Simple Dye Release Assay for Determining Cellulolytic Activity of Fungi

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A colorimetric assay, based on the release of dye from a paper substrate, is described as a simple and rapid means of detecting cellulolytic activity. It is readily adaptable to rapidly screening in situ large numbers of fungal liquid cultures. Since it is a nondestructive assay, samples can be recovered for later use.

An important aspect of the general problem of solid waste disposal is the disposal of paper and paper products. These products make up about 50% of our refuse, and only 25% of this is being recycled (1, 7). No doubt the percentage entering the recycling channel will increase as pressure from ecologically oriented consumers makes it necessary. However, some paper will probably never be reclaimed, such as low-quality paper or paper found in garbage. One approach to removing this unwanted paper is through composting, which can convert objectionable refuse into a desirable end product. Composting presently has the drawback of being slow. This slowness results in large part because paper and its products are highly resistant to cellulose breakdown even in the presence of cellulolytic microorganisms (11).

Many investigators have examined the role of cellulolytic fungi in destroying articles made of cellulose or as plant pathogens, but their object was to develop control methods. Few attempts have been made to increase cellulase production or activity to obtain more rapid rates of breakdown. Mutant strains of cellulolytic fungi which produce highly active cellulases could be useful either for enzyme production to be used for processing compost or as inoculum in processes like those already in use which require the presence of cellulolytic organisms.

Cellulase is not an inducible enzyme. As fungi degrade cellulose, the end product, glucose, acts as a catabolite repressor reducing further enzyme synthesis (3). Mutants that are not subject to catabolite repression could thus be very useful. Whether they could survive competition with other microbes if used as an inoculum is not known.

\textit{Trichoderma viride} is a common saprophyte which produces a stable cellulase complex. A mutant of \textit{T. viride} which produces twice as much cellulase as its wild-type parent strain was recently recovered (6). Our own attempts to find other mutants revealed the scarcity of cellulase assay methods that were convenient and suitable for screening thousands of samples. This paper describes such an assay based upon the rate of dye release from uniformly dyed paper prepared under standard conditions.

**MATERIALS AND METHODS**

Preparation of the dyed paper was adapted from a procedure for dying starch (12) or cellulose powder (2) with Remazol Brilliant Blue R. Two to four white enamel photographic trays (64 by 48 by 8 cm) containing one sheet each of Whatman no. 1 chromatographic paper (38 by 45 cm) were preheated on a steam table. Distilled water (250 ml, 100 C) was poured over each sheet, and a print roller was used to smooth out air bubbles. Remazol Brilliant Blue R (Calbiochem), 1.5 g in 250 ml of distilled water (100 C), was poured evenly over each sheet. The dye was distributed by tipping the tray vigorously, causing the dye to swirl over the paper. At 2-min intervals for 10 min, 30.0 g of sodium sulfate in 100.0 ml of distilled water (100 C) was added in 20.0-ml portions per sheet. During this time, the dye was continuously agitated. Then 1.5 g of trisodium phosphate in 15.0 ml of water (100 C) was evenly dropped into the solution, mixed well, and allowed to remain for 10 min. The fluid was poured off, and excess dye was washed off with distilled water (paper in tray during washing). When the rinse water was clear, the paper was rinsed twice with methanol and air dried. The dry paper was then cut into 1.0- by 6.0-cm strips (50 mg).

During the washing, the paper appeared to be heavily streaked. However, the appearance became
uniformly blue as distilled water was hosed over it. The paper was not removed from the tray until after the methanol wash because of its very low wet strength. A uniform blue color on both sides was produced, but occasionally darker areas occurred, and these were not used. Discoloration is produced by uneven distribution and mixing during the dyeing process.

Quantitative assay for cellulase activity. Two milliliters of 0.05 M sodium citrate buffer (pH 4.8) and 1.0 ml of cellulase in distilled water at appropriate concentrations (0.1 to 1.0% w/v) were added to 50.0 mg of dyed paper (1.0- by 6.0-cm strip) in individual tubes (18 by 150 mm). Commercial cellulase from *T. viride* (Onozuka, Japan Biochemical Co.) was used as a standard. The paper strips were coiled by using a vibratory mixer or were dropped in as a handrolled coil at the start. The tubes were incubated at 50 C for 6 hr, and the reaction was terminated with 0.2 ml of 2 N HCl if they were to be determined the next day. Acidified samples could be stored up to 3 days at 4 C without any deterioration. The solution was decanted into Coleman spectrophotometric tubes (10 by 76 mm), the paper fiber was allowed to settle for 5 min, and the absorbance was determined at 595 nm. The blank was prepared with distilled water in place of the enzyme. Enzyme solutions were kept free from contamination with 0.05% Merthiolate (Eli Lilly, Co.). The above procedure was partly adapted from a test for cellulase activity using filter paper (release of glucose) (5).

Glucose and protein determination. Glucose was released from undyed Whatman no. 1 strips (50.0 mg, 1.0 by 6.0 cm) with cellulase under conditions described by Mandels and Weber (5). The cellulase activity was terminated by boiling in this instance, as acid conditions were not favorable for the following assay. Glucose was determined colorimetrically (Kit 510 from Sigma Chemical Co.) after treatment with glucose oxidase, peroxidase, and o-dianisidine. This procedure was based on the method of Raabo and Terkildsen (10). Protein was determined by the Lowry procedure (4).

Culture filtrates. Two cultures of *T. viride* wildtype QM6a and a derived mutant with enhanced cellulase production (6) QM9123, were obtained from the U.S. Army Natick Laboratories, Natick, Mass. Two unidentified strains of *Trichoderma*, T1 and T6, were isolated from compost. The fungi were cultured on liquid media (25 ml) at 30 C in flasks which were shaken for 24 days. The culture media consisted of an inorganic salts solution and filter paper as the sole carbon source (9).

Rapid screening assay for cellulolytic fungi. Culture tubes (18 by 150 mm) containing one coiled blue paper strip were sterilized by autoclaving. An amount (3.0 ml) of sterile salts solution containing the inoculum was added, and, after incubation at 30 C, the tubes were vigorously diluted to 6.0 ml with distilled water at various time intervals. The absorbance was determined at 595 nm with a Coleman spectrophotometer within 5 min, the time taken for the paper fibers and mycelia to settle. The large cuvette holder was used, with the bottom half (16 mm) of its window length (32 mm) masked to eliminate the turbidity caused by settled paper fibers and mycelia in the tube bottom.

RESULTS

Figure 1 illustrates dye release obtained with the commercial Onozuka cellulase (*T. viride*) as a function of time and enzyme concentration. Concentrations indicated in this and other figures are those of the enzyme solution (1.0 ml) added prior to dilution with citrate.

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**Fig. 1.** Dye release from a paper substrate incubated at 50 C with Onozuka cellulase for selected times at various concentrations.
buffer. A Lowry protein determination (4) of the commercial Onozuka cellulase indicated a 25.0% protein composition. A nearly linear relationship (Fig. 1) holds for concentrations of Onozuka cellulase below 1.0% (w/v) or 2.50 mg of total protein (in final assay mixture) and the amount of dye released over intervals of one to 6 hr. No dye was released or present in solution at zero time for any enzyme concentration.

After 6 hr, only insignificant increases in absorbance occur. It may be that cellulase rapidly digests the more susceptible portion of the paper, and the residue quickly becomes increasingly resistant to further enzymatic action (Fig. 2).

Figure 3 shows that the near linearity of dye release is reproducible in three separate 6-hr incubations for concentrations below 0.6% cellulase. The assay is linear but does not quite obey Beer's Law, since the standard curve does not exactly pass through the origin. This does not affect the results, however, as the values are reproducible. This failure to pass through the origin arises perhaps because the T. viride cellulase is a complex of the C1 and C2 forms of cellulase (5). Once released, the dye was found to be very stable. Provided dye release in vitro was terminated by acidic denaturation of the cellulase (which is not necessary if the samples are to be read the same day), the samples could be stored at 4 C for up to 3 days before being read.

The correlation between dye release and the hydrolysis of glucose was followed in a further experiment, and a linear relationship, as expected, was found (Fig. 4). The relationship was linear up to 0.6% (w/v) Onozuka cellulase, after which both dye and glucose release leveled off, so as to yield a cluster of points at the end of the line. Glucose release was measured after 1 hr because the literature values are expressed on that basis.

Figure 5 shows the cellulolytic activities of the four different strains of Trichoderma determined by the screening technique. The activities determined by chemical analysis and dye release are compared in Table 1. Onozuka cellulase at 0.5% (w/v) was found to hydrolyze 1.49 mg of glucose from the paper substrate per ml (1.25 mg protein/ml). An amount (1 ml) of culture filtrate obtained from a 25-ml liquid culture of T. viride QM9123 released approximately 3.6 mg of glucose and contained 0.96 mg of protein. The screening assay employed inocula of two concentrations, $3 \times 10^3$ and $3 \times 10^4$ spores per ml. The end results were the
same, except the lower spore density prolonged the time required due to a slower initial growth rate.

The screening technique produced optimal results at 18 days. Color development was intense, and it increased with increasing cellulolytic activity. The relative agreement between the chemical and dye screening assays was good (Table 1). Color development, although increasing beyond 18 days, cannot be readily determined beyond that growth time with the screening assay because the tubes become extremely turbid with mycelia and paper fibers, necessitating centrifugation prior to absorbancy determinations.

The disposable tubes used in the screening assay were not a source of error, as the absorbancy at 595 nm differed among tubes by less than 0.01 absorbancy units. Leaving the paper in the tube and using the partially masked cuvette window gave the same absorbancy reading as samples having the paper removed and read in the unmasked cuvette holder.

### DISCUSSION

Cellulose degradation is a slow process. A contributing factor may be catabolite repression by glucose (3). During the past few years, it has become apparent that mutant strains of fungi could be useful in accelerating normal rates of cellulose breakdown (6, 11). *T. viride* is a likely candidate for mutation, both because of its widespread occurrence and its evident adaptation to a range of environments (5, 8). A mutant with increased cellulase production has already been produced (6).

Efficient searches for mutants with increased cellulolytic activity require a simple, rapid, and nondestructive assay which is readily adaptable to screening thousands of samples. Our assay meets these requirements and is also useful for generally measuring cellulase activity.

Since cellulase releases a glucosyl-dye derivative from dyed cellulose (2), we might ask...
whether this hydrolytic product has an adverse effect on the screening assay. Mandels and Weber found that the release of glucose by *T. viride* cellulase in vitro from various cellulose substrates leveled off with time (5). Mandels et al. (6) showed that *T. viride* QM9123 produced twice as much cellulase in liquid culture as *T. viride* QM6a and also gave a twofold greater release of glucose from filter paper (filter paper activity). Our studies showed a similar leveling off for dye release (Fig. 1 and 5) in vitro and in vivo and a linear relationship between glucose and dye release (Fig. 4). Our filter paper activity determinations (Table 1) verified those of Mandels et al. (6). The results of our screening assay were in agreement with those of the filter paper assay (Table 1), i.e., QM9123 was shown to be the superior cellulolytic fungus and to have double the activity (optical density) of QM6a. Results were also comparable for T₁ and T₂. This similarity in results of the two methods suggests the glucosyl-dye derivative has no adverse effect on cellulase activity.

Our assay was tested with organisms possessing C₁ and C₂ forms of cellulase. According to the data of Mandels and Weber (5), C₁ seems to be necessary for the hydrolysis of solid cellulose. Filter paper activity was still found by them even at C₂ levels as low as 1% of that found in *T. viride* QM6a, but the rate of breakdown was slower. Whether our assay would work without the C₁ form is doubtful in view of their data. Since most of the cellulose found in solid wastes or nature appears to require the C₁ form for degradation, this should not be a drawback.

When adapted as a screening assay, the dye release method was found to be especially useful for determination of increased cellulolytic activity among large numbers of samples. The paper substrate requires no weighing (due to the uniformity of Whatman no. 1 paper) and does not have to be removed for the absorbancy determinations. Since the color was developed in situ during growth in liquid culture, it was not necessary to isolate culture filtrates. In tests of the method, *T. viride* QM9123 (mutant) was easily distinguishable from three other forms (Fig. 5). The liquid cultures were read directly in the spectrophotometer, and many could be visualized rejected prior to absorbancy determinations. If a desirable mutant strain was found, it could easily be recultured as the screening assay was found to be nondestructive. Cellulase production by *T. viride* QM9123 levels off after 18 days. The in vivo assay need only be run that long to find more productive forms.

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