as the hydrolysis capacity of the enzymes. There are several issues in this work: it cannot be easily expressed in a quantitative manner, lacks theoretical basis, and does not consider all effective factors, such as concentration of cellulose and cellulase, the hydrolysis time, the ratio of crystalline and amorphous cellulose, and the proportion between different individual components.

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in the enzyme preparations (Wu et al., 2006). Researchers have mainly focused on improving methods for measurement of cellulase activity, which have already been widely used. Developing new sufficient cellulase assays is hampered by the physical heterogeneity and limited enzyme accessibility of cellulosic materials, and the complexity of cellulase enzyme systems (synergy and/or competition) (Zhang et al., 2006). Thus, an accurate and reproducible assay for the measurement of cellulase hydrolysis rate is still required (Wu et al., 2006).

In this review article, total cellulase activity by application of filter paper (filter paper assay, FPA) will be explained and then individual cellulase activities including EGs, exoglucanases, and β-glucosidases will be discussed. Moreover, we will also summarize some novel approaches such as (1) quartz crystal microbalance (QCM), (2) miniaturized colorimetric assay, (3) automated FPA for the measurement of cellulase activity, (4) fluorescent microfibrils, and (5) amperometric cellobiose dehydrogenase biosensor. Figure 1 recaps the different cellulase assays discussed in the article. This review article summarizes and compares past and present cellulase assaying techniques and suggests future directions important for the ever-growing field of biofuel research.

**FPA (FPase activity): total cellulase activity**

To compare the efficacy of cellulase activity between microorganisms or their secreted enzymes, techniques for measuring total cellulase activity are required. The FPA is the key method for analysis of total cellulase activity. In 1976, the FPA was developed by Mandels et al. The FPA became widely used since 1984, when the Commission on Biotechnology of the International Union of Pure and Applied Chemistry (IUPAC) proposed a number of standard procedures for the measurement of cellulase activity. Traditionally, the FPA uses a 1 × 6-cm strip of Whatman no. 1 filter paper as the standard substrate because it is readily available and inexpensive (Coward-Kelly et al., 2003). This standard filter paper method has been reviewed by Ghose (1987). The International Unit (IU) of filter paper activity (FPase) (FPU) is defined as the micromole of glucose equivalent liberated per minute of culture filtrate under assay conditions, where assay conditions refer to the conditions such as pH and temperature at which the enzymes are held at during the assay and depend largely on the properties of the enzyme, varying widely between cellulases and microorganisms. Reducing sugar is estimated as glucose by the Miller method. This assay is performed so that 0.5 mL of diluted enzymes releases about

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**Figure 1.** Different cellulase assays that are classified within two groups: (1) total cellulase activity and (2) individual cellulase activity including endoglucanases, exoglucanases, and β-glucosidases. Filter paper assay can be improved by adding supplemental β-glucosidase, which is indicated by the broken arrow. Released reducing sugars can be measured using different reducing sugar assay methods such as 3,5-dinitrosalicylic acid (DNS), glucose oxidase (GOD), and high-performance liquid chromatography (HPLC). Recently, a few novel assays with ease of operation and high reproducibility have been developed. (See colour version of this figure online at www.informahealthcare.com/bty)
2.0 mg of glucose equivalents in 60 min, as determined by the 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959; Wood and Bhat, 1988).

The DNS reagent is used as a colorimetric method for the determination of reducing sugars, such as glucose. It contains sodium potassium tartrate, which decreases the tendency to dissolve oxygen by increasing the ion concentration in the solution. Phenol increases the amount of color produced during the color-developing reaction. Sodium bisulfite stabilizes the color obtained and reacts with any oxygen present in the buffer. Finally, an alkaline buffer is required for the redox reaction between DNS and glucose, or other reducing sugars. DNS will be added at the last step of the enzyme assay to stop the reaction. To promote full color development, samples will be read at 540 nm (Zhang et al., 2009). One disadvantage of using such a dye for quantification is that some of the reducing sugars are degraded while the analysis is performed (Miller, 1959).

There are several more concerns associated with using the FPA to quantify total cellulase activity. Although the FPA is commonly used, it is also known for being nonreproducible. Difficulties arise from the preparation of the DNS reagent, which is a tedious task, requiring optimal mixing ratios of the different components. Additionally, DNS reagent requires appropriate temperature control to allow for proper color development and color stability (Miller, 1959). Furthermore, it is known that the decomposition of sugars in the alkaline solution recommended by the IUPAC method causes an increase of (measured) enzyme activity to values higher than the actual ones (Gilman, 1943). To summarize, it is time-consuming, labor-intensive, and requires large quantities of reagents. It is also difficult to obtain adequate sensitivity and reproducibility when characterizing newly isolated cellulases using this method. Factors that affect sensitivity and reproducibility often result from the fact that most natural cellulase complexes tend to have a shortage of β-glucosidase activity (Breuil et al., 1986; Coward-Kelly et al., 2003).

Several methods have been developed to improve the FPA for the evaluation of total cellulase activity. Nordmark et al. (2007) designed a modified method for the FPA, which requires the use of protein stabilizers. This method allows the sensitive measurement of cellulase activity below the level required for the detection of reducing sugars using the traditional FPA. The traditional FPA requires a fixed degree of conversion of substrate, i.e., a fixed amount (2.0 mg) of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper within a fixed time (60 min). Because of the heterogeneous (amorphous/crystalline) nature of filter paper, reducing sugar yield during hydrolysis is not a linear function of the quantity of cellulase enzyme in the assay mixture (Zhang et al., 2009). To overcome this limitation, researchers usually measure two enzyme activities (slightly less than and slightly greater than 2.0 mg of reducing sugar equivalents (RSE) in 1 h) (Nordmark et al., 2007). It is difficult to measure activities greater than 2.0 mg RSE in 1 h for all cellulases because cellulase preparations typically have lower cellulase activity due to lower concentration. Protein stabilizers (such as bovine serum albumin) extended the enzyme reaction time thereby allowing a proportionate calculation of cellulase activities on natural cellulosic substrates to those obtained in the IUPAC assay (Nordmark et al., 2007).

Similarly, Coward-Kelly et al. (2003) found that the FPA could be improved by adding supplemental β-glucosidase. If an organism or enzyme complex has low β-glucosidase activity, a high amount of cellobiose will be produced resulting in a lowered or “false” absorbance reading for the DNS assay because it is not a reducing sugar. Adding supplemental β-glucosidase can help to overcome this issue. In this study, supplemental β-glucosidase increased the assay reading by 56%. They also tested the hypothesis that extended boiling time will improve the FPA but failed to find any such benefit. A 5-min boiling time is sufficient; however, they suggest that the water bath boil vigorously to eliminate temperature excursions (Coward-Kelly et al., 2003).

Finally, downsizing the FPA has also been developed as an improvement to the assay, allowing researchers to assay a large number of samples simultaneously. This has been achieved by reducing the volume of the reagents and substrate, so the assay can be done in a 96-well microtiter plate. The overall enzymatic reaction volume was reduced from the IUPAC 1.5 mL standard to 60 µL. An office hole puncher was used to create small disks of filter paper substrate to fit perfectly in the wells. No significant difference was observed between the activities measured using the IUPAC FPA compared with the minimized reactions in the microtiter plate (Xiao et al., 2004).

EGs activity: carboxymethyl cellulase activity

EGs can randomly hydrolyze internal glycosidic bonds in cellulose chains. EGs activities can be measured using a soluble cellulose derivative with a high degree of polymerization (DP) such as carboxymethyl cellulose (CMC). Carboxymethyl cellulose (CMCase) is mainly evaluated based on the procedure described by Mandels et al. (1976). In this method, CMCase activity is measured by determining reducing sugars released after 5 min of enzyme reaction with 0.5% CMC at pH 4.8 and 50°C (Mandels et al., 1976). Also, one unit (IU) of EG is defined as the amount of enzyme that liberates 1 µmol of glucose per minute under assay conditions. Reducing sugar can be estimated by application of different methods such as high-performance liquid chromatography (HPLC) (Fujita et al., 2002) or glucose oxidase (GOD)/peroxidase reagent (Trinder, 1969) or a colorimetric method such as the Somogyi-Nelson method that uses alkaline copper as an inorganic oxidant. Cupric ions (Cu (II)) accept electrons from the donating aldehyde groups of reducing sugars and reduce to Cu (I). In the second step, reduced Cu (I) ions will be oxidized back to Cu (II) using a chromogenic compound. The reduced chromogenic compound produces color that can be measured using a colorimeter and compared with standards prepared from reacting sugar solutions of known concentration to
determine the amount of reducing sugar present (Nelson, 1944; Somogyi, 1952).

Although CMC is commonly used as a substrate to quantify EG activity, there are several concerns associated with using CMC. It is known for being non-reproducible, which is only linear within a limited degree of hydrolysis of CMC to glucose due to interference by substituents. In this case, substituted glucose units available in different CMCs are also accessible to cellulase that caused non-reproducibility (Zhang et al., 2006; Eveleigh et al., 2009). In addition, the quantity of reducing sugars produced and thus the unit values will be highly affected by the particular type of CMC used in the assay (Mandels et al., 1976; Eveleigh et al., 2009).

These difficulties arise from two important variable physical parameters of CMC: (1) the degree of substitution (DS) and (2) the DP that will affect its solubility and viscosity, respectively. It is recommended that a reducing sugar assay or viscosity assay should be limited to the first 2% hydrolysis of substrate when CMC is used as the substrate with DS = 0.7; this is to ensure that only nonsubstituted glucose units are accessible to EG (Zhang et al., 2006). Additionally, the DP of CMC has an important role in determination of viscosity reduction. Therefore, to minimize the influence of some conditions such as pH and ionic strength on DP and thus viscosity, some substituted CMC substrates such as ionic CMC have to be avoided for determining EG activity whereas non-ionic substituted cellulose such as hydroxyethyl cellulose (HEC) is preferred (Guignard and Pilet, 1976; Zhang et al., 2009).

EGs activities can be measured using dye, either by adding dye to soluble cellulose derivatives or by adding it to solid agar plates known as "zymograms." Remazol Brilliant Blue R and Ruthenium Red are two examples of dyes that have been used in CMC assays. Recently in a zymogram assay, Gram's iodine has been used for a fast and easy detection of EG activity, which makes a sharp and distinct zone around the cellulose-producing microbial colonies in a bluish-black background within a short time (3–4 min) (Kasana et al., 2008). This method and other zymogram methods are applicable for screening of a large number of colonies. However, they do not provide a quantitative result for the enzyme activity due to the lacking of a linear relationship between halo zones and enzyme activity. Moreover, EGs activities can be measured using some other dyes by adding them to insoluble cellulose derivatives or substituting insoluble cellulose derivatives chemically to produce chromogenic CMC. Examples of these are Cibacron Blue 3GA (Ten et al., 2004) and chromogenic trinitrophenyl CMC (TNP-CMC) (Huang and Tang, 1976), respectively.

**Exoglucanases activity: avicellulases**

CBH (exoglucanases) are classified as exo-acting based on the assumption that they all cleave β-1,4-glycosidic bonds from chain ends releasing cellobiose and some glucose molecules. Commercial Avicel (also called microcrystalline cellulose or hydrocellulose) is used for measuring exoglucanase activity because it has a low degree of polymerization of cellulose and it is relatively inaccessible to attack by EGs despite some amorphous regions.

Enzymes that show relatively high activity on Avicel and little activity on CMC are identified as exoglucanases (Maki et al., 2009). However, Avicel contains some amorphous cellulose and soluble cellodextrins, which can act as substrates for both exo- and endo-glucanases. There is no highly specific substrate to test exoglucanase activity in cellulase mixtures (Sharrock, 1988; Wood and Bhat, 1988).

Different assays have been reported for selection of exoglucanase activity; nevertheless all of these assays have some sort of limitations. van Tilbeurgh et al. (1985) found that 4-methylumbelliferyl-β-d-lactoside was an effective substrate for assaying CBHII of *Trichoderma reesei*, where hydrolysis of this substrate yields lactose, phenol, and 4-methylumbelliferyl (a fluorescent signal molecule) as products. However, this substrate could not be used to determine CBHII activity of *T. reesei* thus it is not an effective representation of true exoglucanase activity for this strain (van Tilbeurgh et al., 1982, 1985).

Similarly, Deshpande et al. (1984) developed an assay for quantification of exoglucanase activity in the presence of EGs and β-glucosidas (Deshpande et al., 1984). This assay is based on the following: exoglucanases specifically hydrolyze the aglyconic bond of p-nitrophenyl-β-d-cellobioside to yield cellobiose and p-nitrophenol; β-glucosidase activity is inhibited by adding d-glucono-1,5-δ-lactone (Holtzapple et al., 1990); and the influence of exoglucanase hydrolysis activities must be quantified in the assay procedure in the presence of added purified EGs. The limitations for this assay are that: (1) the CBHII activity cannot be measured using p-nitrophenyl-β-d-cellobioside, (2) the specific activity of the available purified EGs may not be representative for all existing EGs in the mixture, and (3) the product ratio from EG actions may be influenced by the presence of exoglucanases (Zhang et al., 2006).

Other less commonly used substrates for measuring or detecting exoglucanase activity for both bacteria and fungi include the following: PNP-β-d-cellobioside (Kohring et al., 1990), bacterial microcrystalline cellulose (BMCC) (Caspi et al., 2008), and MU-β-d-cellobioside (MU-C) (County et al., 2005). Limitations of these substrates are not clearly defined.

**β-Glucosidase assay**

β-Glucosidase activity can be measured using various chromogenic and nonchromogenic substrates and are mainly evaluated based on the procedure of Kubicek (1982). In one chromogenic method, p-nitrophenol-β-glucoside (pNPG) is used as the substrate. The liberated p-nitrophenol will be measured in order to determine the hydrolysis rate in optimal temperature and pH. Reaction conditions such as temperature and pH of different β-glucosidases vary based on the enzyme (Table 1). The compound pNPG, as the
substrate at the optimal concentration (usually 1–5 mM), will be added to an appropriate buffer with optimal pH, containing the enzyme and incubated at the optimal temperature. After 10-min incubation, the reaction will be stopped by adding three volumes of sodium tetraborate saturated solution, and then the absorbance will be read at 405 nm. One unit of β-glucosidase is defined as the amount of enzyme that liberates 1 μmol of p-nitrophenol per minute (Chandra et al., 2009). However, in the case of nonchromogenic substrates different methods can be used depending on the substrates. For example, when oligo- or disaccharides (such as cellobiose) are used as the substrates, the liberated glucose can be evaluated by the GOD method with a commercial kit. Nevertheless, when the substrate is a polysaccharide, reducing sugars liberated will be measured by the DNS method. Using polysaccharides as the substrate, the enzyme unit will be determined as the amount of enzyme required for the liberation of 1 μmol of glucose or reducing sugar per minute. Moreover, substrate specificity of enzymes can be determined using different substrates listed in Table 1 and applying the above-mentioned methods.

β-Glucosidase activity measurement using chromogenic substrates such as pNPG is a common technique used in many different studies (Murray et al., 2004; Bhatia et al., 2005; Karnchanatat et al., 2007; Daroit et al., 2008; Tsukada et al., 2008; Yang et al., 2008; Yoon et al., 2008; Joo et al., 2009; Korotkova et al., 2009). However, correlation between β-glucosidase activity on the analog substrates (e.g., pNPG) and the natural substrate (e.g., cellobiose) is not clear. As a natural substrate, cellobiose has been used in β-glucosidase screening experiments using 96-well microtiter plates (McCarthy et al., 2004). However, this method is not preferred for screening of a large library of enzyme-producing microorganisms due to its disadvantages such as being time-consuming and costly (Liu et al., 2009). Recently, thermostable β-glucosidase mutants (BGLA) from Paenibacillus polymyxa have been identified using novel and fast combinatorial selection/screening approach. In this study a big mutant library including 100,000 clones were generated using error-prone PCR and cloned and expressed in Escherichia coli. Thermostable β-glucosidase mutants have been identified in a two-step process using a natural substrate (cellobiose): (1) selection for mutants with adequate β-glucosidase activity and (2) screening for improved thermostability. In the first step, cells were grown on selection plates containing minimal growth medium plus cellobiose as the sole carbon source and thus, only cells expressing active β-glucosidase could grow on the medium. Colonies on the selection plate were duplicated using a nylon membrane and then incubated at 60°C for 10 min to break the cells and release intracellular β-glucosidase. Also, heat treatment deactivated most of the β-glucosidase mutants and only thermostable β-glucosidase mutants will remain active and will be able to hydrolyze cellobiose to glucose on the screening plate. In the second step, the membrane was overlaid on the soft agar screening plate containing minimal medium with cellobiose as the sole carbon source. In addition to that, the medium contained an indicator strain of E. coli, which was enabled to utilize glucose only (but not cellobiose). After incubation, the growth of the indicator strain on the screening plate was used as an indicator to detect the clones expressing thermostable BGLA mutants. This screening method enabled scientists to screen larger libraries within a shorter time. In this case, a thermostolerant mutant with 11-folds improved thermostolerance compared with the wild-type has been selected (Liu et al., 2009).

<table>
<thead>
<tr>
<th>β-Glucosidase</th>
<th>Source</th>
<th>Optimum temperature/pH</th>
<th>Substrate specificity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus japonicus</td>
<td>65/4.5–5</td>
<td>High toward cellobiose and pNPG and low toward β-glucan from barley</td>
<td>Korotkova et al. (2009)</td>
<td></td>
</tr>
<tr>
<td>Daldinia eschscholzii (Ehrenb.:Fr.) Rehm</td>
<td>50/5</td>
<td>pNPG, cellobiose, sophorose, laminaribiose, and gentiobiose</td>
<td>Karnchanatat et al. (2007)</td>
<td></td>
</tr>
<tr>
<td>Fomitopsis palustris</td>
<td>70/4.5</td>
<td>pNPG and cellobiose</td>
<td>Yoon et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Fomitopsis pinicola KM1812</td>
<td>50/4.5</td>
<td>pNPG and cellobiose</td>
<td>Joo et al. (2009)</td>
<td></td>
</tr>
<tr>
<td>Monascus purpureus NRRL1992</td>
<td>50/5.5</td>
<td>pNPG, cellobiose, salicin, n-octyl-β-D-glucopyranoside, and maltose</td>
<td>Daroit et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Paecilomyces thermophila</td>
<td>75/6.2</td>
<td>Very broad, pNPG, cellobiose, gentiobiose, sophorose, amygdalin, salicin, daidzin, and genistin</td>
<td>Yang et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Penicillium verruculosum</td>
<td>60/5.5</td>
<td>High toward β-glucan from barley and low toward cellobiose and pNPG</td>
<td>Korotkova et al. (2009)</td>
<td></td>
</tr>
<tr>
<td>BGLIB</td>
<td>P. chrysosporium</td>
<td>-/6–6.5</td>
<td>Cellobiose, cellobionolactone, and pNPG</td>
<td>Tsukada et al. (2008)</td>
</tr>
<tr>
<td>BGLII</td>
<td>Pichia etchellsii</td>
<td>45/6</td>
<td>pNPG, sophorose, gentiobiose, cellobiose, laminaribiose, and salicin</td>
<td>Bhatia et al. (2005)</td>
</tr>
<tr>
<td>Cel3a</td>
<td>Talaromyces emersonii</td>
<td>71.5/5</td>
<td>pNPG, pNPC, salicin, cellobiose, and barley β-glucan</td>
<td>Murray et al. (2004)</td>
</tr>
<tr>
<td>T. reesei</td>
<td>70/5</td>
<td>High toward β-glucan from barley and low toward cellobiose and pNPG</td>
<td>Korotkova et al. (2009)</td>
<td></td>
</tr>
</tbody>
</table>
Novel approaches for measurement of cellulase activity: automated measurement of cellulase activity

Traditionally, enzymatic assays, particularly the widely used FPA, are recognized for their complexity and sensitivity to operator error and are generally deemed tedious and time-consuming. It appears obvious that new and/or improved assays are required; however, there are several difficulties to be addressed when developing assays to quantify the activity of enzymes such as cellulases. Is the assay reproducible, reliable, and can it be applied in a time- and cost-efficient manner? The aphorism “You get what you screen for” implies that the screening method/assay is crucial for accurate, reproducible, and high-quality results. Thus, researchers have focused on developing a wide variety of novel techniques for the measurement of cellulase activity in hopes of addressing some of these difficulties with currently used assays.

There are a variety of different techniques used to evaluate cellulase activity as previously mentioned, such as colorimetric detection of reducing groups, chromatographic, fluorogenic group release, chromatographic substrates, and viscometric detection methods, to highly sophisticated mainly research-based methods such as ion, liquid, and anion-exchange chromatography and HPLC (Schwald et al., 1988). Recently, a few novel assays with ease of operation and high reproducibility have been developed. Table 2 summarizes some cellulase assays using novel techniques.

One of the most recent novel assay methods uses a QCM piezoelectric-sensing technique, for measuring cellulase activity, and relates crystallinity of different substrates to the cellulase activity (Hu et al., 2009). The piezoelectric property of quartz crystal allows the production of an ultrasensitive mass balance. Changes in frequency of a quartz crystal can be used to measure viscosity and density changes in a solution used to incubate a given cellulose substrate, after enzymatic hydrolysis. The results can be used to quantify the enzyme activity. Here, the quantification of cellulase activity using QCM was closer to those results obtained by measuring the actual reducing sugars (IUPAC assay). QCM is advantageous to use because it allows operators to reduce reagents, thereby reducing costs, aliquoting errors, and ultimately the time for quantification.

Similarly, the possibility of complete automation of a cellulase assay was fully exemplified by Decker et al. (2003). This group created an automated version of the traditional FPA using a Cyberlabs C400 robotics deck equipped with customized incubation, reagent storage, and plate-reading capabilities. The goal of such an automated assay was to reduce operator error during determination of cellulase activity and to reduce the amount of reagent usage as well as lower reagent disposal costs, while allowing for a high throughput of samples to be assayed. The maximum throughput of samples of the automated procedure is 84 enzymes per day. After the initial cost associated with the purchase of such a piece of equipment, the high efficiency and low reagent usage will allow this technology to be successful; however, at its current stage this automated assay is not sufficiently comparable with the traditional FPA (Decker et al., 2003).

Furthermore, a more sensitive cellulase assay was developed using fluorescent microfibrils from bacterial cellulose prepared using 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) as a grafting agent. Fluorescent dyes, such as DTAF, which bear dichlorotriazinyl groups are known to react with hydroxyl groups of polysaccharides making DTAF a good candidate. A protocol to graft microfibrils with DTAF was developed, which does not modify the physical integrity of the substrate. This grafted DTAF-cellulose was created by

Table 2. Some cellulase assays using novel techniques.

<table>
<thead>
<tr>
<th>Cellulase assay</th>
<th>Substrate</th>
<th>Advantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz crystal microbalance</td>
<td>FP, Avicel, CMC</td>
<td>Various substrates, Easy to implement.</td>
<td>Hu et al. (2009)</td>
</tr>
<tr>
<td>Miniaturized colorimetric</td>
<td>FP, Avicel, corn stalk,</td>
<td>High-throughput, comparable results, various substrates</td>
<td>King et al. (2009)</td>
</tr>
<tr>
<td></td>
<td>switchgrass, CMC, arabinoxylan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Automated FPA</td>
<td>FP</td>
<td>Reproducible, high-throughput, reduced reagent usage</td>
<td>Decker et al. (2003)</td>
</tr>
<tr>
<td>Fluorescent microfibrils</td>
<td>Bacterial cellulose</td>
<td>Possible automation, native cellulose, sensitive cellulose detection</td>
<td>Helbert et al. (2003)</td>
</tr>
<tr>
<td>Amperometric cellulobiose dehydrogenase biosensor</td>
<td>Avicel</td>
<td>Rapid, readily implemented, nondestructive, comparable precision</td>
<td>Hilden et al. (2001)</td>
</tr>
</tbody>
</table>

*Comparable results—results of assay are comparable with traditional methods.
dissolving 10–70 mg of DTAF into 10 mL of a suspension containing 100 mg of cellulose microfibrils in 0.1 N NaOH. These mixtures were stirred at room temperature for 24 h. Cellulose digestion resulted in the release of fluorescent celldextrins and reducing sugars. This method allowed for a comparison between the amount of released fluorescence and that of released reducing sugar from which one could differentiate between processive exo- and endocellulase activities. This research group also casted films of DTAF-grafted microfibrils to the bottom of microwell titer plates producing sensitive cellulase detection and allowing for possible automation. Sensitivity of detection can be increased by optimization of the grafting conditions, which maximizes the quantity of soluble products. The main advantage for using fluorescent microfibrils is it allows for measurement of minute amounts of cellulase activity and it reduces the dependency on using substrates such as CMC, which are far different from native cellulosic substrates. Cellulose microfibrils produced by algae and bacteria have been well characterized and shown to contain most of the structural and morphological characteristics of “real” cellulose materials. Being in a dispersed state, these cellulose microfibrils reduce cellulase–substrate accessibility problems (Helbert et al., 2003).

Moreover, Hilden et al. (2001) set out to create a faster, more convenient, yet equally reliable method for determining cellulase activities of a series of samples. They achieved this by using an amperometric redox polymer-based biosensor to determine the total concentration of soluble oligosaccharides. The biosensor was produced based on cellobiose dehydrogenase from Phanerochaete chrysosporium wired by a redox polymer. This newly applied method of measuring cellulase activity provides several advantages over traditional methods. First, it is rapid, allowing analysis of a maximum 30 samples in an hour. In addition, the biosensor can be readily used without prior planning because it can be stored in water in flow injection analysis. Furthermore, the enzyme solution may be recovered after passing the electrode due to its nondestructive nature. Not to mention, no harmful chemicals, boiling, or cooling is required with this method simplifying implementation. Finally, the precision of the method is equivalent to traditional methods such as the Somogyi-Nelson technique with high sensitivity detection to the same order of magnitude for cellobiose, cellotriose, and cellotetraose (Hilden et al., 2001).

Despite the newly emerging cellulase activity assays, the FPA is still the most widely used method. Perhaps automation of the FPA will help researchers achieve reproducibility while reducing costs. However, biosensors are becoming more popular and may offer a similar promising solution to the evaluation of cellulase activity, which will give results comparable with the direct measurement of reducing sugars via FPA.

Concluding remarks
Many different cellulase activity assays have been used and developed over the last few decades. However, only a few of them have been used consistently and they are discussed in this article. Among these assays, total cellulase activity by application of filter paper and methods for measuring individual cellulase activities for EGs, exoglucanases, and β-glucosidases are the major cellulase assays. Some of the major obstacles for cellulase assays are heterogeneity of insoluble cellulose, complicated synergy/competition among endo- and exoglucanases, and changes of enzyme-substrate ratio (Zhang et al., 2009). More recently, some novel approaches such as QCM, miniaturized colorimetric assay, automated FPA, fluorescent microfibrils, and amperometric cellobiose dehydrogenase biosensors have been developed for the measurement of cellulase activity. Among these novel approaches, biosensors are recently attracting more attention.

Amperometric biosensors measure the changes of current of a working electrode resulting from biochemical and electrochemical reactions. In amperometric biosensors, the potential at the electrode is held constant while the current is measured. The overall performance of the biosensor will mainly depend on the properties of biosensing (enzyme) membrane and to a lesser extent on the instrumentation system used to acquire the signal generated by biochemical reaction at the biosensing membrane (Gopel and Heiduschka, 1995; Chaubey and Malhotra, 2002; Baronus et al., 2003). The combination of modern electrochemical techniques with enzymatic biosensors may potentially increase demands for investigation on cellulase assays to design high-performance biosensing systems in terms of selectivity, sensitivity, reliability, durability, and low cost. An example of an amperometric biosensor with potential application in cellulase assays is the glucose oxidase biosensor. The enzyme GOD is incorporated in the membrane of the electrode to detect glucose and ultimately relay glucose concentration. The GOD biosensor cannot detect small oligosaccharides such as cellobiose and cellotetraose, which may be products of cellulase activity relating to endo- and exoglucanases. However, the previously discussed cellobiose dehydrogenase containing amperometric biosensor is capable of measuring such products. For an accurate analysis of total cellulolytic activity, we propose the production of a mixed enzyme membrane for biosensor detection. Combining GOD with an additional enzyme such as cellobiose dehydrogenase would allow the detection of all cellulose hydrolysis products.

Declaration of interest
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