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METHODS AND PROTOCOLS

Quantitative colorimetric measurement of cellulose degradation under microbial culture conditions

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Abstract We have developed a simple, rapid, quantitative colorimetric assay to measure cellulose degradation based on the absorbance shift of Congo red dye bound to soluble cellulose. We term this assay "Congo Red Analysis of Cellulose Concentration," or "CRACC." CRACC can be performed directly in culture media, including rich and defined media containing monosaccharides or disaccharides (such as glucose and cellobiose). We show example experiments from our laboratory that demonstrate the utility of CRACC in probing enzyme kinetics, quantifying cellulase secretion, and assessing the physiology of cellulolytic organisms. CRACC complements existing methods to assay cellulose degradation, and we discuss its utility for a variety of applications.

Keywords Cellulase · Cellulose degradation · Enzyme secretion · Bioprocessing · *Cellvibrio japonicus*

Introduction

Cellulose is the most abundant organic carbon source on earth (Leschine 1995). Many bacteria and fungi rely on cellulose for nutritive purposes; the cellulose-degrading enzymes (cellulases) encoded by these organisms are of great ecological interest as they play key roles in global carbon cycling (Heimann and Reichstein 2008; Wilson 2011). Cellulases also play a prominent role in bioenergy efforts, providing important tools for the conversion of lignocellulosic biomass to fermentable sugars (Carere et al. 2008; Lynd et al. 2002; Carroll and Somerville 2009).

Cellulose is a repeating polymer of glucose subunits connected by β-1,4 glycosidic linkages. Most enzymes that cleave long-chain cellulose fibrils are characterized as either exoglucanases (also called cellobiohydrolases), which cleave the disaccharide cellobiose from either the reducing or non-reducing ends of the polymer, or endoglucanases, which cleave within the polysaccharide chain (Béguin and Aubert 1994; Wilson 2008). A third class of enzyme, β-glucosidase, hydrolyzes the small products of endoglucanase or exoglucanase activity to glucose. Measuring the activity of these various enzymes is a central tool for basic research and bioengineering.

Diverse methods exist to measure cellulase activity, each with strengths and weaknesses that suit it to particular applications. Viscometry provides sensitive measurement of soluble cellulose digestion, while chromatography and quartz crystal microbalance methods allow greater flexibility in substrate selection (Hu et al. 2009; Vlasenko et al. 1998). These methods are slow and not easily scalable, however. Fluorescent methods are more convenient but require modified substrates and conditions that minimize background fluorescence (Helbert et al. 2003; Du et al. 2010). The most popular methods measure the release of reducing sugars from polymerized cellulose (Marais et al. 1966; Anthon and Barrett 2002; Doner and Irwin 1992; Lever 1972). Reducing sugar assays such as the popular dinitrosalicylic acid (DNS) assay are versatile in that they can be used to measure multiple classes of enzymatic activities on natural, pretreated, or solubilized forms of cellulose. The DNS assay has poor sensitivity, however, and is most

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useful when used to assay purified and highly active enzymes. In addition, this assay can display significantly nonlinear behavior depending on the enzymatic breakdown products (Lindner et al. 1983; Ghose 1987). Other reagents such as bicinchoninic acid (BCA) provide lower limits of detection for enzymatic activity but are sensitive to the presence of proteins in the reactions and often require purification of the enzyme prior to measurement (Hu et al. 2009; Kongruang et al. 2004). The use of any reducing sugar assay may lead to intolerably high background in cases where reducing sugars are already present in a sample (i.e., in cell culture media).

For our own studies on the physiology of cellulase expression and secretion in bacteria, we needed a rapid and highly sensitive cellulase assay that was compatible with multiple classes of culture media and did not require purification of enzymes from culture supernatants or cell lysates. We therefore developed a simple, quantitative colorimetric assay for cellulase activity, which we term "Congo Red Analysis of Cellulose Concentration," or "CRACC." CRACC is adapted from the popular plate-based non-quantitative screening technique introduced by Teather and Wood (1982). In the Teather and Wood technique, degradation of carboxymethyl cellulose (CMC) in an agar plate is visualized by staining with the dye Congo red in the presence of NaCl. Congo red preferentially binds to long-chain biopolymers, and when bound, increases its absorbance of green light (Wood 1980), allowing the visual differentiation of bound and unbound dye. In CRACC, the absorbance shift following the binding of Congo red is used to quantify the amount of CMC in a solution.

Here, we show validation of CRACC using a commercial cellulase solution, as well as extracts of cellulolytic bacteria. In doing so, we demonstrate three important applications of CRACC: (1) following time-dependent CMC degradation by a cellulolytic microbe, (2) quantifying the secreted fraction of a model endoglucanase, and (3) analyzing the effects of mutations and growth conditions on the production of cellulases by a cellulolytic bacterium.

Materials and methods

Overview of the CRACC assay

CRACC determines the amount of soluble cellulose that is present in a solution via its binding to Congo red and the resultant increase in Congo red absorbance at 530 nm. Enzymatic digestion of cellulose is quantified by a four-step procedure. First, the enzyme of interest is incubated with CMC. Second, enzymatic digestion is halted with NaOH at one or more time points. Third, samples are incubated with Congo red and NaCl to allow color development. Finally, the A_{530} of the solution is determined with a spectrophotometer; a decrease in A_{530} is used to quantify CMC degradation. As will be discussed below, the assay can measure the endoglucanase activity of purified enzymes, culture supernatants, and cellular extracts. However, the assay is not suitable for the assay of cellobiohydrolases (exoglucanases).

Details of the CRACC assay and important considerations

Sample preparation and CRACC procedure

Digestion reactions were performed by incubating enzyme-containing samples with 0.2% CMC in 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–HCl (pH 7). At indicated time points, material was removed from reactions or cultures and 40 mM NaOH was added to terminate enzymatic activity. For short time course experiments (<60 min), NaOH-treated samples were generally stored at room temperature prior to Congo red staining. To stain, 0.25 mg/ml Congo red and 0.5 M NaCl were added, and samples were incubated at room temperature for 30 min. A_{530} values were measured in 96-well plates containing 200-µl sample aliquots using a Tecan M1000 plate reader (Durham, NC).

Activity measurements for Cellvibrio japonicus

Three independent cultures of wild-type Ueda107 and a *gspD* mutant of *C. japonicus* (Gardner and Keating 2010) were grown at 30 °C with aeration in 30-mm culture tubes for 12 h and the cells collected by centrifugation. The cell supernatants were filtered through 0.45-µm Spin-X columns (Corning, Lowell, MA). The filtered supernatants were then subjected to CRACC assay as described above, alongside a positive control (Accellerase 1500 from Genencor) and a no-enzyme control. CMC digestion reactions were performed without shaking at 30 °C for 15 h.

Important considerations for sample preparation

Enzyme assays can be performed at any pH or salt concentration necessary, without affecting the detection of CMC by Congo red. However, the pH of samples must be adjusted to 7 or above, and the sodium chloride concentration adjusted to 0.5 M, prior to Congo red addition. In our experience, pH values greater than 7 did not affect Congo red detection of CMC. Congo red has been shown to alter its spectral properties in the presence of amyloid protein fibers (Howie et al. 2007; Klunk et al. 1989), so these should be absent from assay mixtures. In addition, pre-staining the CMC with Congo red prior to the addition of enzyme dramatically reduced the sensitivity of the assay. Finally, it is

important not to mix the Congo red and NaCl solutions prior to use as the dye gradually precipitates from the mixed solution. Extended staining of samples with Congo red and NaCl (greater than 30 min) also results in precipitation of the dye and should be avoided.

Materials and reagents

Carboxymethyl cellulose was medium viscosity sodium salt material prepared by MP Biomedicals (Solon, OH). Congo red was purchased as a 1-mg/ml aqueous solution manufactured by Ricca Chemical Company (Arlington, TX). The control enzyme cocktail Accellerase 1500 was obtained from Genencor (Beloit, WI). *Escherichia coli* cell lysates were generated via treatment of cells with the PopCulture reagent from Novagen (Madison, WI).

Reducing sugar assays

BCA and 3-methyl-2-benzothiazolinone hydrozone (MBTH) assays were performed as previously described (Anthon and Barrett 2002; Vlasenko et al. 1998). Briefly, reaction material was added to 1/4 volume of 0.7 M sodium carbonate buffer (pH 10) for BCA analysis or to 1 volume of 0.5 M NaOH for MBTH analysis. Samples were heated in BCA assay mix (2 mM BCA, 2 mM $CuSO_4$, and 5 mM L-serine) or MBTH assay mix (1 mg/ml MBTH and 0.33 mg/ml DTT); samples treated with MBTH were then supplemented with MBTH development mix (0.2% $FeNH_4(SO_4)_2$, 0.2% sulfamic acid, 0.1 M HCl) to promote color development. A Tecan M1000 plate reader was used to measure A_{620} for MBTH-treated samples or A_{560} for BCA-treated samples.

Growth media, strains, and plasmids

Wild-type *C. japonicus* Ueda107 and the *C. japonicus gspD*::pJGG1 mutant were cultured as described previously (Gardner and Keating 2010). *E. coli* strains were grown in lysogeny broth (LB; Bertani 1951, 2004) supplemented with 50 μg/ml spectinomycin and 100 μg/ml ampicillin at 37 °C. *E. coli* strains secreting *Dickeya dadantii* enzymes Cel5Z or PelB were constructed by electroporation of plasmid pRH104 encoding Cel5Z or pRH100 encoding PelB into DH5α (K12 λ^- F^- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1*) carrying the pCPP2006 plasmid encoding the *D. dadantii* type II secretion system (He et al. 1991). Plasmid pRH100 was constructed by amplifying the *pelB* gene from pPL3 (Keen et al. 1984) using the following primers: upstream, 5′-AAACAGGTACCTGACTCATGAAATCACTC-3′; downstream, 5′-GACGGGATCCGGATTATTTACAGGCTGAG-3′. Plasmid pRH104 was constructed by amplifying the *cel5Z* gene from *D. dadantii* 3937 genomic DNA using the following primers: upstream, 5′-AATAGGGTACCTAATGCCGCTCTCTTATTTGGA-3′; downstream, 5′-AATAGGATCCTTAGTTACAGCTACCAACCTGC-3′. For both pRH100 and pRH104 construction, PCR products were digested with *Acc*65I and *Bam*HI, and ligated into the large *Acc*65I/*Bam*HI pTrc99A (Amann et al. 1988) fragment.

Results

Rationale for assay

We developed CRACC because we needed a simple, rapid, and highly sensitive cellulase assay that did not require enzyme purification and would function in the presence of diverse growth media. The dye Congo red is widely used as a qualitative tool for assessing cellulolytic activity of microbes grown on agar plates. We reasoned that the blue shift in absorbance of Congo red when bound to CMC in solution could be used to quantify CMC degradation in a simple spectrophotometric assay.

Assay development

Spectral properties of Congo red binding in the presence of soluble cellulose

The optimal wavelength for monitoring CMC degradation was determined by measuring absorbance spectra of a constant amount of Congo red incubated with a dilution series of CMC in the presence of 0.5 M NaCl (Fig. 1a). As reported previously (Wood 1980), the binding of Congo red to CMC results in an increase and blue shift in absorbance, the effect of which correlated positively with CMC concentration. Similar spectra were obtained using CMC dissolved in the rich bacterial medium LB (which has a propensity to interfere with cellulase assays due to its yellow color, fluorescent properties, and chemical heterogeneity; data not shown). The optimal wavelength for CRACC was determined to be 530 nm because it represented maximal absorbance for ≥0.25% CMC and allowed discrimination between CMC concentrations. Examination of the correlation between A_{530} and CMC concentration (Fig. 1b) showed that absorbance increased linearly with CMC concentration between 0% and 0.15% CMC (lines fit to these data had R^2 values of 0.97 in LB and 0.93 in MOPS), plateauing thereafter and showing no further increase beyond 0.4% CMC. Extinction coefficients for Congo red ranged from 8,200 (no CMC) to 23,500 M^{-1} cm^{-1} (0.5% CMC) in MOPS buffer and from 7,800 (no CMC) to 20,800 M^{-1} cm^{-1} (0.5% CMC) in LB. To ensure that absorbances fell within the linear range, all kinetic experiments

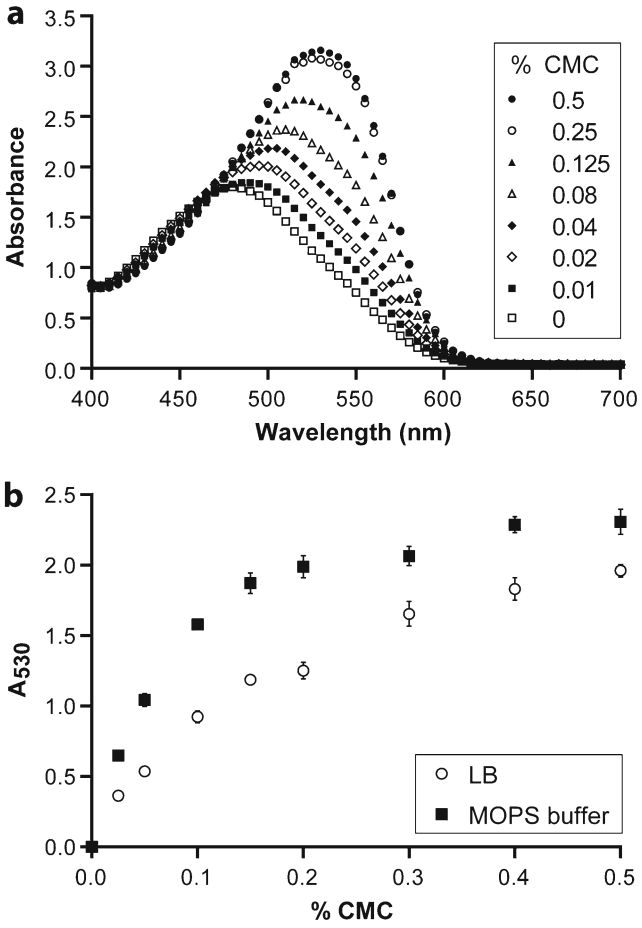

Fig. 1 Spectrophotometric evaluation of Congo red binding to CMC. **a** Absorbance spectra of Congo red dye in the presence of varying concentrations of CMC. Congo red (0.25 mg/ml) was added to a concentration series of CMC containing 50 mM MOPS pH 7 and 0.5 M NaCl as described in "Materials and methods" section. The absorbance spectra between 400 and 700 nm was then determined for each sample. **b** A_{530} measurements for Congo red dye in the presence of varying concentrations of CMC in 50 mM MOPS pH 7 and LB. A concentration series of CMC solutions was prepared in 50 mM MOPS pH 7 and LB, as well as blanks that lacked CMC. Congo red (0.25 mg/ml) and 0.5 M NaCl were then added and the absorbance determined at 530 nm. The values reported represent the absorbance values after subtraction of the absorbances for the corresponding blank (no CMC) controls. Mean values are shown ±SEM

were performed at ≤0.2% CMC to ensure that absorbance signals were linearly correlated to CMC concentration.

The optimal NaCl concentration to distinguish between CMC-containing and CMC-free solutions was found to be 0.5 M in a panel of NaCl concentrations (data not shown). CRACC was performed at pH 7 or higher because under acidic conditions, absorbance of Congo red did not scale linearly with CMC concentration (data not shown). CRACC activities were equivalent at pH values greater than 7. In our experience, enzymatic assays can be performed at any salt concentration or pH prior to CRACC (see "Materials and methods" section).

Colorimetric measurement of Congo red binding during degradation of soluble cellulose

To assess the utility of Congo red spectral changes in quantifying cleavage of cellulose, changes in absorbance at 530 nm were measured at various time points during enzymatic digestion of CMC. The commercial cellulase cocktail Accellerase 1500 was employed due to its high activity and common usage by researchers in the field. Varying dilutions of Accellerase 1500 were therefore incubated with CMC prior to addition of Congo red and NaCl and A_{530} measurement (Fig. 2, open circles). The relationship between absorbance change and enzyme concentration was initially linear, saturating at higher concentrations of Accellerase 1500. For the two highest enzyme concentrations tested, A_{530} decreased to near-blank levels, indicating robust digestion of long-chain CMC. Thus, CRACC could be used to quantitatively monitor CMC breakdown and to distinguish between different concentrations of cellulases.

Comparison of CRACC to reducing sugar methods using purified cellulases

Sensitivity and dynamic range are key determinants of the applicability of any cellulase assay. These characteristics were evaluated for CRACC by comparison to a conventional reducing sugar assay using the BCA reagent, which has a good linear range and has been reported to be the most sensitive of the reducing sugar assays (Doner and Irwin 1992; Vlasenko et al. 1998; Kongruang et al. 2004). As an initial basis for comparison, CMC was incubated with Accellerase 1500 and activity determined by both techniques

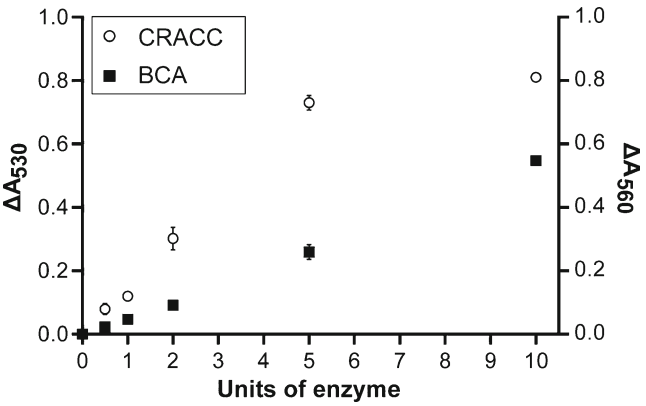

Fig. 2 Comparison of BCA and CRACC assays. Aqueous dilutions of Accellerase 1500 were incubated with 0.2% CMC for 10 min at 22 °C. The amount of CMC degradation was measured by appearance of reducing sugar via the BCA assay or by determining the decrease in CMC via the CRACC assay, as described in "Materials and methods" section. ΔA_{530} represents the change in absorbance at 530 nm after addition of enzyme and detection by Congo red staining. ΔA_{560} represents the increase in absorbance in the BCA assay, associated with the appearance of reducing sugar. Mean values are shown ±SEM

(Fig. 2). Both CRACC and the BCA assays detected degradation of CMC, which increased as the amount of enzyme increased.

The BCA assay signal correlated linearly with the amount of enzyme present over the 20-fold dilution series shown, indicating a reducing-end production of 0.2 μM unit enzyme^{-1} min^{-1}. The relationship between reaction rate and enzyme concentration became nonlinear when reactions produced >25 μM reducing ends (data not shown), which is consistent with previously reported data (Waffenschmidt and Jaenicke 1987). The dynamic range of CRACC was smaller than that of the BCA assay. CRACC signal was linear over a tenfold range of enzyme concentration (Fig. 2), but signal levels reached a plateau at higher concentrations of enzyme. The extended linear range of the BCA assay at higher concentrations likely reflects the ability of the reducing sugar assay to detect endoglucanase cleavage of oligosaccharides undetectable by CRACC. Characterization of Congo red interaction with CMC has shown that oligosaccharides with a degree of polymerization less than 5 undergo a reduced degree of binding with Congo red (Wood 1980). Because CRACC signal depends on polymer chain length while BCA signal does not, and because it is impossible to determine the molarity of the complex mixture of polymers in CMC, enzymatic rates calculated by the CRACC and BCA methods are non-commensurable. Though specific activities could not be compared, the data did indicate that both assays were equally successful at determining relative quantities of enzyme over their respective linear ranges, with CRACC giving greater colorimetric signal at low enzyme concentrations.

Application of CRACC to *E. coli* expressing a model cellulase

The results described above demonstrated the utility of CRACC in measuring cellulase activity of purified enzymes. To determine if the assay could measure cellulase activity in the presence of enzymes in complex solutions containing contaminating medium components, proteins, and metabolites, CRACC was used to quantify CMC breakdown by cell extracts and cell supernatants derived from cellulose-degrading microbes. A simple system employing a bacterium expressing a single cellulolytic enzyme was characterized as an initial test. Though not naturally cellulolytic, *E. coli* K-12 strains carrying the *D. dadantii* type II secretion system can express and secrete the model *D. dadantii* endoglucanase Cel5Z (He et al. 1991; Chapon et al. 2000). This model system was used to investigate the utility of CRACC in three microbiological applications: (1) measuring cellulase activity in culture media, (2) quantifying cellulase secretion, and (3) following degradation of cellulose by a bacterial culture.

Determination of relative Cel5Z concentration and kinetics of enzyme activity in LB

In preparation to perform CRACC under culture conditions, it was first necessary to demonstrate that the assay would function in culture medium. Cultures of Cel5Z-secreting *E. coli* were grown in the rich medium LB, and cell-free supernatants were used as a source of enzyme in CMC degradation assays. As predicted from spectral analysis, CRACC signal was robust in LB. Undiluted supernatant material digested 60% of long-chain CMC in 10 min, consistent with the high CMCase activity reported for Cel5Z (Park et al. 2002; Py et al. 1991). As with Accellerase 1500, enzyme concentration and CRACC signal displayed a linear relationship across a 16-fold dilution series of supernatant material. CRACC was also able to deliver kinetic information by measuring rates of CMC degradation by culture supernatant material harboring differing amounts of Cel5Z [Fig. 1 in Electronic supplementary material (ESM)]. In contrast, the BCA assay resulted in high background and prevented accurate determination of Cel5Z activity when applied to culture supernatants derived from cells grown in LB (data not shown). Although the MBTH-based reducing sugar assay has been reported to be more resistant to the presence of protein than the BCA assay (Anthon and Barrett 2002), MBTH detection of LB-containing samples resulted in high background similar to BCA (data not shown).

Quantifying the extent of cellulase secretion

Because CRACC can quantify Cel5Z activity in culture supernatants, this method provides a rapid, sensitive, and quantitative means to measure subcellular localization of a cellulase. A culture of *E. coli* cells secreting Cel5Z was harvested by centrifugation, the culture supernatant was removed, and the cells were lysed in fresh broth using the PopCulture reagent (see "Materials and methods" section). Cell lysates and cell-free supernatants were then added, without purification, to solutions of CMC, and CMC concentration measured by CRACC at varying time points thereafter. The mean initial rates of A_{530} decrease were 0.003 min^{-1} for cell lysates and 0.049 min^{-1} for cell-free supernatants (thus, the total rate for the culture was 0.052 min^{-1}). These data demonstrate that 94% of the Cel5Z-dependent activity was located extracellularly, in accordance with previously published data showing robust Cel5Z secretion by this strain (Chapon et al. 2000).

Measurement of cellulose degradation during growth of a bacterial culture

Because CRACC can be performed directly in culture media, it can be useful in assessing soluble cellulose

degradation by growing microorganisms. As a proof-of-principle experiment, CRACC was used to follow time-dependent cellulose degradation by Cel5Z-secreting *E. coli* grown in CMC-containing broth. The assay was successful in measuring CMC degradation in the culture. Furthermore, the assay appeared specific for cellulase activity in that cultures secreting the pectin-degrading *D. dadantii* enzyme PelB (which has not been reported to degrade cellulose) did not show CMC degradation by CRACC (Fig. 2 in ESM).

Analysis of the effects of mutations and growth conditions on a cellulolytic organism

To investigate the breadth of CRACC utility, we used it to characterize endoglucanase activity in the well-studied cellulolytic bacterium *C. japonicus*, which encodes 154 enzymes predicted to function in the degradation of polysaccharides, including 15 predicted cellulases (DeBoy et al. 2008). Mutational analysis demonstrated that the majority of extracellular *C. japonicus* cellulase activity is secreted via a type II secretion system (T2SS) (Gardner and Keating 2010). Culture supernatants containing the complex cellulolytic secretome of *C. japonicus* represented an excellent test case for CRACC. Wild-type *C. japonicus* and a *gspD* mutant, which lack the functional T2SS, were cultured in minimal broth with glucose, and endpoint CRACC assays were performed on cell-free supernatants. A paired control reaction with concentrated Accellerase 1500 was used to define 100% CMC digestion under the conditions tested. In accordance with previous results, cell supernatants of wild-type *C. japonicus* cultures displayed robust endoglucanase activity, degrading a mean of 80% of the available CMC in three replicate experiments. In contrast, the *gspD* mutant, which has previously been reported to display reduced cellulase secretion (Gardner and Keating 2010), degraded less than 5% of the available CMC. Repeating this experiment with alternative carbon sources revealed that the CRACC assay was unaffected by the presence of xylose, xylan, or the disaccharide cellobiose in the growth media (data not shown), consistent with previous data on the spectral properties of Congo red (Wood 1980). Thus, CRACC can be applied to various bacteria to screen for mutations or growth conditions that affect cellulase expression or secretion, even under conditions where reducing sugar assays would not be possible (e.g., when glucose is present).

Discussion

We present here CRACC, a simple, rapid, high-throughput capable, quantitative assay for the breakdown of soluble cellulose in aqueous solution based on the qualitative Congo red-based assay of Teather and Wood (1982). The assay has two major strengths. First, it is sensitive, showing greater colorimetric signal per unit enzyme than the most sensitive reducing sugar assay. Second, the assay is unaffected by reducing mono- and disaccharides, and is less sensitive than reducing sugar assays to background issues in complex media. The latter properties allow CRACC to be performed directly in culture media without purification of enzymes. We demonstrate the utility of CRACC in several important applications: enzyme quantification and kinetics, following enzymatic activity in cultures, and assessing mutations that affect cellulolytic activity.

As with all assays for enzymatic activity, CRACC has several limitations. Its dynamic range is relatively small, so proper enzyme dilutions and incubation times must be empirically determined if rigorous quantification is desired. Like reducing sugar assays and viscometric measurements, CRACC cannot be used as a continuous assay because Congo red inhibits enzymatic degradation of bound cellulose (Maglione et al. 1997). Because Congo red will not bind to microcrystalline cellulose, CRACC cannot be used to measure breakdown of insoluble substrates. CRACC is also of limited utility in quantifying cellobiohydrolases (exoglucanases) that display poor activity on CMC. For measuring cellobiohydrolase activity and the breakdown of microcrystalline forms of cellulose, reducing sugar assays remain critical tools.

Though not optimal for every application, CRACC has significant advantages that make it preferable for certain lines of experimentation. We commonly use CRACC to compare intracellular and extracellular levels of endoglucanases in bacterial cultures. The ease of performing the assay, combined with its functionality in cell lysate material, makes it extremely useful for this purpose. CRACC also works well in microtiter plates, and we expect CRACC can easily be adapted for use as a high-throughput assay. As shown by our data, CRACC can be used under conditions where reducing mono- and disaccharides (glucose, cellobiose, and xylose) are present. Removing the necessity for protein purification prior to activity measurements, often required for standard reducing sugar assays, increases the speed and accuracy of activity determinations. Eliminating the need for purification and heat treatment of samples facilitates high-throughput applications. Furthermore, the high sensitivity of CRACC allows detection of dilute or low-activity enzymes.

In short, CRACC is a versatile tool that will complement previously described cellulase assays. In reporting our CRACC method, we hope to broaden the toolkit available to scientists studying cellulose degradation.

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Conflict of interest The authors declare that they have no conflict of interest.

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