Measurement of Cellulase Activity in Brown- and White-rot Fungi on Dyed Cellulose

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1. Introduction

Numerous assay procedures have been developed to screen for cellulolytic activity of fungi in culture tests (J. DINGLE et al., 1953; G. S. RAUTELA and E. B. COWLING, 1966; D. W. STRANKS and J. BIENIADA, 1971; F. WABNEGG et al., 1980). One of the most common culture methods for determining cellulolytic activity of fungi has been to monitor the clearing of cellulose in an opaque agar medium (G. S. RAUTELA and E. B. COWLING, 1966). As the fungi grow, they secrete cellulolytic enzymes that act to create a clear zone in the opaque medium beneath the growing culture. Clearing is due to dissolution of the cellulose substrate.

Some cellulolytic wood-decay fungi, however, are unable to produce clearing in cellulose agar medium, even with phosphoric acid-swollen cellulose (T. NILSSON, 1974; T. NILSSON and J. GINNS, 1979). In particular, certain brown-rot wood-decay fungi, such as *Poria placenta*, *Lentinus lepideus*, and *Gloeophyllum trabeum*, are unable to clear cellulose agar. In a previous paper (T. L. HIGHLEY, 1980), it was proposed that the inability of these wood-decay fungi to clear cellulose agar medium may be due to (a) unfavorable culture conditions, (b) retarded movement of cellulolytic enzymes through the agar medium, or (c) indistinct zone formation.

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M. LEISOLA and M. LINKO (1976) showed that measurement of color intensity produced by release of dyed-cellulose breakdown products from cellulose dyed with Remazol brilliant blue R was a useful method for determination of cellulase activity. With dyed-cellulose substrates they found a linear correlation between the release of colored compounds and the solubilization of cellulose. The main colored hydrolysis product was dye-cellobiose.

R. E. SMITH (1977) found that dyed-cellulose powder as the substrate in agar medium greatly facilitated the detection of cellulolytic activity in several mold fungi. As cellulose bonds were broken, the dyed products diffused into the agar and the extent of color developed indicated cellulolytic activity.

The purpose of this work is to determine if cellulolytic activity of brown-rot fungi unable to clear cellulose agar can be detected using a dyed-cellulose agar method, and to determine the effect of various cultural conditions on breakdown of dyed cellulose by brown- and white-rot fungi.

2. Methods

Fungi. – The following fungi were used: Brown rot: *Poria placenta* (Fr.) Cke. (Mad-698), *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. (Mad-617), *Coniophora puteana* (Schum. ex Fr.) Karst. (Mad-515), *Lentinus lepideus* Fr. (Mad-534). *Leucogyrophana arizonica* Ginns (RLG-9902), *Leucogyrophana olivascens* (Berk. et Curt.), Ginns et Weresub (FP-104339); White rot: *Coriolus versicolor* (L. ex Fr.) Quél. (Mad-697), *Phanerochaete chrysosporium* Burds. (ME-446), *Ganoderma applanatum* (Pers. ex Wallr.) Pat. (Mad-708).

Cellulase assay. – The assay procedure was similar to that of R. E. SMITH (1977). The basal salts medium was the same as that used previously (T. L. HIGHLEY, 1973), except 0.02% NH_4SO_4 was the nitrogen source. Basal salts medium, 3 ml, adjusted to pH 4.5, was poured into test tubes. After sterilization at 120°C for 15 minutes, the medium was allowed to solidify in a vertical position. Sterilized cellulose azure, approximately 10 mg (Type I, Sigma, an acid treated cellulose dyed with Remazol brilliant blue R), was then asceptically layered on the surface of the agar. Tubes were inoculated with 0.2 ml of washed mycelial suspension and incubated in the dark at 27°C and 80% relative humidity.

Release of dyed-cellulose degradation products was assessed quantitatively as follows. Dye released into agar was extracted by adding 5 ml of H_2O to tubes with thorough mixing, followed by agitation on a vortex mixer for 30 seconds. Tubes were allowed to set for 1 hour, and then the contents were filtered under vacuum. The absorbance of filtrates was determined at 595 nm, and this value was used to indicate cellulolytic activity.

Effect of culture conditions on breakdown of dyed cellulose. - The effect of glucose and length of incubation on agar medium coloration by dyed breakdown products was determined by incorporating 1% glucose into the agar medium and measuring color development at 7, 14, and 21 days.

The effect of other soluble carbohydrates on cellulose degradation was determined at a 1% concentration as above. Insoluble carbon sources, 10 mg, were layered on the agar surface along with the cellulose azure.

Various nitrogen sources at 0.02% and 0.2% were added to the agar medium and their effect on cellulolytic activity determined.

Cellulolytic activity at different pH levels was determined by adjusting the pH of the agar medium from pH 2 to 9 with 4N HCl or 4N NaOH.

3. Results and Discussion

The brown-rot fungi, *P. placenta*, *L. lepideus*, and *G. trabeum*, previously reported unable to clear phosphoric acid-swollen cellulose in agar medium (T. NILSSON, 1974, T. NILSSON and J. GINNS, 1979), were able to break down cellulose azure and release dyed-cellulose degradation products into the agar medium below (Table 1). Thus, inability of these fungi to break down phosphoric acid-swollen cellulose in opaque or semi-opaque agar medium apparently cannot be attributed to failure to produce the required enzymes in axenic culture. In the dyed-cellulose assay used here, the cellulose was layered on top of the agar, rather than suspended in the agar as in the cellulose-clearing assay. This could account for the differences observed in the two assay methods. For cleared zones to be visible in cellulose-agar, the cellulolytic enzymes must diffuse beyond the edge of the fungal mycelium. Thus, the agar may retard movement of, may dilute, or may inactivate the

Table 1. Effect of glucose on breakdown of cellulose azure by brown- and white-rot fungi at 7, 14, and 21 days.

Fungus	Clearing capability[a]	7 Days		14 Days		21 Days	
		+Glucose	-Glucose	+Glucose	-Glucose	+Glucose	-Glucose
		Absorbance at 595 nm × 100					
Brown rot							
P. placenta	NC	0	0	20	0	32	0
G. trabeum	NC	26	25	30	36	30	36
L. lepideus	NC	0	0	26	22	34	30
C. puteana	C	14	30	19	36	20	56
L. arizonica	C	0	0	20	30	22	80
L. olivascens	C	0	0	22	50	40	75
White rot							
C. versicolor	C	0	39	0	25	0	7
G. applanatum	C	0	25	0	24	0	11
P. chrysosporium	C	0	49	0	42	0	26

a) NC = Not able to clear swollen cellulose in agar. C = Clear swollen cellulose in agar

cellulolytic system of the brown-rot fungi unable to clear cellulose agar. G. A. SOMKUTI (1974) found that the clearing of cellulose-agar is not sensitive enough to detect the activity of weakly cellulolytic organisms. Therefore another explanation for failure of certain cellulolytic fungi to produce visible clearing zones may be that cellulase activity is too weak to produce detectable clearing under the culture conditions of the assay.

Table 1 shows the effect of glucose on breakdown of cellulose azure at 7, 14, and 21 days. The brown rotters differed from the white rotters in that the brown rotters exhibited cellulolytic activity in the presence of glucose. This agrees with what is commonly observed for the effect of glucose on cellulase production by brown- and white-rot fungi in liquid culture (T. L. HIGHLEY, 1973). P. placenta was the only brown-rot fungus that required glucose in the medium to degrade cellulose. Without glucose in the medium, *L. lepideus* and *G. trabeum* produced about the same cellulolytic activity. Glucose reduced cellulase activity of *C. puteana, L. arizonica,* and *L. olivascens.*

Release of dyed-cellulose degradation products into the agar medium maximized at 21 days for the brown-rot fungi (Table 1). Maximum coloration of the agar medium by the white-rot fungi occurred at around 7 days and decreased thereafter. Possibly the white-rot fungi utilized dyed-cellulose degradation products in the agar. Thus, in comparing cellulolytic activity of fungi with the dyed-cellulose assay, care must be taken to monitor the assay periodically.

Table 2 shows the effect of various carbohydrates on cellulolytic activity by white- and brown-rot fungi. With the exception of *P. placenta,* the fungi exhibited good growth with all of the carbohydrates added to the cellulose azure medium. P. placenta did not grow on cellulose azure medium with glucono-a-lactone, cellobiose octaacetate, and xylan. All of the brown-rot fungi examined broke down cellulose azure in the presence of simple sugars. The cellulolytic system of the white-rot fungus, *G. applanatum,* was particularly repressed by simple sugars, being unable to degrade cellulose azure in the presence of any of the mono- or disaccharides. Only glucose, mannose, and cellobiose prevented breakdown of cellulose azure by the other white-rot fungi. The ß-glucosidase inhibitor, glucono-a-lactone, had little effect on cellulolytic activity. In most cases cellulolytic activity of both brown- and white-rot fungi was less in the presence of hemicelluloses, ball-milled sawdust, and holocellulose. These materials could affect release of dyed-cellulose degradation products into the agar medium by the tying up of either degradative enzymes or breakdown products of dyed cellulose.

Nitrogen sources had a variable effect on cellulolytic activity (Table 3). Relatively good activity and fungal growth were obtained on all sources; in most cases, concentration did not greatly affect activity. A different nitro-

Table 2. Breakdown of cellulose azure by brown- and white-rot fungi in the presence of different carbon sources.

Carbon source	Brown rot[a)]			White rot[b)]		
	P. placenta	*G. trabeum*	*C. puteana*	*C. versicolor*	*G. applanatum*	*P. chrysosporium*
	Absorbance at 595 nm × 100					
Monosaccharides						
Glucose	24	28	20	0	0	0
Mannose	22	26	42	0	0	0
Galactose	38	32	24	35	0	36
Xylose	32	28	42	38	0	37
Arabinose	23	24	38	14	0	38
Disaccharides						
Cellobiose	28	30	30	0	0	0
Lactose	20	21	20	14	0	14
Maltose	29	30	38	33	0	32
Miscellaneous						
Glucono-σ-lactone	0	23	20	48	34	32
Cellobiose octaacetate	0	20	57	45	38	28
β-methyl-xyloside	30	32	42	38	0	12
Xylan	0	17	38	20	0	32
Glucomannan	24	24	42	32	28	44
Holocellulose (aspen)	18	9	48	30	0	30
Holocellulose(hemlock)	24	0	40	32	15	36
Ball-milled aspen	0	12	26	24	15	39
Ball-milled spruce	0	13	20	24	0	36
None	0	32	42	39	25	49

a) Activity determined at 21 days.
b) Activity determined at 7 days.

gen source produced optimum coloration for each fungus. It was interesting that the three brown-rot fungi degraded cellulose azure without the addition of nitrogen to the medium, whereas the white-rot fungi did not. According to the manufacturer, the agar contains 0.17% nitrogen. The concentration of nitrogen in the agar medium without nitrogen added would therefore be 0.0026%. How much of this is available nitrogen is unknown. Apparently the three brown-rot fungi require only trace amounts of nitrogen for cellulolytic activity, while more nitrogen is required for the cellulolytic system of the examined white-rot fungi to be operative.

The effect of pH on cellulolytic activity is illustrated in Figure 1. The pH optimum for cellulolytic activity of all of the fungi was in the range of 3 to

Table 3. Breakdown of cellulose azure by brown- and white-rot fungi in the presence of different nitrogen sources.

Nitrogen source	Conc. (%)	Brown rot[a)]			White rot[b)]		
		P. placenta	*G. trabeum*	*C. puteana*	*C. versicolor*	*G. applanatum*	*P. chrysosporium*
		Absorbance at 595 nm × 100					
NH_4NO_3	0.2	28	28	45	40	26	52
	0.02	24	35	44	32	28	52
NH_4SO_4	0.2	25	27	51	44	28	54
	0.02	28	31	44	42	24	54
NH_4Cl	0.2	36	22	56	50	32	54
	0.02	26	31	52	23	34	48
NH_4HPO_4	0.2	36	37	50	30	25	42
	0.02	32	47	55	36	25	42
Asparagine	0.2	19	54	54	44	23	34
	0.02	28	56	44	66	32	54
Casein	0.2	32	64	54	58	36	52
	0.02	40	48	50	36	34	48
None	–	20	14	20	0	0	0

a) Activity determined at 21 days. 1% glucose added to *P. placenta* assays.
b) Activity determined at 7 days.

5. The brown-rot fungi were active on cellulose azure at pH 2, but not above pH 7. *P. placenta* was particularly sensitive to a rise in pH; its cellulolytic activity dropped sharply above 5. The white-rot fungi, on the other hand, were inactive on cellulose azure at pH 2, and high pH was not as inhibitory to cellulolytic activity as in the case of the brown-rot fungi. Similar effects of pH are reported for cellulases isolated from filtrates of brown- and white-rot fungi (T. L. HIGHLEY, 1975).

Measurement of cellulolytic activity by color development produced by release of dyed-cellulose breakdown products into agar medium has the advantages discussed by G. S. RAUTELA and E. B. COWLING (1966) for the clearing method: (a) is simple and rapid, (b) correlates with capacity to utilize native cellulose, and (c) ensures minimal risk of loss of cellulolytic activity due to binding, inhibition, or denaturation. In addition, the dyed-cellulose assay appears to be more sensitive than the clearing method because it demonstrated cellulolytic activity in wood-decay fungi that the clearing method failed to detect (T. NILSSON, 1974; T. NILSSON and J. GINNS,

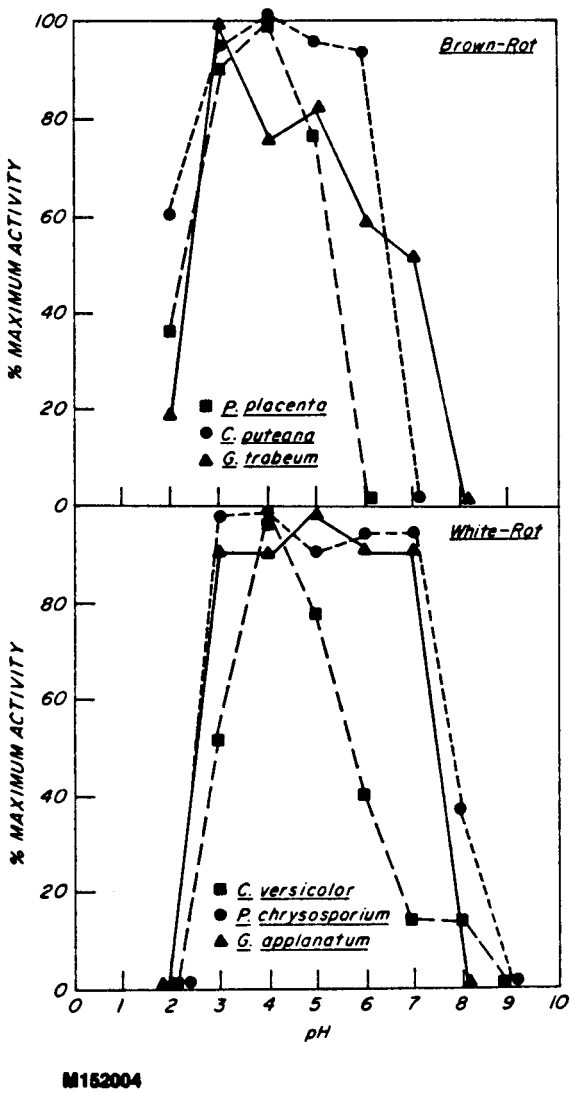

Fig. 1. The effect of pH on dye release by brown- and white-rot fungi. One percent glucose added to *P. placenta* assays.

1979). The dyed-cellulose assay also has the advantage that it can be quantified by colorimetric determinations while the clearing method cannot. This cellulase assay should prove particularly useful in screening for cellulase inhibitors that might serve as a basis for development of new preservatives against decay fungi.

4. Summary

Cellulolytic activity in 6 brown-rot fungi and 3 white-rot fungi was evaluated by their ability to release dyed breakdown products from dyed cellulose over an agar medium. Dyed-cellulose demonstrated cellulase activity in brown-rot fungi that the cellulose-clearing assay failed to detect. The dyed-cellulose assay must, however, be carefully monitored because the 3 white-rot fungi caused dyed-cellulose degradation products to disappear soon after maximum color development. The effect of pH and simple sugars on cellulase activity determined by the dyed-cellulose assay was similar to that found on cellulase activity in fungal culture filtrates. This cellulase-assay method should be a valuable research tool for study of chemical and culture effects on the enzymatic breakdown of cellulosic materials by wood-decay fungi.

Zusammenfassung

Messung der Cellulase-Aktivität von Braun- und Weißfäulepilzen auf angefärbter Cellulose

Die cellulolytische Aktivitat von 6 Braun- und 3 Weißfäulepilzen wurde an ihrer Fähigkeit, gefärbte Abbauprodukte aus angefärbter Cellulose an einen Agar-Nährboden abzugeben, bestimmt. In angefärbter Cellulose wurde eine Cellulase-Aktivität bei Braunfäulepilzen festgestellt, die mit der Klar-Zonen-Methode nicht nachgewiesen werden konnte. Der Versuch mit der angefärbten Cellulose muß allerdings genau kontrolliert werden, da die von den 3 Weißfäulepilzen gebildeten Abbauprodukte kurz nach der starksten Farbentwicklung wieder verschwanden. Die in diesem Versuch bestimmten Einflüsse des pH-Wertes und der einfachen Zucker auf die Cellulase-Aktivität waren denen ähnlich, die Pilzkulturfiltrate auf die Cellulase-Aktivität ausübten. Dieses Cellulase-Prüfverfahren sollte ein wichtiges Hilfsmittel sein zur Untersuchung der chemischen und von der Kultur ausgehenden Einflüsse auf den enzymatischen Abbau von cellulosehaltigem Material durch holzzerstörende Pilze.

Resume

Mesure de l'activité cellulasique des champignons de pourritures brune et blanche sur cellulose colorée

L'activité cellulolytique de 6 champignons de pourriture brune et de 3 champignons de pourriture blanche a été évaluée par leur capacité à produire des composes de degradation colorés à partir de cellulose colorée sur milieu gelose. La cellulose colorée a démontré l'activité cellulasique des champignons de pourriture brune que la méthode d'éclaircissement de la cellulose n'avait pas pu détecter. La méthode a la cellulose colorée doit cependant être soigneusement contrôlée car les 3 champignons de pourriture blanche provoquent la disparition des produits de degradation de la cellulose colorée aussitôt après le développement maximum de la couleur. L'effet du pH et des sucres simples sur l'activité cellulasique determinee par la méthode à la cellulose colorée était semblable à celui trouvé sur l'activité cellulasique des filtrats de culture fongiques. Cette méthode cellulasique devrait être un outil de recherche valable pour l'étude de la degradation enzymatique des matériaux cellulosiques par les champignons de pourriture du bois.

Resumen

Medición de la actividad celulàsica en hongos de la pudrición parda y blanca sobre celulosa coloreada

Se procedió a evaluar la actividad celulolítica en seis hongos de la pudrición parda y tres de la blanca por su habilidad de excretar productos de degradación coloreados, procedentes de celulosa coloreada, sobre un cultivo de agar. En los hongos de la pudrición parda tuvo lugar una actividad celulásica con celulosa coloreada que, sin embargo, no pudo ser comprobada mediante el análisis de claros. Es preciso, no obstante, controlar minuciosamente el expenmento con celulosa coloreada, puesto que los tres hongos causantes de la pudrición blanca hicieron desaparecer los productos de degradación de la celulosa coloreada a1 poco tiempo de alcanzar éstos el mayor grado de coloración. Los efectos del valor pH y de los azúcares simples sobre la actividad celulásica observados en este expenmento fueron similares a los ejercidos por filtrados de cultivos de hongos sobre la actividad celulásica. Este método de prueba de celulasa constituye una valiosa ayuda para el estudio de los efectos químicos y del cultivo sobre la degradación enzimática de materias con contenido de celulosa por hongos destructores de la madera.

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