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Quantification of Cellulase Activity Using the Quartz Crystal Microbalance Technique

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The development of more efficient utilization of biomass has received increased attention in recent years. Cellulases play an important role in processing biomass through advanced biotechnological approaches. Both the development and the application of cellulases require an understanding of the activities of these enzymes. A new method to determine the activity of cellulase has been developed using a quartz crystal microbalance (QCM) technique. We compare the results from this technique with those from the IUPAC (International Union of Pure and Applied Chemistry) dinitrosalicylic acid (DNS) standard method and also from biccinchoninic acid and ion chromatography methods. It is shown that the QCM technique provides results closer to those obtained by measuring the actual reducing sugars. The elimination of the use of color development in the standard redox methods makes the QCM platform easier to implement; it also allows more flexibility in terms of the nature of the substrate. Finally, validation of the proposed method was carried out by relating the crystallinity of different substrates to the cellulase activity. Numerical values of cellulase activities measured with the QCM method showed that celluloses with higher crystallinity indices were hydrolyzed slower and to a lower extent than those of lower crystallinity indices for the cellulase mixtures examined.

Cellulases are a group of enzymes mainly consisting of different isozymes, including endoglucanases (EGI and EGII), exoglucanse or cellobio-hydrolyases (CBHI and CBHII), and β-glucosidases, which work synergistically to hydrolyze cellulose into reducing sugars. Cellulases play an important role in the conversion of biomass into energy and chemicals. Significant efforts are being made to develop cellulases of higher efficiency, both from different sources and by transgenic modification, and to explore new fields for their application.

The activity of cellulases is an important parameter since it characterizes their performance and efficiency. A widely used method to measure cellulase activity is the protocol recommended by the International Union of Pure and Applied Chemistry (IUPAC), which was published in 1987.[1] According to this method, cellulase activity is determined from the enzyme concentration required to produce a certain amount of reducing sugar in 1 min. The activity is expressed as the micromoles of glucose produced by 1 mL of enzyme in 1 min. The amount of sugars produced is typically determined using a spectrophotometric determination under the assumption that all reducing sugars produced after incubation are glucose.

The filter paper activity assay (FPA), which uses Whatman no. 1 filter paper (pure cellulose) as substrate, can be applied to determine the overall activity of cellulases. Dinitrosalicylic acid (DNS) is used in the IUPAC-FPA assay as an agent to quantify the amount of reducing sugars released from 50 mg of filter paper (Whatman no. 1) after incubation with the cellulase mixture. In order to obtain reliable results this method requires the preparation of DNS reagent (dinitrosalicylic acid together with phenol are both toxic chemicals[2]) and also demands careful sampling and accurate control of color development.[1]

DNS preparation demands optimal mixing ratios of the different components involved and proper temperature control for color development and color stability.[3] 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.[4] Despite efforts to improve the efficiency of IUPAC's filter paper assay,[5,6] the issues indicated before are considered important drawbacks in its utilization.

An alternate method, based on the bicinchoninic acid (BCA) reagent, was proposed by Johnston et al.[7] Here color development from a redox reaction, as in the case of DNS, is used to measure enzyme activity. Many other methods have been used in the determination of cellulase activity including a viscometric method, measurement of reducing groups, chromophore or fluorescent group release, chromatographic substrate or product measurement, and coupled enzymatic protocols.[7] These methods, including the one based on liquid chromatography for analysis of carbohydrates, have been deemed effective.[8,9] Lacourse et al. provided a technical note covering the use of anion-exchange

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separation of carbohydrates by pulsed amperometric detection with a pH-selective reference electrode.[10] The use of ion chromatography (IC) to quantify the amount of different sugars produced during cellulase activity measurement provides an accurate way to evaluate the sugar-producing ability of cellulases, i.e., the measured sugar content of the hydrolysis product can be used to determine a true cellulase activity in terms of the strict definition of cellulase activity.[11] This method is more used in research due to the sophistication and skills involved in the sample preparation and operation of the instrument.

With an expanded interest in developing reliable protocols to measure enzyme activity, a piezoelectric-sensing technique, the quartz crystal microbalance (QCM), was introduced.[12] The QCM utilizes the piezoelectric property of a quartz crystal as an ultrasensitive mass balance. Sauerbrey reported that the changes in frequency of a quartz crystal can be used to measure the mass or thickness of vacuum-deposited metal, and researchers have commonly used his equation (the Sauerbrey equation), to quantify changes in such parameters in a number of applications:[12−15] material properties and theoretical models,[16,17] thin film deposition,[18,19] electrochemistry,[20,21] biological/biochemical research,[22−24] as well as adsorption and adhesion. Rojas and co-workers[15,17,25,26] and Josefsson et al.[27] have reported recently on the measurement of cellulase behaviors (binding and hydrolysis) with QCM. These efforts have shown that quartz crystal resonators coated with different types of thin films of cellulose can provide excellent capability for in situ monitoring of enzyme activity, under controlled conditions of temperature, pH, etc. The most common substrate used in these efforts has been regenerated cellulose which involves the application of a polar solvent to dissolve cellulose before spin coating on the QCM sensors;[14,17,25−28] the cellulose crystallinity is therefore completely disrupted in this case. Evaporation of solvent may bring back some hydrogen bonds between cellulose chains, but the morphology of the native cellulose is altered; cellulose II is formed from NMMO/DMSO solutions,[29] whereas the DMAc/LiCl procedure produces more amorphous materials.[30] The activity information obtained after using regenerated cellulose may therefore reflect only part of the information that could be obtained from native cellulose substrates. Recently, nanofibrillar cellulose (NFC) coated on QCM sensors was used to partially overcome this issue taking advantage of the fact that NFC manifests the crystallinity and morphology at the nanoscale of the native cellulose.[15] However, finding a substrate that truly mirrors the chemical composition, morphology and other characteristics of the cell wall at the micro- and nanoscale continues to be challenging. Furthermore, empirical models should be used to obtain numerical activity results for enzymes acting on surfaces such as those indicated above, and these data should be compared with results from other methods.

Although changes in film mass were monitored in the efforts cited above, no attempt was made to monitor the changes in solution properties (such as density or viscosity) as the degradation evolved under different incubation conditions. It should be noted, however, that in 2000, He et al. reported on the use of quartz crystal sensors to measure the changes of viscosity of CMC solutions incubated with cellulase; they also pointed out the possibility of studying cellulose hydrolysis by cellulase as a new assay method.[31] Nevertheless, this work did not contribute to actual development of cellulase activity measurement; instead it focused on the kinetic aspects of CMC hydrolysis. Ash et al. also used the droplet quartz microbalance technique to measure the viscosities of industrial oils for the study of their viscosity properties.[32] Saluja and Kalonia reported on measurements of fluid viscosity with the QCM and made suggestions for improving the circuit for the QCM.[33] The above efforts have taken advantage of the fact that changes in liquid properties affect the shift of the resonant frequency, as was modeled by Kanazawa and Gordon using a system with wave propagation in the shear mode.[34,35] Kanazawa and Gordon's model states that the change of resonance frequency due to changes in liquid properties is proportional to the square root of the product of dynamic viscosity and density, as is indicated by eq 1:[34,35]

$$\Delta f = -\frac{f_0^{3/2}}{\sqrt{\pi\rho\mu}}\sqrt{\rho_L\eta_L} \qquad (1)$$

where Δf is the resonant-frequency shift, f_0 is the resonant fundamental frequency of the quartz crystal, μ is the shear modulus of the quartz crystal, η_L is the dynamic viscosity of the liquid, and ρ and ρ_L are the densities of the quartz crystal and the liquid, respectively. A similar effect was observed and reported 20 years earlier by Stockbridge[36] when exploring the

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effect of gas pressure on the quartz crystal microbalance. Stockbridge formulated an expression similar to that in eq 1 but considered the resonance overtones (overtone number n):

$$\Delta f = -\sqrt{\frac{n}{\pi}}\frac{f_0^{3/2}}{\upsilon\rho}\sqrt{\rho_L \eta_L} \quad (2)$$

where υ is the wave propagation velocity in quartz (3340 m/s) and the other variables have the same meaning as stated before (eq 1). Ward and Buttry have pointed out that the fundamental frequency f_0 is a function of tension and mass of the quartz crystal used;[23] also, the gold electrode coating may cause alterations to f_0. Most relevant to our work, Ward and Buttry reported on the QCM frequency response caused by the interaction between glucose and hexokinase bound on polyacrylamide films.[23]

In the present work we propose an alternate protocol to measure cellulase activity by using the QCM. In this method QCM frequency change is used to measure the solution viscosity and density changes in the solution used to incubate the given cellulose substrate, after enzymatic hydrolysis. The results are then used to quantify the enzyme activity. In order to validate the proposed method and to compare it with the traditional ones, two cellulase enzymes and three different substrates were used. It is worth noting that the proposed method is not limited to any particular type of substrate, in contrast to most standard protocols for which only a given type of cellulose substrate can be used. For the purpose of illustrating this fact we document here the cases of three cellulose sources, namely, microcrystalline cellulose powder (MCC), filter paper (FP), and carboxylmethyl cellulose (CMC). The measured activities for the various cellulase enzymes were then compared to values obtained with the DNS, BCA, and chromatographic methods.

EXPERIMENTAL SECTION

Materials and Methods. Microcrystalline cellulose (AvicelPH-101) was purchased from Fluka. Carboxylmethyl cellulose (MW of ca. 90 000 and D.S. of 0.7) and no. 1 Whatman filter paper (FP hereafter) were purchased from Sigma. Dyadic experimental cellulase (DEC) was supplied by DyAdic (Jupiter, FL), and a commercial cellulase mixture in powder form was purchased from MP Biomaterial Inc. (MPC thereafter). The modified Lowry assay kit (to determine the protein content) was purchased from Pierce (Rockford, IL). DNS, biccinchoninic acid, and cupric sulfate were from Fisher Scientific. Glacial acetic acid and sodium acetate were used in the preparation of buffer solutions (pH 4.8 and ionic strength of 100 mM). QCM quartz sensors coated with gold (QSX303) were purchased from Q-Sense (Q-sense, Baltimore, MA).

An HP 8453E UV−vis spectrophotometer was used to determine light absorbance in the DNS and BCA methods. Dionex ion chromatographs ICS-3000 and IC-500 were used for sugar analysis. CarboPac PA-1 columns and electrochemical detectors were used with both chromatographic instruments. A Q-300 quartz crystal microbalance with dissipation, QCM-D (Q-Sense, Sweden) was used with Q-tools software for data processing.

X-ray diffraction (Philips XLF ATPS XRD 1000) was used to determine the crystallinity index of the cellulose substrates. The instrument used an OMNI Instruments Inc. customized Automount with a Cu target with $\lambda = 1.5$ Å. The quantification of the crystallinity index was based on the method proposed by Segal et al.[37] Finally, a Cannon−Fenske capillary viscometer was used to determine the viscosity of glucose solutions while their densities were measured by volumetry using a 500 mL volumetric flask at 25 °C.

Activity Test of Cellulase. 1. Protein Assay with Lowry Kit. On the basis of the work by Lowry et al.,[38] the modified Lowry protein assay kit (product no. 23240) with bovine serum album (BSA) was applied as a standard for the assay of protein content. Two samples as well as all standard calibration solutions (with a series of different protein concentrations) were treated with the protein assay and Folin−Ciocalteu reagents. For this purpose the assay kit was used following the recommended procedures and conditions noted by Lowry et al.[38] The absorbance was measured at 750 nm with an HP spectrophotometer.[38] Test samples and protein standards were diluted with sodium acetate (pH 4.8 and ionic strength 100 mM).

2. Enzyme Activity Using DNS and BCA Methods. Whatman no. 1 FP, Avicel PH-101 MCC, and CMC were used as substrates to test cellulase activities. The procedures for filter paper activity (FPase) and CMC activity (CMCase) were applied following the IUPAC method. Activity assays on MCC followed a similar procedure using a 50 mg substrate mass in each assay. In the case of the BCA assay, incubation was done with test tubes wrapped in aluminum foil to prevent light interference. In order to get accurate results, the BCA agent, a mixture of bicinchoninic acid and cupric sulfate, was freshly prepared before addition to either the standard calibration solution or hydrolysis products. Due to the extreme high sensitivity of BCA agent to reducing end groups in a mixture, the concentrations of standard solutions were in the milligram per liter range and the hydrolysis products were therefore diluted by a factor of 500−2000. Incubation conditions and the spectrophotometric protocols followed the procedures reported by Johnston et al.[7] Both DNS and BCA methods were used to determine the amount of reducing sugars. The calculation of enzyme activity followed the IUPAC method.[1]

3. Enzyme Activity Using Ion Chromatography. Two sets of standards were prepared to identify all the sugars that were produced after incubation of the substrates. One set included monosaccharides only (arabinose, xylose, glucose, mannose, galactose, and fructose). The other set of calibration solutions contained only glucose and cellobiose. In the case of monosaccharide analysis, pure water was the eluent (1.2 mL/min elution rate), whereas for samples containing cellobiose the eluent consisted of 50 mM potassium hydroxide aqueous solution (elution rate of 1.0 mL/min). In all cases fucose was used as an internal standard.

After hydrolysis, the samples for IC were vigorously boiled for at least 30 min right after the incubation, and then they were filtered through a 0.45 μm nylon membrane syringe filter. Collected samples were diluted by a factor of 50 in a volumetric flask, and the fucose internal standard was added. The amount of glucose and glucose equivalent of other reducing sugars were

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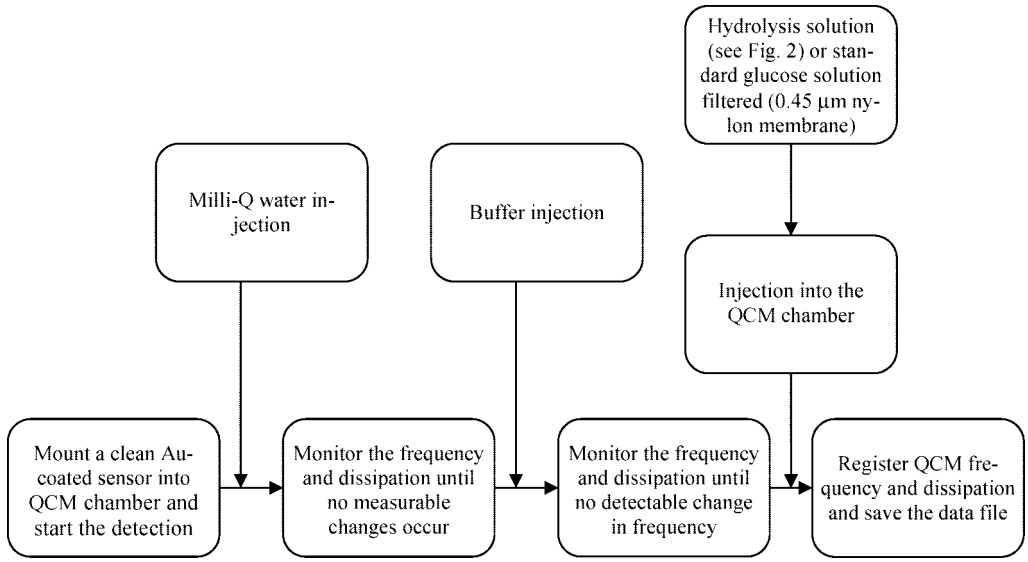

Figure 1. QCM protocol to obtain the calibration curves or to test enzymatic activity from measurements with incubation (hydrolysis) solutions.

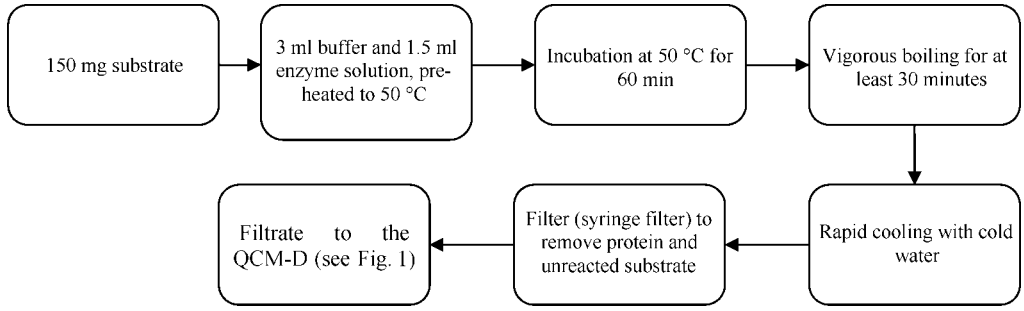

Figure 2. General procedure followed to measure enzymatic hydrolysis of the substrate in the QCM method.

measured as the sugars produced by enzymatic hydrolysis. At least two concentrations of cellulases were used so that release sugars were below and above 2.0 mg which is the reference concentration used to calculate cellulase activity.[1]

4. Enzyme Activity Using QCM-D.

4.1. Standard Curve. Glucose solutions were prepared at five different concentrations in buffers of identical pH and ionic strength and used to measure QCM frequency in triplicate runs. The temperature of the QCM chamber was set to 25 °C for all tests, and the sequence used in the measurements was randomized. The test procedure is summarized in Figure 1. Note that in the case of the standard calibration curves discussed here the third injection shown in Figure 1 consisted of standard glucose solutions instead of the solutions from hydrolysis generally used (see next section).

4.2. Substrate Hydrolysis. A modified procedure for hydrolysis of the substrates (MCC, CMC, and FP) was applied (see Figure 2). A sample of 150 mg of each substrate was incubated with enzyme (instead of the 50 mg used in the other methods, see later sections). The relative amounts of both buffer and enzyme solutions were kept the same as in the other methods. After the incubation of the mixtures, vigorous boiling for 30 min was applied to ensure the enzymes were completely denatured and deactivated. Enzyme blanks for each cellulase concentration were boiled together with their corresponding hydrolysis mixtures. No subsequent chemical treatments were adopted for color development (as in the DNS and BCA methods). The boiled reaction mixtures were then filtered through a 0.45 μm hydrophilic nylon syringe filter to remove protein and residual cellulose. The filtrates were introduced directly in the QCM-D chamber for frequency detection which was then used to determine the enzyme activity using the standard curves obtained in section 4.1. Standard Curves. (See also Figure 1.)

For each enzyme, solutions of four different concentrations were prepared to show that the produced amount of reducing sugars was linearly related to the respective enzyme concentration. The chosen concentrations were also in a range which was selected to produce 2 mg of reducing sugars (using 0.5 mL of enzyme solution and a 60 min incubation time). In other situations, the dilution was different in order to have the correct amount of sugar equivalent (0.5 mg in the case of CMC).

4.3. Test of Hydrolysis Product with QCM-D. Quartz crystal sensors coated with gold were used for testing both the standard solutions and the hydrolysis products at 25 °C. At the beginning of each run, Milli-Q water was used to ensure the cleanliness of the sensors and the stability of the baseline. After stabilization of the instrument, an injection of buffer solution with a syringe pump followed. All fluids were introduced at very low flow rates to avoid temperature and stress disturbances in the sensor area. After instrument stabilization for at least 5 min, the

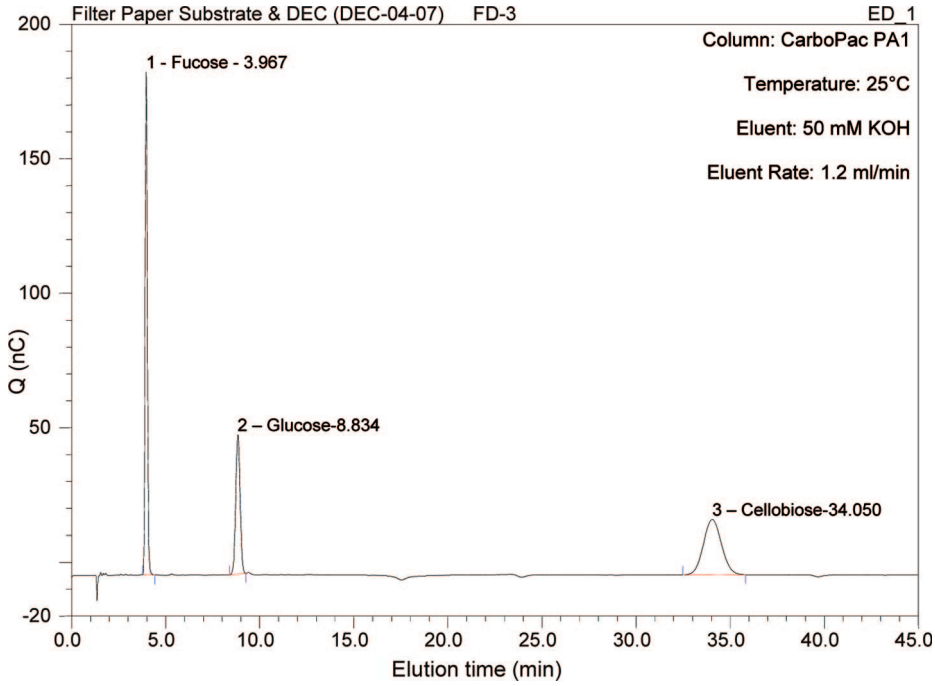

Figure 3. Chromatogram of hydrolysis solution after boiling and filtration. Incubation was performed at 50 °C for 60 min using Whatman no. 1 filter paper substrate and Dyadic experimental cellulase (DEC).

Table 1. Cellulase Activity (μmol per mg per min) for DEC and MPC Determined from the Four Techniques Used, DNS, BCA, IC, and QCM-D

enzyme and method	Dyadic experimental cellulase (DEC)				commercial cellulase from MP (MPC)			
substrate	DNS	BCA	IC	QCM-D	DNS	BCA	IC	QCM-D
MCC	286	130	84	173	72	failed to eliminate protein contribution[a]	60	120
FP	417	252	389	329	185		84	171
CMC	5873	2482	1082	6458	1779		837	3244

[a] Protein interference in the case of MPC cellulase (with the BCA method) prevented accurate determination of activity. The units for activity were units per milligram of cellulase instead of units per milliliter, as recommended by Ghose (ref 1).

injection of the hydrolysis products was initiated. Each run was terminated once the frequency variation was less than 0.1 Hz over a 5 min period. Finally, a surfactant solution was injected to clean the tubing and QCM chamber (see Figure 1).

RESULTS AND DISCUSSION

Verification of Separation Interferences. Ion chromatography was used to verify which sugars were contained in the filtrates from incubation of Whatman no. 1 filter paper used in the QCM experiments (see also Figures 1 and 2). In addition to the first negative noise signal, three peaks were detected (see Figure 3), which corresponded to fucose (internal standard), glucose, and cellobiose. Therefore, only these two sugars were present in the filtrate of the hydrolysis products. It is apparent that the process of boiling the reaction mixtures successfully coagulated the cellulase proteins and did not produce any interfering species passing through the syringe filter that could be detected. Since the cellulase enzymes used in this research were received as powders, no stabilizing reagents, such as sorbitol, were expected as was supported by their absence in the IC chromatograms. The same experiments were performed using the substrates CMC and MCC and a second cellulase (MPC). Only peaks for the internal standard and glucose were obtained after hydrolysis using the MPC cellulase. Several experiments were also performed for up to 2 h elution time under the same operating conditions as show in Figure 3. No peaks other than those for fucose, glucose, and cellobiose were observed (chromatograms not shown).

Protein Content and Enzyme Activity from DNS and BCA Methods. As noted before, we used a protein standard consisting of BSA to measure the protein content of the enzymes used in this investigation. The average protein content, expressed as micrograms of protein/milligram of the crude cellulases from triplicate measurement was 520 ± 28.7 and 210.5 ± 20.5 for DEC and MPC, respectively.

For the purpose of comparison, filter paper (Whatman no. 1), which has a high degree of crystallinity, MCC, a cellulose with higher crystallinity, and CMC were used as substrates in our experiment. The measured activities are presented in Table 1 which includes results from all methods used, as will be discussed below.

Cellulase Activity Using Ion Chromatography. IC results showed that products of hydrolysis from DEC and MPC treat-

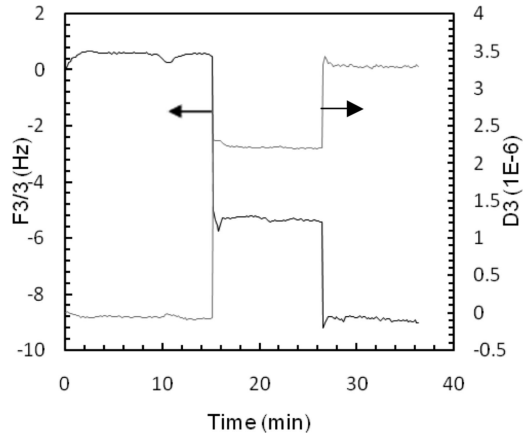

Figure 4. Frequency profile following the injection of buffer (15 min time) and glucose solution (26 min). The primary vertical axis on the left indicates the normalized third-overtone frequency, while the one on the right indicates the corresponding change in dissipation. The first plateau corresponds to water in the QCM chamber, the second one to buffer solution, and the third to a 5.0 mg/mL glucose solution in buffer. Tests were performed at 25 °C.

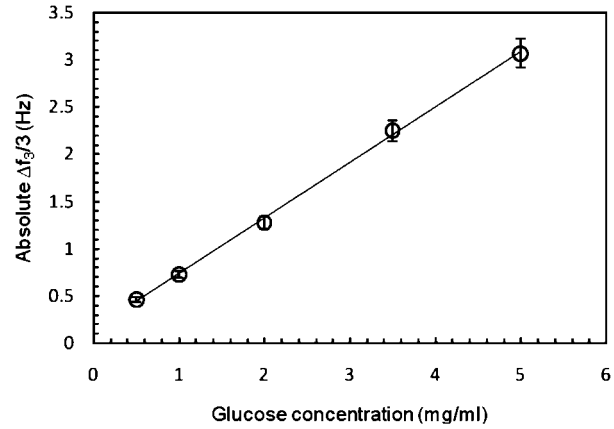

Figure 5. Calibration curve of absolute change of normalized frequency vs glucose concentration. Measurements were performed at 25 °C using a gold-coated quartz crystal sensor. Each concentration was run in triplicate, and the fitting equation is $\Delta f = 0.59C_{gl} + 0.15$ with $R^2 = 0.99$. The experimental error is depicted by the error bars, which in most cases are smaller than the data symbols used in the figure.

ments yield different carbohydrate species. DEC produced both glucose and cellobiose, whereas MPC produced only glucose. This distinctive difference is explained by the fact that MPC contains relatively larger amounts of β-glucosidase which was capable of degrading cellobiose units into glucose as soon as it is produced during incubation. The amount of total carbohydrates produced (glucose and cellobiose) were factored in obtaining cellulase activities, as explained earlier. Cellulase activity was calculated following the IUPAC method.[1]

Cellulase Activity Using QCM-D. 1. QCM Frequency and Glucose Concentration. Figure 4 shows a time profile of the QCM third-overtone frequency after introduction of different solutions into the resonator chamber (see Figure 1). The first flat section of the QCM frequency corresponds to Milli-Q water in contact with the sensor. Upon injection of buffer solution, the frequency dropped abruptly to a plateau while dissipation increased. These changes may be explained by the change in bulk density and viscosity of the buffer solutions relative to the Milli-Q water. The overshoot signal is the result of temporal stresses in the resonator upon injection and is disregarded in any calculation. Injection of buffered glucose solution followed which produced a second drop in the QCM frequency (shown in Figure 4). Once the temperature stabilized at 25 °C, both the frequency and dissipation curves leveled out. The frequency and dissipation changes were calculated numerically by subtracting the value corresponding to the buffer from that corresponding to the buffered glucose solution. Glucose solutions of five different concentrations, each run in triplicate, were tested following the procedure explained above. The normalized change in QCM frequency (Δf) and dissipation (ΔD) as a result of changes in glucose concentrations (C_{gl}) were fitted using linear regression, yielding the following expressions: $\Delta f = 0.59C_{gl} + 0.15$ for frequency change with an $R^2 = 0.99$ and $\Delta D = 0.22C_{gl} + 0.03$ for dissipation change with an $R^2 = 0.98$. See Figure 5 for the frequency calibration curves.

2. Cellulase Activity Using the QCM-D Method. The protocols followed for standard glucose solutions explained before

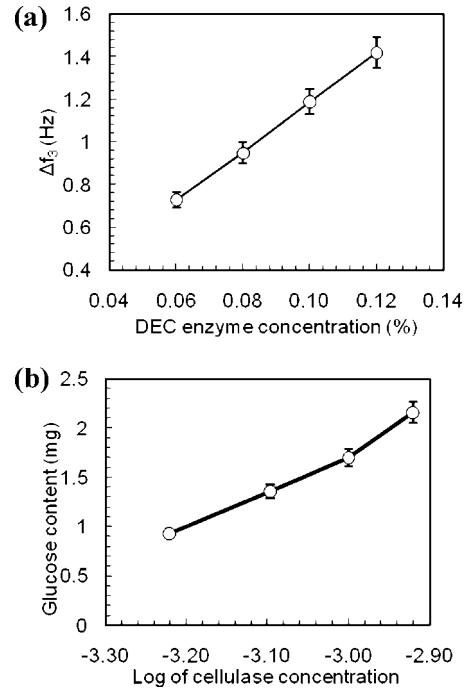

Figure 6. (a) QCM frequency change as a function of DEC cellulase concentration. Δf is the absolute value of adjusted frequency change. Best fitted curve is $\Delta f_3 = 1189.4C_{dec}$. (b) Glucose content as a function of the logarithmic value of DEC enzyme on FP substrate. The best fitted equation: glucose content = 3.98 Log(C_{dec}) + 13.73 with a correlation coefficient of 0.98, where C_{dec} is the cellulase concentration.

were applied to the hydrolysis products after incubation of filter paper with DEC enzymes at different concentrations, and the QCM changes in frequency corresponding to these experiments were collected. The difference between Δf from filtrates from enzymatic hydrolysis and their corresponding blanks were used as the net Δf contribution from hydrolysis products, Δf_3. The observed changes in QCM frequency for the various hydrolysis solutions are the result of different concentrations of glucose

Table 2. Density, Viscosity, and Predicted Δf Based on the Stockbridge Equation

glucose concn (mg/mL)	density (g/mL)[a]	dynamic viscosity (N s/m^2)[a]	calcd Δf (10^6 Hz)	calcd Δf relative to buffer (Hz)[b]	exptl Δf (Hz)[c]
0	1.00166	1.132×10^{-3}	457.9 ± 0.1	0.0	0
0.5	1.00178	1.133×10^{-3}	458.2 ± 0.4	0.3	0.46 ± 0.07
1	1.00202	1.135×10^{-3}	458.6 ± 0.5	0.7	0.73 ± 0.05
2	1.00246	1.137×10^{-3}	459.1 ± 0.4	1.2	1.29 ± 0.06
3.35	1.00304	1.141×10^{-3}	460.1 ± 0.5	2.2	2.25 ± 0.10
5	1.00376	1.142×10^{-3}	460.4 ± 0.4	2.5	3.07 ± 0.17
6.67	1.00450	1.146×10^{-3}	461.4 ± 0.4	3.5	

[a] Both density and dynamic viscosity values are the average of three replicates. [b] The relative confidence intervals for calculated Δf relative to buffers are less than ±5% and are not shown in the table (the ±5% relative intervals have included the accumulative property of errors). [c] The experimental Δf is shown in the table with 95% confidence intervals. The design of experiments for the experimental Δf was generated using a software package JMP from SAS Institute Inc.

produced and therefore different densities and viscosities of the bulk solution measured by the QCM sensor. It should be noted, however, that any other monomeric or oligomeric degradation product that may be small enough to pass through the syringe filter can also contribute to the measured signal. The contribution from these units is accounted for by subtracting the frequency change for an enzyme "blank". Figure 6a shows a plot of Δf_3 against DEC concentration.

The frequency values in Figure 6a were converted to glucose content using the calibration curve (Figure 5) and then plotted against the logarithmic values of the respective cellulase concentration used during the incubation (see Figure 6b for DEC enzyme used to hydrolyze filter paper substrate). The glucose concentration measured in the solution after incubation of the substrate with the enzyme was shown to have a logarithmic relationship with the cellulase concentration.[1] The dilution factor of the enzyme solution that would be required to produce 2 mg of glucose in 60 min was obtained from Figure 6b, and from this value the cellulase activity was calculated according to Ghose.[1] The resulting activity values are shown in Table 1 which includes DEC and MPC activities on various substrates.

3. Density and Viscosity Effects on QCM Frequency. Table 2 includes values for the density and viscosity of glucose solutions; it also shows the expected QCM frequency changes if such solutions were tested with the QCM in response to bulk liquid density and viscosity, as calculated from eq 2 (using a quartz density and wave velocity of 2649 kg/m^3 and 3340 m/s, respectively).[36] The fundamental frequency used in the calculations is 2.5 MHz, which equals one-half of the first overtone as indicated by the QCM-D used in this research.[39] The two slopes in Figure 7 are approximately the same within experimental errors. The pronounced difference at 5.0 mg/mL glucose concentration may come from experimental error produced in determining its dynamic viscosity. In comparison to the description by Ward and Buttry,[23] the fundamental frequency of a 1 mm thick AT-cut quartz crystal sensor would be 3.3 MHz, with shear modulus of 2.947×10^{11} dyne/cm^2 and density of 2.648 g/cm^3. The difference between these fundamental frequencies is probably due to the gold coating on the crystal sensors because gold has different shear modulus and density from crystal. A plot of the experimental values versus the predicted frequency changes for different glucose concentrations gives an excellent linear relationship ($R^2 = 0.99$), and the plot of calculated Δf versus glucose concentration is also very linear with $R^2 = 0.98$ (Figure 7).

Enzymatic Activity from the Different Methods. Table 1 summarizes the enzyme activities for the different substrates. These results demonstrate that the activity of enzymes applied on CMC was by far the highest, followed by FP and MCC activities. This observation is probably explained by the difference in substrate crystallinity, as measured by X-ray crystallography: The crystallinity index of Whatman no. 1 FP was 78%, whereas Avicel PH-101 MCC was 84% (Figure 8). The crystallinity index of MCC was similar to that obtained by Fan et al.,[40] whose crystallinity index for MCC was 84.5%.[36] In comparison to the crystallinity index obtained by Nidetzky et al. for filter paper (45%),[41] our experimental result shows a higher value (78%). However, Banka and Mishra showed a wide range of values between 57% and 96% for the same substrate after being ball-milled for different times.[42] Our results for enzyme activities for the different substrates are consistent with the fact that a substrate with higher crystallinity is expected to be hydrolyzed slower.[40,42] However, Grethlein has claimed that surface area is more important than crystallinity in determining cellulose hydrolysis rate.[43] The large variance of cellulase activity on CMC is the result of its complete solubility (noncrystalline nature) in water.

Examination of Table 1 also shows that DNS and QCM-D yielded the highest enzyme activity values, whereas IC gave the lowest values. The difference in the measured activity is explained by the fact that the DNS method is sensitive to all reducing end groups, including those from substrate, cellulase enzyme, and product, whereas QCM-D detects only contributions from sugars (since it is expected that the enzyme blank can eliminate effects from other small molecules that might pass through the syringe filter) Ion chromatography, however, detects the contribution from sugars depending on the species used in the preparation of the standard calibration. Here the results are based on the contribution from glucose and cellobiose only. Finally, the BCA technique is very sensitive to any possible protein contained in the test solutions.

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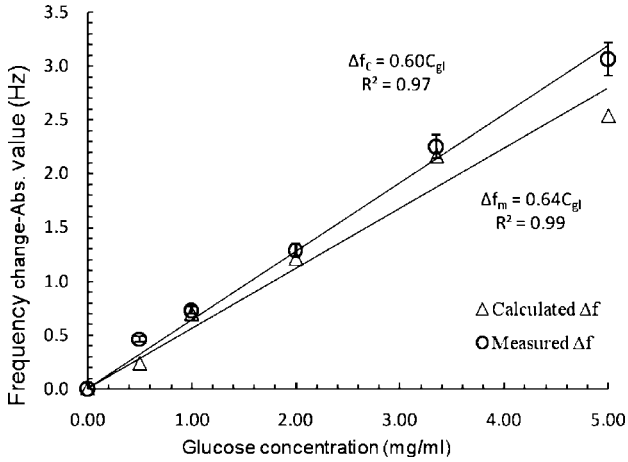

Figure 7. Plot of calculated Δf using eq 2 and experimental Δf against glucose concentration. Both have a very good linear relationship. The fit equation for calculated frequency change is $\Delta f_C = 0.60C_{gl}$ with a correlation coefficient of 0.97, whereas that for measured frequency change is $\Delta f_m = 0.64C_{gl}$ with a correlation coefficient of 0.99.

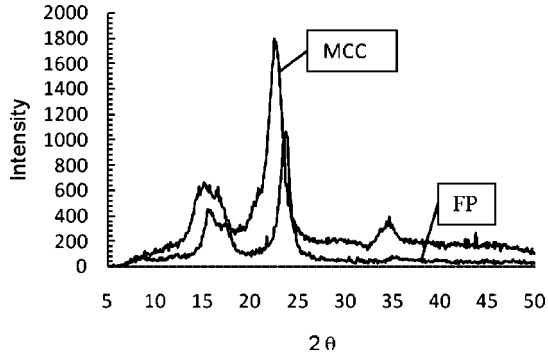

Figure 8. X-ray diffractogram for microcrystalline cellulose (MCC) and Whatman no. 1 filter paper (FP). The calculated MCC crystallinity index was 84%, whereas that for FP was 78%.

Figure 9 shows the correlation of cellulase activities from QCM-D, DNS, and BCA with respect to those obtained from the IC method. It is reasonable to believe the IC results more closely represent the actual, overall ability of a cellulase enzyme to produce reducing sugars, especially based on the definition from Ghose.[1] The IC result combines both the contribution of glucose and cellobiose, whereas it eliminates other interferences such as proteins, under the premise that those substances were removed by the nylon filter, as demonstrated in the chromatograms such as that presented in Figure 3. A higher correlation coefficient between QCM-D and IC activities was found compared to that of the DNS method. This can be explained by the fact that the syringe filter used in the QCM-D and IC methods was able to remove most interfering substances that are produced during hydrolysis and the major components detected by both QCM and IC are sugars. Since the DNS method has been the most widely used one to measure cellulase activity, we compared the IC, QCM, and BCA methods against it and noted that QCM-D gave a relatively good correlation ($R^2 = 0.955$). However, the correlation between IC and DNS is not as good, which can be explained

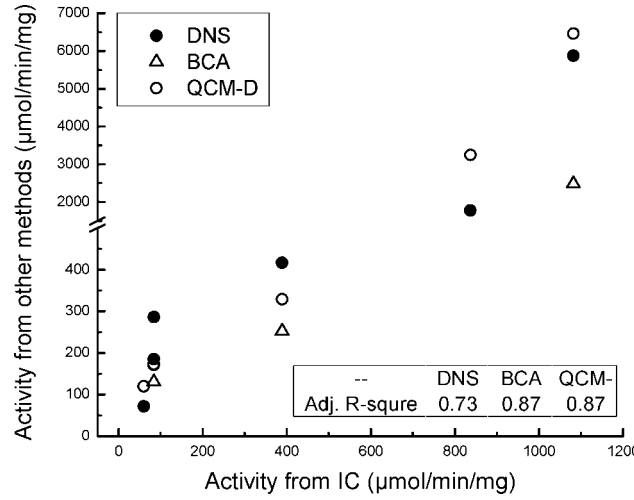

Figure 9. Correlation of enzyme activity measured with DNS, BCA, and QCM-D methods compared to IC results.

by the different relative amounts of glucose and cellobiose produced for the different substrates. This was in fact observed in experiments with DEC after analyzing the chromatograms (not shown). A high correlation between BCA and DNS was observed, as expected from the fact that both methods are based on redox reactions and also because they use the same detection technique (UV−vis spectroscopy).

QCM-D is an advanced technique that involves simple operation and does not involve application and disposal of color-developing (redox) chemicals. In comparison to the more sophisticated IC, it involves less advanced skills. The standard calibration curve shows good reproducibility with a correlation coefficient as high as 0.99 (see section 1. QCM Frequency and Glucose Concentration). When hydrolyzing complex substrates such as lignocellulosics, the DNS and BCA methods can suffer from interfering substances in color development or measurement. This is in contrast to QCM that has no limitation in terms of the type of substrate used. The IC technique gives unambiguous sugar identification, but sample preparation may be involved, and with complex substrates possible column contamination could cause interference.

CONCLUSION

A new method to measure enzyme activity is proposed that measures the change in frequency of a gold-coated quartz sensor when exposed to hydrolysis solutions after substrate incubation. The quantification of cellulase activity is based on the Stockbridge relation for different concentrations of degradation products. Validation of the proposed method was accomplished by the use of three different substrates, with different crystallinities. Lower enzyme activities were measured in the case of substrates with high crystallinity. Comparisons were carried out with other methods, including IUPAC's DNS method.

In contrast to methods requiring color development and possible exposure to toxic reagents, the QCM activity protocols do not entail the use of any chemical that would be critical to its reproducibility. Results from the four different techniques tested (QCM, DNS, BCA, and IC) showed that the QCM-D method produced results closer to the true sugar production activity of

cellulase enzymes as measured by IC. The skill required and the cost and maintenance needed in IC prevent its use as a routine method to quantify cellulase activity, whereas QCM-D is a promising application in quantification of cellulase activity with a simple operation principle. Finally, the QCM technique can be applied to situations where substrates other than pure cellulose are to be used. This opens the possibilities for analysis of complex lignocellulosic matrixes such as whole biomass substrates.

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