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Quantification of cellulase activity using cellulose-azure

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Abstract

Despite wide application of cellulose-azure as a substrate for measuring cellulase activity, there is no quantification of hydrolysis rate or enzymatic activities using this substrate. The aim of this study was to quantify the hydrolysis rate in terms of product formation and dye released using cellulose-azure. The amount of dye released was correlated with the production of glucose and the enzyme concentrations. It is shown that the lack of correlation can be due to (1) repression of the release of the azure-dye when azure-dye accumulates, (2) presence of degradable substrates in the cellulase powder which inflate the glucose measurements and (3) the degradation of cellulose which is not linked to the dye in the cellulose-azure. Based on the lack of correlation, it is recommended that cellulose-azure should only be applied in assays when the aim is to compare relative activities of different enzymatic systems.

Keywords: Hydrolytic activity; Cellulose-azure; Hydrolysis; Cellulase; Glucose

1. Introduction

Cellulase activity measurements have been used to quantify the cellulose hydrolysis rate in different biological systems. Cellulolytic activities of municipal solid waste samples from landfills [1–3] and of anaerobic digester sludges [4] have been studied. The complexity of the multi-component cellulase enzyme system and the physical heterogeneity cellulosic substrates makes it extremely difficult to predict the hydrolytic potential of any cellulase mixture. The wide range of methods used for determining cellulase activity and the numerous ways of expressing enzyme activity also make it difficult to compare data between laboratories quantitatively.

To quantify cellulase activities, enzyme assays have been carried out mostly on pure cellulose or cellulose derivatives [5]. Enzyme unit values are based on limited conversion of the substrate. Cellobiose and carboxymethyl cellulose are used as the substrate when quantifying β-glucosidase and endoglucanase activity, respectively [5]. Cotton and cellodextrins can be used as the substrate when measuring exoglucanase activities [6]. Filter paper is normally used as the substrate when the saccharifying cellulase activity is of interest [7]. When applying the above assays, reducing sugars are monitored as products released using either high performance liquid chromatography (HPLC) or a colourimetric (Nelson–Somogyi) method [8].

The above methodologies involve lengthy procedures and if the overall cellulase activity is the major concern, rather than individual cellulase activity, then assays using cellulose-azure as a substrate could be employed. Cellulose-azure has been widely used for cellulolytic activity measurement by different research groups [1–3,9,10]. The preparation of cellulose-azure was described by Fernley [11]. This substrate is basically comprised of insoluble cellodextrins coupled with water-soluble reactive dyestuffs.

This cellulose-azure assay has been applied on municipal solid waste samples [1–3]. In addition, the cellulose-azure substrate has been used to monitor starch, amylose and keratin-degrading enzymes [9], cellulose solubilising activity [10] and cellulose activity in desert soils [12]. Similarly, keratin-azure was used to study keratin degradation by fungi [13]. In the above studies, cellulase activity of a sample was determined by extracting enzymes from the sample, and adding this extract to an appropriate buffer with cellulose-azure in suspension. Incubation was performed at constant temperature until dye release was detectable. The cellulolytic activity in these previous studies was quantified either in terms of dye released per unit time

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when a colour change became detectable [1–3], as a relative activity compared to a control [10] or by preparing a standard curve of cellulose-azure degrading activity versus activity in international filter paper units (IFPU) for a commercial cellulase enzyme preparation [9]. The linear portion (low enzyme concentrations, 0–0.01 IFPU) of that curve was used to calibrate cellulose-azure hydrolysis for the determination of apparent cellulase activity.

Despite the wide application of cellulose-azure for hydrolysis studies, there is no actual quantification of hydrolysis rate or enzymatic activities for this substrate. It was assumed in all these previous studies that the rate of hydrolysis or the hydrolytic activity in a sample is proportional to the amount of dye released. This may not be true because the particles of the cellulose-azure substrate are not uniform in size and therefore do not have a constant surface area to volume (and mass) ratio e.g., particle attrition in storage will increase dye release from the cellulose-azure when applied to a standardized concentration of cellulase. Also, the amount of dye attached to each particle of the substrate will vary according to the preparation procedure and the surface area available. If the azure dye was not chemically bound to the substrate particles in a uniform fashion, the release of dye will not be a function of hydrolysis rate. It was therefore the aim of this study to quantify the hydrolysis rate in terms of product formation and dye release when using cellulose-azure as the substrate. By measuring both of these quantities, the dye release (increase in adsorbance) and the actual extent of hydrolysis (glucose concentration) can be correlated. The hydrolytic activity of any inoculum source can be estimated using cellulose-azure as either a rate of glucose formation or the total hydrolytic enzyme available provided that the correlation between (i) the dye released and glucose produced and (ii) the rate of glucose production and the concentration of commercial cellulase are both available. The hydrolysis step of wastewater or solid waste treatment process can thus be quantified directly as the hydrolytic activity based on the standard curves acquired.

2. Experimental

Cellulose-azure (product number: C1052, Sigma) which is an insoluble cellodextrin coupled with water-soluble reactive dyestuffs was used as the substrate in this study. Prior to using it in the assay, cellulose-azure was washed by milli-Q (MQ) water a number of times to remove the loosely (physically) attached dye. This procedure involves making a cellulose-azure solution with MQ water at a concentration of 20 g/L, centrifuging the solution at 2800 g for 20 min at room temperature, then measuring the absorbance of the supernatant at 595 nm by using a distilled water blank. The process was repeated until the absorbance of the supernatant was below 0.03 absorbance unit, A. After drying in an oven set at 60 °C overnight, the washed cellulose-azure was then used as the substrate in the assay.

A commercial enzyme extracted from *Aspergillus niger* (EC3.2.1.4 Cellulase, product number: C-1184, Sigma) with an estimated activity of 0.45 units/mg was used. According to the manufacturer of the enzymes, one unit liberates 1.0 μmol of glucose from cellulose in 1 h at pH 5.0 and 37 °C (with 2 h incubation time). Cellulose-azure solutions (1.0 g/L) were prepared in 250 ml of acetate buffer at pH 5.0. These solution mixtures were kept in 250 ml Schott bottles and kept at 38 °C in a water bath during the experiment. The effect of various enzyme concentrations (0.5, 1.5 and 4.0 units/ml) on cellulose hydrolysis was investigated. The experiment using an assay with enzyme concentration of 1.5 units/ml was repeated four times while triplicates were carried out for the experiments using assays with enzyme concentrations of 0.5 and 4.0 units/ml. The solutions were gently stirred by magnetic stirrer. Aliquot (3 ml) was withdrawn from the solution at different time intervals for absorbance measurement at 595 nm. The sample was filtered using a 0.45 μm membrane filter prior measurement. A control experiment with no enzyme addition was carried out and the absorbance values measured from this experiment were used as blank values. The blank value was subtracted from the activity measurement. The cellulase activities were measured in terms of the amount of dye released, i.e. the increase in colour intensity with time, which can be measured at 595 nm by a photometer as absorbance units at different time intervals.

After the absorbance measurement, the extracted samples were further treated to remove residual colours so that the colour did not interfere with the analysis of glucose and cellobiose using HPLC. The further treatment involved filtering the extracted sample through a Sep-Pak C18 cartridge (Part number: WAT051910 Waters) for colour removal. Each Sep-Pak cartridge was preconditioned by 5 ml of high purity methanol and then rinsed by 10 ml of MQ water. No extra steps were carried out to deactivate the enzymes prior to glucose analysis beyond removing the cellulose-azure by filtration because there was no substrate available in the final solution after two filtration processes. Six samples of the two standard glucose and cellobiose solutions (500 and 1000 ppm) were used to test if there was any adsorption of glucose onto the Sep-Pak cartridges. The results indicated that the Sep-Pak cartridges did not adsorb any glucose or cellobiose and it was therefore concluded that the application of this cartridge has no effect on glucose and cellobiose analysis. The HPLC was equipped with a Waters' M45 pump (flow rate of 0.6 ml/min), a Perkin-Elmer series 200 RI detector and a ISS 200 auto-sampler and a Biorad HPX-87H column and a compatible guard column. The operational temperature of the column was 65 °C. The solvent used was 0.008 N sulfuric acid and the sample size was 50 μL.

Azure-dye (product number: R8001, Sigma), the water-soluble reactive dyestuff used in the formulation of cellulose-azure, was employed to study the effect of dye inhibition on the solubilisation of cellulose-azure. Prior to using it in the assay, the relationship between the dye concentration and absorbance was determined by formulating a calibration curve, which is shown in Fig. 1. The effect of dye inhibition was studied by monitoring glucose formation after the addition of a fixed amount of azure-dye (22.1, 44.2 or 88.4 mg/L) to a set of cellulose hydrolysis experiments. These three azure-dye concentrations were chosen as they corresponded to be within an expected range of absorbance readings.

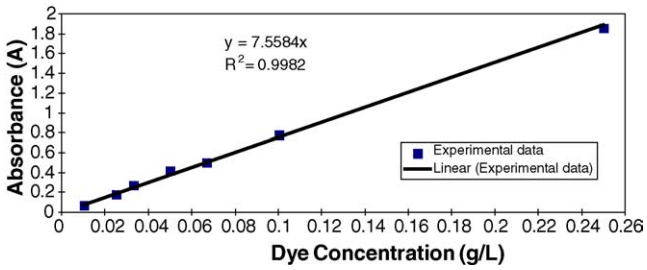

Fig. 1. Azure-dye calibration curve.

Avicel (microcrystalline cellulose type PH-101, FMC Corp.), a cellulosic substrate, was used as an alternative substrate in this study. Two volumes of Avicel solution (1.0 g/L) were prepared in 250 ml of acetate buffer at pH 5.0. These solution mixtures were kept in 250 ml Schott bottles and kept at 38 °C in a water bath during the run. The enzyme concentration investigated was 1.5 units/ml. The solutions were gently stirred by magnetic stirrer. Aliquot (3 ml) of sample was withdrawn from each solution mixtures after 2 h of incubation and the samples were then boiled for 20 min to ensure enzyme deactivation. The boiled samples were analysed for glucose and cellobiose content using the HPLC method as described previously.

3. Results and discussion

Table 1 shows the average absorbance units and average glucose concentration as a function of time at different enzyme concentrations. Standard deviations based on repeated runs are shown in parentheses. Cellobiose was not detected in any sample. Fig. 2 shows the accumulation of released dye (in absorbance unit) with time for each enzyme concentration. Fig. 3 shows the accumulation of glucose with time for each enzyme concentration. The rate of dye accumulation increases with enzyme concentration and for each enzyme concentration, dye accumulates more or less linearly with time (Fig. 2). As shown in Fig. 3, the rate of glucose accumulation also increases with enzyme concentration. When considering the lower enzyme concentration (0.5 units/ml), the glucose concentration increases linearly with time but for the other two enzyme concentrations (1.5 units/ml and 4.0 units/ml), the increase in glucose concentration slows down after an initial rise within the first 5 min. Fig. 4 shows the relationship between the dye concentration (absorbance) and glucose concentration, which are the dependent variables of Figs. 2 and 3, respectively.

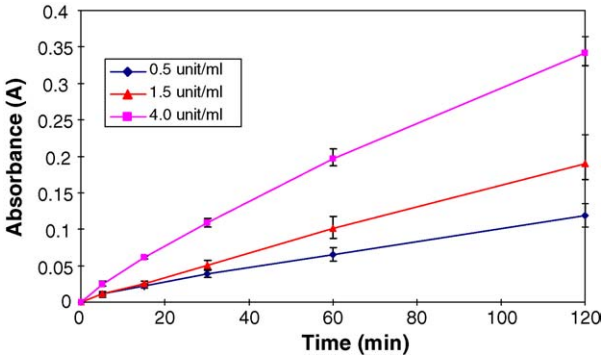

Fig. 2. Absorbance profile as a function of enzyme concentration.

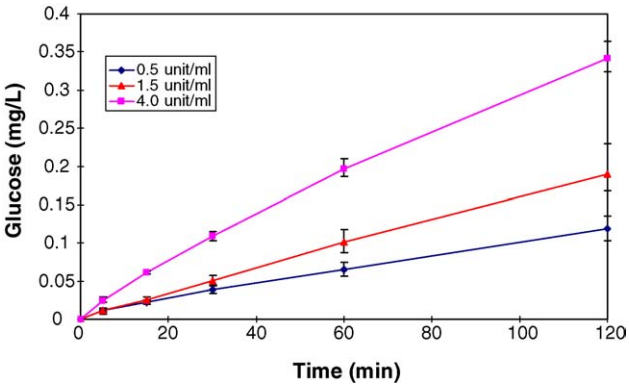

Fig. 3. Glucose profile as a function of enzyme concentration.

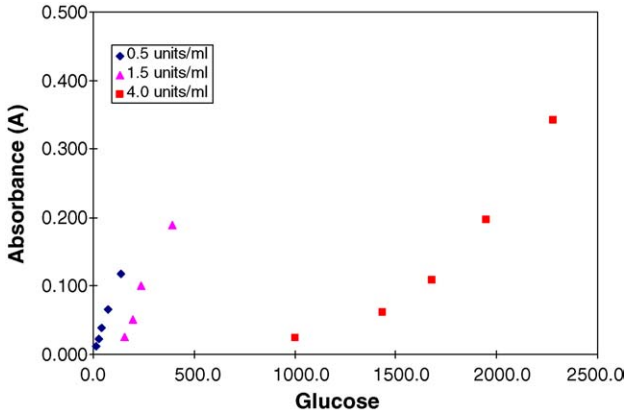

Fig. 4. Relationship between dye released and glucose formation.

Table 1
Experimental results of assays at different enzyme concentrations—glucose formation and dye released at different time intervals

Time (min)	0.5 enzyme units/ml		1.5 enzyme units/ml		4.0 enzyme units/ml	
	Absorbance (A)	Glucose (mg/L)	Absorbance (A)	Glucose (mg/L)	Absorbance (A)	Glucose (mg/L)
0	0	0	0	0	0	0
5	0.012 (0.0015)	14 (1.5)	0.011 (0.0033)	155 (25)	0.025 (0.0038)	152 (2)
15	0.022 (0.0031)	27 (4.8)	0.026 (0.0030)	194 (62)	0.061 (0.0020)	273 (4)
30	0.039 (0.0050)	42 (4.4)	0.051 (0.0052)	236 (45)	0.109 (0.0060)	458 (13)
60	0.065 (0.0095)	75 (14)	0.101 (0.013)	393 (35)	0.197 (0.012)	823 (28)
120	0.118 (0.016)	135 (14)	0.189 (0.029)	544 (67)	0.342 (0.020)	1450 (12)

If the amount of dye released is to be used as a quantitative measure of the extent of hydrolysis, then the amount of dye released should be proportional to the glucose concentration and this relationship should be independent of the initial enzyme concentration. However, it can clearly be seen from Fig. 4 that this is not the case. Instead, it can be concluded that the correlation between the absorbance and glucose concentration is indeed a function of initial enzyme concentration. It can also be seen in Fig. 4 that at a low initial enzyme concentration (0.5 units/ml), there exits a linear relationship between the absorbance and glucose formation. By proposing a linear model on the data set of 0.5 enzyme units/ml using the Trendline function of Excel, a linear relationship between absorbance (X) and glucose (Y) was predicted with R^2 (coefficient of determination) equals to 0.9992 and the predicted function is $Y = 1140X$. However, when considering the other two enzyme concentrations (1.5 and 4.0 units/ml), this simple linear relationship between the release of dye (absorbance) and the formation of glucose does not exist. In those cases, the release of dye could not be used as an indicator in predicting the production. This lack of correlation may be due to the following reasons:

1. Not all cellulose in the cellulose-azure had the dye attached. At the larger enzyme concentrations, the amount of dye released per unit mass of solubilised cellulose increased with time. This suggests more readily accessible cellulose chains, possibly being shorter chain polymers, had lower densities of azure attachment. As the reaction proceeded, more azure-dye was released as the cellulosic-substrate chains with higher densities of dye attachment (possibly the longer cellulose chains) were hydrolysed.
2. The commercial enzyme used in this research study may contain substrates, which were hydrolysed during the experiment.
3. The azure dye may repress the hydrolysis.
4. The structural characteristics of the substrate used in the assay may affect hydrolysis.

The extent to which factors 2, 3 and 4 affect hydrolysis were further investigated. When considering the experiments using an assay with enzyme concentrations of 0.5, 1.5 and 4.0 units/ml, the glucose production closely matches the specified activity (in terms of mg glucose/unit enzyme) of the cellulase used. At the end of the analysis run, there were 135, 544 and 1450 mg/L of glucose produced in the 0.5, 1.5 and 4.0 units/ml experiment, respectively. Based on the specification of the cellulase used, the amount of glucose produced after 2 h of incubation should have been 180, 540 and 1440 mg/L for the 0.5, 1.5 and 4.0 units/ml experiment, respectively. However, it should be noted that the glucose concentration of 1450 mg/L for the 4.0 units/ml experiment exceeded the theoretical glucose production based on the fact that there was only 1.0 g/L of cellulose-azure in the assay. By applying the stoichiometry of the hydrolysis reaction of cellulose, the maximum amount of glucose produced should not have been more than 1110 mg/L if all the cellulose was converted. This observation may be explained by the fact that other substrates were present in the assay initially. Based on personal communications with Sigma (Australia), it was found out that the commercial enzyme (EC 3.2.1.4 Cellulase, Sigma, product number: C-1184) used in this research study did contain substrates such as maltodextrin (ranging from 2 glucose units up to 12 glucose units) and other simple sugars such as glucose. Excess glucose could also have been produced at the other enzyme concentrations (0.5 and 1.5 units/ml), which ultimately resulted in an over-estimation of glucose yield. However, the extent of these over estimations could not be quantified based on the existing information.

It was shown experimentally that the accumulation of azure-dye did not affect the production of glucose from cellulose-azure, but decreased the release of the azure-dye. Table 2 shows the amount of glucose formed at different initial azure-dye concentrations. All the assays contained 1.0 g/L of cellulose-azure and 1.5 enzyme units/ml in 250 ml acetate buffer at pH 5.0. Despite the presence of excess azure-dye, the amount of glucose produced was similar. However, the amount of azure-dye released after 2 h of incubation was reduced when the initial azure-dye concentration was increased. Since the range of absorbance readings listed in Table 2 are well within the linear range of Fig. 1, the amount of azure-dye measured after 2 h of incubation should be the sum of extra azure-dye added initially and the azure-dye released as a result of hydrolysis. However, the dye concentration measured was lower than the expected value.

The structural characteristics of the substrate did not significantly affect the specific activities. When the concentration of enzyme was set at 1.5 units/ml and 1.0 g/L of Avicel was used instead of cellulose-azure as the substrate, the amount of glucose produced in 2 h was 416 and 464 mg glucose/L in repeat assays (or 0.27 and 0.31 mg glucose/unit enzyme). These specific activities are slightly less than that of cellulose-azure, which was 544 ± 67 mg glucose/L (or 0.36 ± 0.044 mg glucose/unit enzyme).

Table 2
Experimental results of azure-dye inhibition experiment—glucose formation and dye released after 2 h of incubation

Description	Azure-dye added initially (mg)	Absorbance expected from extra dye (A)	Absorbance after 2 h of incubation (A)	Net Absorbance (from dye released) (A)	Glucose concentration after 2 h of incubation (mg/L)
1a	22.1	0.167	0.362	0.195	400
1b	22.1	0.167	0.362	0.195	445
2a	44.2	0.334	0.470	0.136	445
2b	44.2	0.334	0.468	0.134	400
3a	88.4	0.668	0.759	0.091	461
3b	88.4	0.668	0.782	0.114	501

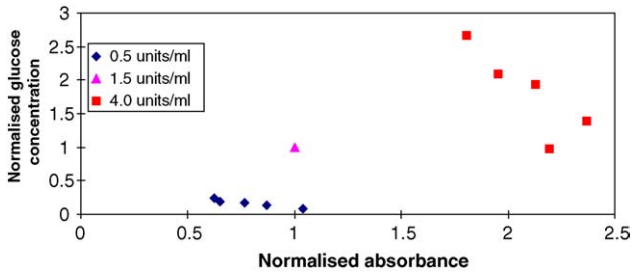

Fig. 5. Relationship between normalised glucose concentration and normalised absorbance data.

The hydrolytic activity of a given inoculum (e.g. activated sludge or leachate samples of landfills) can be estimated by using cellulose-azure assay once the standard curves of the cellulose-azure substrate are formulated. However, the initial enzyme concentration affects the amount of dye released by hydrolysis and therefore the relationship between dye release and glucose production. This dependence is complex. If it were only a matter of the additional substrates in the cellulase, then the absorbance versus glucose concentration plots in Fig. 4 should have the same slope, but be offset by a x-intercept equal to the solubilised carbohydrate in the cellulase. Fig. 4 suggests that the absorbance versus glucose concentration plot will be close to linear for both the 1.5 and 4 units/ml case if the plots are not constrained to go through zero. Regardless of these considerations, the slope of the 4 units/ml plot is clearly different to that of the 0.5 and 1.5 units/ml. Maximum slope of 4 units/ml plot is 5.17×10^{-4} and that of 0.5 and 1.5 units/ml are 1×10^{-3} and 6.32×10^{-4}, respectively. There is no clear theoretical basis why the amount of dye released per unit mass of hydrolysed cellulose should be less in the 4 unit/ml case.

Further both the absorbance and glucose concentration data sets of the 0.5 and 4.0 units/ml were normalised against their respective value at an enzyme concentration of 1.5 units/ml. The normalised absorbance data was plotted against the normalised glucose data as shown in Fig. 5. Despite the attempt to normalise the data set, it is obvious that the correlation between the normalised absorbance and normalised glucose is again a function of initial enzyme concentration. This is because no simple functions can be derived from the complete set of normalised data but instead, there exists two different linear trends for the data set of the low (0.5 units/ml) and high (4.0 units/ml) enzyme concentrations.

4. Conclusions

This study examined the use of cellulose-azure as a method of quantifying cellulolytic activity in a sample. The correlation between the amount of dye released from cellulose-azure and glucose formation was shown to be dependent on the concentration of the enzyme. Additionally, the measured glucose production at all three enzyme concentrations was overestimated by the presence of carbohydrates in the commercial cellulase used in this study. These extra substrates introduce an offset in the correlation between the extent of hydrolysis (product formation) and absorbance.

It was shown that the production of glucose from cellulose-azure was not inhibited by the accumulation of azure-dye. However, the release of azure-dye during the hydrolysis process was repressed by the accumulation of azure-dye. It was also shown that structural characteristics of the substrate used in the assay did not affect hydrolysis. Based on the above findings, it is therefore concluded that cellulose-azure should only be applied in assays when relative comparisons among different sources of enzymes are the major focus and may not be suitable for quantifying the hydrolysis rate.

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