Semiquantitative Plate Assay for Determination of Cellulase Production by *Trichoderma viride*¹

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A plate clearing assay was devised to screen for high-producing cellulase mutants of *Trichoderma viride*. The method employs (i) the use of either rose bengal or oxgall to limit colony size and (ii) Phosfon D (tributyl-2,4-dichlorobenzylphosphonium chloride) to enhance cellulase detection, in combination with acid-swollen cellulose on agar plates. The method was used to isolate constitutive cellulase mutants of *T. viride* and should prove useful for isolating high-producing mutants from a range of organisms. This technique has been also used to determine the concentration at which glucose and glycerol inhibit cellulase synthesis by catabolite repression in the wild-type strains.

Current forecasts for world energy sources coupled with the rapid utilization of nonrenewable oil supplies, have led to the search for alternative energy sources. Since forest wood (cellulose) is a naturally abundant and replaceable resource, and is also paramount among waste products, it has been proposed to convert wood to glucose. From glucose, a wide range of useful products may be synthesized either by fermentative or chemical methods. Considerable attention has been focused recently on proposals for the conversion of cellulose to glucose, and these have been summarized in the proceedings of several symposia (1, 21, 23). The most attractive approach is through the enzymatic hydrolysis of cellulose. The source of cellulase currently favored is *Trichoderma viride*, as it was realized early that this fungus produced a stable extracellular cellulase complex capable of attacking crystalline cellulose. The latter action appears to be absent in many other microbial cellulases (11). Other microbial cellulases are under study (2, 4, 5, 19). Since a high proportion (60%) of the overall cost of the conversion of cellulose to glucose is the expense of the cellulase itself (24), it is desirable to develop methods of producing large amounts of cellulase inexpensively. Considerable detail is known regarding the optimal cultural conditions for the production of the maximal amounts of cellulase, and these methods have been reviewed (1, 23, 24). However, the yields obtained are low in comparison with microbial

enzymes such as amylase (6), β -galactosidase (7), invertase (12), and protease (13, 14), which have been obtained through selection of hyper-secreting mutants. Some of these high-producing mutants are catabolite repression-resistant strains (6, 7, 12). Since all known *T. viride* enzymes involved in the digestion of cellulose are catabolite repressed (10, 15), it would be extremely useful to obtain a series of cellulolytic *T. viride* mutants whose cellulase complex is no longer repressed by glucose or cellobiose. For many years, workers have been attempting to devise alternatives to mass screening for the selection of high-producing cellulase mutants, without success. This has been due to the fast-spreading growth form of *T. viride* and also to the recalcitrant nature of the cellulose. Methods reported to date for observing cellulolytic microbes are inappropriate for mass screening (18, 20). This report describes a simple plate assay that allows the simultaneous screening of large numbers of mutants. The technique also distinguishes between constitutive producers and normally repressed organisms.

MATERIALS AND METHODS

Organisms. The test organisms used in these experiments were *T. viride* QM6a and QM9414, obtained from Emory Simmons, Natick Laboratories Culture Collection of Fungi (QM), Department of Botany, University of Massachusetts, Amherst, Mass. These strains were selected because they are reported to be the best fungal cellulase producers known. Strain QM9414 is a mutant derived in a two-step mutation from QM6a and produces two to four times more enzyme than the wild type (8).

Method of culture. Stock cultures of *T. viride*

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QM6a and QM9414 were maintained on potato dextrose agar (Difco). Spore suspensions were obtained by adding sterile water and glass beads to a stock slant and shaking. *T. viride* is a fast-spreading soil fungus, which rapidly overgrows petri dishes in 36 to 48 h. To select single-colony isolates, it became necessary to restrict the colony size. To achieve this, rose bengal (50 $\mu\text{g}/\text{ml}$) (Allied Chemical) (16) or oxgall (1.5%) (Difco) (17) was added to the medium. Figure 1 shows a comparison of *T. viride* QM6a grown in the presence and absence of 1.5% oxgall. In the presence of oxgall, each individual colony remains distinct, and separation can be achieved for as long as 10 days after inoculation. In contrast, in the absence of oxgall, the plate is completely overgrown in 96 h. This method of restriction allows screening of 70 to 100 colonies simultaneously on a single plate, with the added advantage of being able to replicate the plates.

Plate-clearing assay. The assay plates contain Vogel salts (22) modified as follows. Vitamins added included (milligrams per liter) biotin, 0.005; inositol, 2.0; calcium pantothenate, 0.2; pyridoxine hydrochloride, 0.2; thiamine, 0.2. Trace minerals added were (milligrams per liter) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.4; ZnCl_2 , 1.7; CoCl_2 , 2.0. Either 50 μg of rose bengal per ml or 1.5% oxgall, 500 μg of Phosfon D (tributyl-2,4-dichlorobenzylphosphonium chloride) per ml (Virginia Carolina Chem. Corp.) 2% agar (Difco), and 0.5% phosphoric acid-swollen cellulose (Whatman, CC-41), prepared by the method of Tansey (20), were also added. Agar plates were seeded with spores and incubated at 27°C for 96 h. The cultures were then incubated further at 50°C for 16 h to accelerate the action of extracellular cellulases and thus rapidly develop clearing zones around the cellulase-producing colonies. Figure 2 shows

representative plate clearing with *T. viride* QM9414, using either rose bengal or oxgall as inhibitors. In both cases, distinct clearing zones are evident around the peripheral edges of the colonies where the extracellular cellulases have digested the cellulose in the medium. Figure 2 also illustrates the different effect of the two inhibitors. Oxgall, 1.5%, is superior to rose bengal in controlling hyphal spreading. However, the clearing zones are distinctly sharper on the rose bengal medium.

Conditions for selecting constitutive mutants. Spore suspensions are submitted to ultraviolet irradiation to give a 0.1% survival rate, and then plated as described above with the addition of 5% glycerol in the plate screening medium. This condition is sufficient to completely suppress enzyme formation in the wild-type *T. viride* QM6a and QM9414 in liquid culture when enzyme activity is measured by the filter paper assay of Mandels and co-workers (9). Figures 3A and B show strain QM9414 in the presence (A) and absence (B) of 5% glycerol with 1.5% oxgall as the inhibitor. Figures 3C and D represent the same conditions with rose bengal as the inhibitor. In both cases no clearing is visible in the presence of 5% glycerol, whereas distinct clearing zones are evident in the absence of a catabolite repressor. After mutation and screening on 5% glycerol plates in this manner, mutants that no longer exhibit catabolite repression can easily be distinguished from parent strains by their production of clearing zones. An example of this visualization of a catabolite-derepressed mutant is shown in Fig. 4. As many as 100 colonies per plate can be screened if oxgall is used as the inhibitor. However, if rose bengal is the inhibitor, the number of irradiated spores per plate must be reduced to 10 to 15 due to the differences in colony size. With efficiency in mind, we have se-

FIG. 1. Approximately 100 spores of *Trichoderma viride* QM6a were seeded on Vogel medium modified as described in Materials and Methods. (A) No oxgall; (B) 1.5% oxgall. Incubation is at 27°C for 96 h.

FIG. 2. *Trichoderma viride* QM9414 spores were plated on agar containing Vogel medium, modified as described, and 0.5% acid-swollen cellulose, plus 500 μ g of Phosfon D per ml, with 50 μ g of rose bengal (A) per ml or 1.5% oxgall (B). Incubation is at 27°C for 96 h followed by overnight at 50°C.

lected the oxgall-Phosfon combination as the method of choice since a greater number of potential mutants can be screened simultaneously.

In addition, a comparison of the two strains of *T. viride* QM6a and QM9414 on oxgall-Phosfon cellulose plates without glycerol shows a distinct difference in the size of the clearing zones around each colony. After 96 h of growth, the zones around QM9414 are two to three times larger than those around the parent QM6a. This correlates well with the reported differences in enzymatic activity of the two organisms (10) and suggests that this assay may be used as a semiquantitative measure of cellulase activity.

DISCUSSION

The development of this method is dependent on being able to restrict the growth of *T. viride* and to provide conditions that will allow rapid digestion of the cellulosic substrate.

Many compounds have been recommended for use in fungal plate counting procedures, for their ability to restrict the size of fungal colonies and to prevent rapid overgrowth of the plates. Among several of these compounds, we found rose bengal and oxgall best for our use. Phosfon D has also been recommended as an inhibitor of colony size (3) but we found it to have little effect on *T. viride* in this respect.

However, during the trials, we noticed that it enhanced the clearing in the cellulase plate clearing assay, when used in combination with rose bengal or oxgall.

The biochemical effect of the Phosfon D on the production of cellulase by *T. viride* has not yet been evaluated. If either QM6a or QM9414 is grown in liquid culture in the absence of a catabolite repressor, the addition of Phosfon D has no effect upon cellulase formation or activity as measured by the filter paper assay. However, if the Phosfon D is omitted from the plate screening assay, no clearing zones appear, even in the absence of a catabolite repressor. This suggests that the Phosfon D is acting in a positive way, either by releasing cell wall-bound enzyme, which is normally achieved by shaking in liquid culture, or affecting the rate of diffusion of the enzyme in agar to permit increased digestion of the cellulose.

The use of acid-swollen cellulose as a substrate in the plate clearing assay is a compromise, since in the final analysis it will be necessary to produce a constitutive cellulase that hydrolyzes crystalline cellulose. However, since we are starting with a wild-type organism, QM6a, which produces a complete cellulase complex that degrades crystalline cellu-

FIG. 3. *Trichoderma viride* QM9414 spores plated on Vogel medium modified as described, 0.5% acid-swollen cellulose, and 500 μ g of Phosfon D per ml. (A) and (B) Plates contain 1.5% oxgall with 5% glycerol (A) and without 5% glycerol (B). (C) and (D) Plates contain 50 μ g of rose bengal per ml with 5% glycerol (C) and without 5% glycerol (D). Clearing of cellulose is evident in those plates lacking glycerol.

lose, there is no reason to think that all mutants derived from this parent will not have similar capabilities. Mutant QM9414, which degrades crystalline cellulose, readily attacks acid-swollen cellulose. Our choice of acid-swollen cellulose is a matter of expediency. These organisms will produce clearing zones on crystalline cellulose after several weeks of growth, but the process is complete after 96 h when

acid-swollen cellulose is the substrate.

The overnight incubation of the cultures at 50°C has a twofold effect on clearing zone formation. First, the higher temperature allows a more rapid hydrolysis of the cellulose, while also ensuring that only heat-stable cellulases are selected. Second, 50°C is inhibitory to the growth of *T. viride*. Thus, the fungus stops growing into and obscuring zones of hydrolysis

FIG. 4. Approximately 100 viable irradiated spores of *Trichoderma viride* QM6a were plated as in Fig. 3A and B with 500 μ g of glycerol per ml as the repressor. This concentration of glycerol is sufficient to repress cellulase formation by all of the colonies except the mutant, indicated by the arrow.

around the colony, i.e., a more distinct clearing zone is obtained by enhanced cellulolysis combined with subsequent inhibition of fungal growth. There is also the chance of release of cell-bound cellulases by autolysis as a result of the heating process. This does not appear to occur with *T. viride*, since all isolates, after the heating stage, have proved viable. Overnight exposure to 50°C may kill some microbes. Hence, it is advisable, before the final higher incubation step, to replicate the colonies and grow them at ambient temperatures to ensure against loss of strains.

As previously mentioned, this method may be used semiquantitatively. Suzuki (19) reported a range of 4 to 6 mM glucose as the concentration at which the transition between repression and derepression occurred in *Pseudomonas fluorescens* var. *cellulosa*. In the case of *T. viride*, the range is higher, 10 to 15 mM glucose, as measured by this plate clearing assay, and correlates well with the range described by Nisizawa and co-workers (15) for *T. viride*.

Furthermore, it is possible, using this method, to distinguish between the two standard strains of *T. viride*. Mutant strain QM9414 produces two to four times more enzyme than the wild-type QM6a, and the differ-

ence is reflected in the size of the clearing zones in the absence of a catabolite repressor. The subsequent use of medium containing increased concentrations of the cellulose substrate (i.e., a more opaque medium) will allow the sequential isolation of cellulolytic strains producing more of the enzymatic complex.

We are in the process of isolating a series of mutants of *T. viride* QM6a whose cellulase production is no longer catabolite repressible. In addition, we are screening other cellulolytic fungi and bacteria in this system, in the hope of finding organisms that can digest cellulose and lignocellulosic wastes more efficiently.

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