Measurement of filter paper activities of cellulase with microplate-based assay

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Abstract
It is always a challenge to determine the total cellulase activity efficiently without reducing accuracy. The most common total cellulase activity assay is the filter paper assay (FPA) established by the International Union of Pure and Applied Chemistry (IUPAC). A new procedure to measure the FPA with microplate-based assay was studied in this work, which followed the main idea of IUPAC to dilute cellulase preparation to get fixed glucose release. FPAs of six cellulase preparations were determined with the microplate-based assay. It is shown that FPAs of cellulase Youtell, RCconc, R-10, Lerkam, Yishui and Sinopharm were 67.9, 46.0, 46.1, 27.4, 7.6 and 8.0 IU/ml respectively. There was no significant difference at the 95% confidence level between the FPA determined with IUPAC and the microplate-based assay. It could be concluded that the FPA could be determined by the microplate-based assay with the same accuracy and much more efficiency compared with that by IUPAC.

1. Introduction

Lignocelluloses are the most abundant renewable bioresource in the world. The cellulase, which has been widely used in the food processing, is the critical enzyme which could catalyze cellulose to oligosaccharides and glucose (Patindol et al., 2007; Kapasakalidis et al., 2009; Renouard et al., 2010; Abbès et al., 2011). In fact, the cellulase is a system consisting of endoglucanases, exoglucanases, and β-D-glucosidases, all of which hydrolyze crystalline cellulose synergically. The cellulase activities are always measured using insoluble cellulose. The heterogeneity of insoluble cellulose and the complexity of the cellulase system cause formidable problems in measuring total cellulase activity (Mullings, 1985; Criquet, 2002; Helbert et al.,...
2003; Eveleigh et al., 2009; Farnet et al., 2010; Dashtban et al., 2010). The most common total cellulase activity assay is the filter paper assay (FPA) using Whatman No. 1 filter paper as the substrate, which was established and published by the International Union of Pure and Applied Chemistry (IUPAC) (Zhang et al., 2006; Batool et al., 2015). The main idea of the IUPAC method is that cellulase must be diluted until the amount of product plotted against cellulase concentration is reasonably linear. The assay requires a fixed amount (2 mg) of glucose released from a 50-mg filter paper (1 × 6 cm). A series of cellulase dilution solutions is required to achieve a fixed degree of hydrolysis (Ghose, 1987; Butt et al., 2015).

Though the IUPAC method is accepted worldwide, there are still some shortcomings for FPA assays, such as labor-intensiveness, low-throughput, and requiring a large quantity of substrate, cellulase and chemicals. Several methods were developed for the purpose of high-throughput cellulase activity screening these years (Boyer et al., 2002; Goddard and Reymond, 2004; Xiao et al., 2005; Kasana et al., 2008; King et al., 2008; Peralta et al., 2008). Decker found that replacing the filter paper with Solka-floc, SigmaCell-20, Avicel and cotton linters, the assay for a rather similar substrate in hydrolytic properties to the filter paper could be automated on a Cyberlains C400 robotics deck (Ashraf et al., 2013; Decker et al., 2003). Berlin used the disk made from yellow poplar to estimate the hydrolysis of cellulose to glucose in a 96-well microplate. The assay shows considerable time and cost benefits over the standard assay (Berlin et al., 2005). Chundawat developed a procedure with the 96-well Biomass Conversion Research Lab microplate method for the high-throughput

![Fig. 1](image-url)  The curves for fixing the cellulase concentration that produce 2 mg of glucose release with IUPAC. (A) Youtell, (B) RCconc, (C) R-10, (D) Lerkam, (E) Yishui, (F) Sinopharm. *Only a series of results within 14 parallel tests of each cellulase are shown in Figs. 1 and 2 and Tables 1 and 2.
of diluted cellulase was added to the tube. At least two dilutions must be made of each cellulase sample. One dilution should release slightly more and the other one slightly less than 2.0 mg of glucose. The tubes were incubated at 50 °C for exactly 60 min. At the end of the incubation, each tube was removed from the 50 °C bath and the cellulase reaction was stopped by immediately adding 3.0 ml of DNS reagent. All tubes were boiled for exactly 5.0 min in a vigorously boiling water bath. Finally, after the colored solution was diluted with 20 ml of H₂O, the absorbance at 540 nm was measured.

The microplate-based assay was performed in a 96-well PCR plate. The cellulase preparations were diluted gradually to get a fixed amount of 80 µg glucose released between two dilutions. Then, the FPA was calculated with the Formula B.

The other part of the assay follows MFPA process basically. A 20 µl aliquot of each diluted cellulase was added into wells containing a 7-mm diameter filter paper disk and 40 µl of 50 mM Na-citrate buffer, pH 4.8. After 60 min of incubation at 50 °C, 120 µl of DNS was added into each reaction and incubated at 95° for 5 min. All incubations were performed in a Peltier Thermal Cycler (MJ Research, PTC-200). Finally, a 36-µl aliquot of each sample was transferred to the wells of a flat-bottomed plate containing 160 µl of H₂O, and the absorbance at 540 nm was measured.

2.4. Calculations

2.4.1. IUPAC

Estimation of the concentration of cellulase which would have released exactly 2.0 mg of glucose by means of a plot of glucose liberated against cellulase concentration was done. To find the required cellulase concentration, two data points were taken, which are very close to 2.0 mg, and a straight line was drawn between them to find the cellulase dilution that would produce exactly 2.0 mg glucose equivalents of reducing sugar. Then, the dilutions were translated into concentrations:

Cellulase concentration to release 2.0 mg glucose = 1/Dilution FPA was calculated as

\[
\text{FPA}(I) = \frac{0.37}{\text{Cellulase concentration to release 2.0 mg glucose}(A)}
\]

2.4.2. Microplate-based assay

Relative to the amount of glucose equivalents expected to be produced in IUPAC, the absolute amount of glucose released in the microplate-based assay at the critical dilution is 80 µg. The estimated amount of cellulase which releases 80 µg glucose contains 0.37 units, the same as that of IUPAC. So the FPA could be calculated as

| Cellulase       | Cellulase dilution | Cellulase concentration | FPA (IU/ml) |
|-----------------|--------------------|-------------------------|-------------|
| Youtell         | 189                | 0.0055                  | 69.9        |
| RConc           | 130                | 0.0077                  | 48.1        |
| R-10            | 109                | 0.0092                  | 40.7        |
| Lerkam          | 63                 | 0.0159                  | 23.3        |
| Yishui          | 24                 | 0.0442                  | 8.4         |
| Sinopharm       | 21                 | 0.0476                  | 7.7         |
FPA(M) = 0.37 / Cellulase concentration to release 80 μg glucose

3. Results and discussion

3.1. Measurement of FPA with IUPAC method

FPAs of six commercial cellulases were measured with the IUPAC method in this work. After each cellulase was diluted gradiently, the Whatman No. 1 filter paper was hydrolyzed with diluted cellulase in a 1.5 ml reaction system. Then the concentrations of cellulase which released exactly 2.0 mg of glucose were determined by plotting glucose liberated against cellulase concentration. The FPA of each cellulase preparation was measured fourteen times. The activities of six cellulase preparations were obtained with the Formula A. The results of one series of cellulase preparation are shown in Fig. 1 and Table 1. The results of other thirteen parallel samples are not shown here. It is shown that 2 mg of glucose could be obtained by plotting glucose against cellulase concentrations at two suitable dilutions. The cellulase Youtell has the highest FPA (69.9 IU), while the cellulase Sinopharm has the lowest FPA (7.7 IU/ml). The average activities of fourteen parallel tests of six cellulase preparations are shown in Table 3. It is shown that the activities of six cellulases ranged from 7 IU/ml to 70 IU/ml, which cover the scope of PDA of common commercial cellulases.

3.2. Measurement of FPA with microplate-based assay

After the cellulase preparations were diluted gradiently in microplate, the FPA were measured with microplate-based assay. The amount of cellulase releasing 80 μg of glucose in

Fig. 2 The curves for fixing the cellulase concentration that produce 80 μg of glucose with the microplate-based assay. (A) Youtell, (B) RCconc, (C) R-10, (D) Lerkam, (E) Yishui, (F) Sinopharm.
the well of microplate was fixed. Each FPA of six cellulases was measured fourteen times. PDAs of a series of six cellulase preparations are shown in Table 3. It is shown the cellulase concentration releasing exactly 80% of FPA of cellulase could be determined accurately with IUPAC. It would be an alternative method to measure the FPA of cellulase with the microplate-based assay in future.

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dilution and fix a certain cellulase concentration. Formula B, which is different from glucose could be obtained by means of plotting glucose against it is shown the cellulase concentration releasing exactly 80% of cellulase preparations. The calculated t-values which are below the critical t-value of 2.06 at the 95% confidence level and DF = 26.

Table 3 Comparison of FPAs of fourteen parallel tests determined with IUPAC and microplate-based assay.

| Sample   | FPA with IUPAC (IU/ml) | FPA with microplate-based assay (IU/ml) | t-Value |
|----------|------------------------|----------------------------------------|---------|
| Youtell  | 67.4 ± 2.6             | 67.9 ± 3.1                             | 0.46    |
| RCconc   | 45.7 ± 0.9             | 46.0 ± 1.6                             | 0.61    |
| R-10     | 46.4 ± 1.5             | 46.1 ± 1.8                             | 0.48    |
| Lerkam   | 26.9 ± 2.0             | 27.4 ± 1.9                             | 0.68    |
| Yishui   | 7.0 ± 1.2              | 7.6 ± 1.1                              | 1.38    |
| Sinopharm| 8.9 ± 1.3              | 8.0 ± 1.0                              | 2.05    |

T-test: data obtained from the microplate-based assay were compared with those obtained from IUPAC among six cellulase preparations. The calculated t-values which are below the critical t-value of 2.06 at the 95% confidence level and DF = 26.

4. Conclusion

The procedure and calculation for measuring the FPA with microplate-based assay were studied in this work. The FPAs of six cellulases were determined following the idea of IUPAC in microplate. The microplate-based assay has the same accuracy and much more efficiency compared with that of IUPAC. It would be an alternative method to measure the FPA of cellulase with the microplate-based assay in future.

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Table 2 A series of FPAs of six cellulase preparations with the microplate-based assay.

| Cellulase dilution | Cellulase concentration | FPA (IU/ml) |
|-------------------|-------------------------|-------------|
| Youtell           | 179                     | 0.0056      | 69.8        |
| RCconc            | 122                     | 0.0082      | 47.4        |
| R-10              | 128                     | 0.0078      | 44          |
| Lerkam            | 77                      | 0.01299     | 24.6        |
| Yishui            | 16                      | 0.0625      | 8.2         |
| Sinopharm         | 21                      | 0.0476      | 7.7         |

Table 3 Comparison of FPAs of fourteen parallel tests determined with IUPAC and microplate-based assay.

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