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Use of Dyes in Solid Medium for Screening Ligninolytic Activity of Selective Actinomycetes

Scientific Note

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INTRODUCTION

Lignin, a three-dimensional biopolymer, not only encrusts the cellulose microfibrils in a sheath-like manner, but is also bonded physically and chemically to the plant polysaccharides (*1*). Unless the lignin is depolymerized, solubilized, or removed, the cellulose and hemicelluloses cannot be easily hydrolyzed by respective enzymes for their bioconversion into biofuels and chemicals. By now it has been established that lignin peroxidase (LiP) of white-rot fungus *Phanerochaete chrysosporium* is responsible for degradation of lignin (*2,3*). It has been reported that LiP is produced during secondary metabolism under carbon or nitrogen limitation by this organism (*4*). In literature, usually low yields (per unit volume) of LiP with *P. chrysosporium* have been reported (*5,6*). The reasons for low yields may be attributed to insufficient nitrogen in production media, which ultimately affects the synthesis of LiP protein. Therefore, it necessitated a search for an organism that can produce a ligninolytic enzyme system during its primary metabolism, without any effect of nitrogen limitation in the fermentation medium and without supply of extra oxygen to the cultures.

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Glenn and Gold (*7*) were the first to report that decolorization of polymeric dyes in liquid cultures is related to the lignin degradation system. They demonstrated that like lignin degradation, the decolorization of polymeric dyes by the white-rot basidiomycete *P. chrysosporium* occurred during secondary metabolism, was suppressed in cultures grown in the presence of high levels of nitrogen, and was strongly dependent on the oxygen concentration in the cultures. The evidence presented by them indicated that these dyes serve as substrates for the fungal lignin degradation system and also have the value of the determination of the onset of secondary metabolism in the organism. Pasti and Crawford (*8*) also reported that anthrone-type dyes are suitable substrates for analyzing production of peroxidases by streptomycetes in liquid cultures. The present study was undertaken to establish if certain dyes in solid media could be used to screen ligninolytic activity of selective actinomycetes during their primary metabolism without the limitation of nitrogen in the medium.

MATERIALS AND METHODS

Microorganisms

First of all, it was necessary to establish that decolorization of polymeric dyes in the solid agar medium is a good indicator of the activities of ligninolytic enzyme system. Therefore, *P. chrysosporium* ATCC-24725, the most studied wood-rotting fungus for ligninolytic enzyme system, was taken up as a model ligninolytic organism. *Pleurotus sajor-caju* NRRL-18757, another wood-rotting fungus, was included in this study because of its ability to degrade lignin of high molecular weight (mol wt) into oligolignols of low mol wt (*9*). Moreover, a weak activity of veratryl alcohol oxidase in this organism has also been reported by Bourbonnais and Paice (*10*). In addition to these two ligninolytic fungi, *Trichoderma reesei* QMY-1, NRRL-18760 and *Chaetomium cellulolyticum* NRRL-18756, cellulose degraders (*11*), and a bacterium, *E. coli* DH 11 S (Gibco, Mississauga, Ontario), were included as nonligninolytic organisms.

Culture Media

All the fungi were grown on Mandels and Weber's medium (*12*) containing different dyes (methylene blue 0.0005%, dextran blue 0.05%, remazol blue 0.005%, and Coomassie blue 0.005%) with 1.5% agar in Petri plates of 8.5-cm diameter. One set of Petri plates containing a complete dose of nitrogen (1.4 g $[NH_4]_2SO_4$ + 0.3 g urea/L) and another set of Petri plates containing a one-tenth dose of nitrogen were prepared and labeled as *N*-complete and *N*-limited medium, respectively. The cultures of *P. chrysosporium* and *C. cellulolyticum* were incubated at 37°C, whereas those of *P. sajor-caju* and *T. reesei* were incubated at 30°C.

Streptomyces lividans 1326, the parent strain, and its clones *S. lividans* X-18 and *S. lividans* X-42 (*13*) and one unidentified organism (preliminary identification reveals that it is close to mycelial form of actinomycete) were included as test organisms. These test actinomycetes and *E. coli* were grown on ATCC medium #399 containing xylan or glucose as a source of carbon. The unidentified actinomycete was grown on ATCC medium #174 with glucose and xylose as carbon sources. The cultures of the actinomycetes were incubated at 37°C. To find out the width of decolorized halo, the diameter of colony was substracted from the diameter of complete decolorized circular area, including the colony.

RESULTS AND DISCUSSIONS

Decolorization by *P. chrysosporium* (Table 1)

In general, decolorization of all the four test dyes started early, i.e., within 2–4 d of incubation of *P. chrysosporium* on *N*-limited media and decolorized the whole Petri plate faster than on *N*-complete media.

Methylene blue was completely decolorized in both the *N*-combinations within 7 d of incubation of *P. chrysosporium*, but mycelium and spores of *P. chrysosporium* turned blue because of adsorption of the dye. However, the decolorization of the dye beyond the vicinity of mycelium, which was confined only on the surface of solid agar medium, indicated that it had sufficient extracellular enzymatic activity that decolorized the methylene blue throughout the depth of the solid agar medium. Adsorption of methylene blue by the mycelium and spores had also contributed for decolorization of the dye.

Decolorization of dextran blue started after 4 and 7 d of incubation of *P. chrysosporium* on *N*-limited and *N*-complete media, respectively. There was complete decolorization of dextran blue in case of *N*-limited medium after 7 d of incubation of *P. chrysosporium*. On *N*-complete medium, complete decolorization was not noticed even up to 23 d of incubation.

Decolorization of remazol blue started after 4 and 8 d of incubation of *P. chrysosporium* on *N*-limited and *N*-complete media, respectively. Complete decolorization was observed after 7 d of incubation on *N*-limited medium, whereas some blue patches were still noticeable even after 23 d of incubation of *P. chrysosporium* on *N*-complete medium. Decolorization of coomassie blue by *P. chrysosporium* followed almost the same pattern of remazol blue.

Decolorization by *P. sajor-caju* (Table 1)

The color of methylene blue changed to purple after 19 d of incubation of *P. sajor-caju* on *N*-limited medium, whereas there was no change in color on *N*-complete medium. The color of dextran blue changed completely to

Table 1
Effect of Growth of Ligninolytic and Nonligninolytic Fungi on Various Dyes

Microorganisms/dyes	*N*-limited medium	*N*-complete medium
P. chrysosporium:		
Methylene blue (0.0005%)	Decolorization started after 2 d; complete in 7 d Mycelium and spores turned blue	Decolorization started after 3 d; complete in 7 d Mycelium and spores turned blue
Dextran blue (0.05%)	Decolorization started after 4 d; complete in 7 d	Decolorization started after 7 d; incomplete decolorization up to 23 d
Remazol blue (0.005%)	Decolorization started after 4 d; complete in 7 d	Decolorization started after 8 d; some blue color remained after 23 d
Coomassie blue (0.004%)	Decolorization started after 4 d; complete in 7 d	Decolorization started after 7 d; no complete decolorization in 23 d
P. sajor-caju		
Methylene blue (0.0005%)	Color changed to purple after 19 d	No decolorization up to 23 d
Dextran blue (0.05%)	Color changed completely to pink in 15–23 d	Color only in the center of Petri plate changed to pink in 15–23 d
Remazol blue (0.005%)	Decolorization started after 8 d and was complete after 10 d	Color changed to gray completely between 10 and 23 d
Coomassie blue (0.004%)	No change in color up to 23 d	No change in color up to 23 d
T. reesei	No change in color of any dye	
C. cellulolyticum	No change in color of any dye	
E. coli	No change in color of any dye; methylene blue and dextran blue were absorbed by the cells	

pink within 15–23 d on *N*-limited medium, whereas it changed to pink only in the center of the plate during this time on *N*-complete medium.

Decolorization of remazol blue by *P. sajor-caju* started after 8 d of incubation on *N*-limited medium, and it was complete after 10 d. The color of remazol blue changed to gray between 10 and 23 d of incubation by this organism on *N*-complete medium. No change in color of Coomassie blue was noticed on both the *N*-combinations up to 23 d of incubation.

Decolorization by Nonligninolytic Organisms

T. reesei and *C. cellulolyticum*, nonligninolytic fungi, and *E. coli*, a nonligninolytic bacterium, failed to decolorize any dye on any media up to 23 d of incubation. However, *E. coli* adsorbed some color from methylene blue and dextran blue (Table 1).

Decolorization by Lignin Peroxidase (LiP) (Table 2)

When the LiP (Tienzyme, Inc.) (1.15–2.3 U/mL) was used in an in vitro test, all the four dyes were decolorized completely within 1–2 min of incubation. On the other hand, when the horseradish peroxidase (Boehringer Mannheim) was used in an in vitro test, it did not decolorize methylene blue even with a high dose of 160 U/mL. However, dextran blue was completely decolorized in 30 min with 40 U/mL. The color of remazol blue first changed to light purple and then to light green within 5 min with a low dose of 20 U/mL. The color of coomassie blue changed to yellowish green in 1 min with a high dose of 160 U/mL.

LiP produced with *P. chrysosporium* (0.3 U/mL) on Tien and Kirk (*14*) medium (modified by Laplante [*15*]: wood chips 2%, glucose 0.5%, and yeast extract 0.2%) was also used in the test for decolorization of these dyes. Methylene blue and dextran blue were not decolorized by this LiP, but their color changed to bluish green. The color of remazol blue and Coomassie blue was changed to yellow within 1 min of incubation, indicating good activity of this LiP on these dyes. After 3 min of incubation, the yellow color of remazol blue turned to pink, whereas that of Coomassie blue remained yellow.

Thus, it can be deduced from the information recorded in Tables 1 and 2 that decolorization of these dyes in solid agar media by the test organism will indicate the presence of strong ligninolytic or LiP activities as shown by *P. chrysosporium*, and change of blue color of these dyes to other hues will indicate the presence of weak ligninolytic or LiP activities as shown by *P. sajor-caju*. No decolorization of these dyes by the test organism will indicate the absence of ligninolytic or LiP activities as shown by the nonligninolytic fungi, *T. reesei* and *C. cellulolyticum*, and nonligninolytic bacterium, *E. coli*.

Screening of Ligninolytic Activity of Selective Actinomycetes

The data presented in Table 3 indicate that all the *S. lividans* test organisms decolorized methylene blue and remazol blue beneath their colonies as well as beyond their colonies producing a very clear-cut decolorized halo irrespective of *N*-combinations and source of carbon. However, these test organisms failed to decolorize dextran blue under any nutritional conditions of the medium, except on *N*-complete medium with glucose as carbon source where it produced a halo of 3-mm width.

Table 2
Decolorization of Various Dyes with LiP and Horseradish Peroxidase

Dyes	Quantity of peroxidase, U/mL	Decolorization[a]
LiP (Tienzyme, Inc.)		
Methylene blue (0.004%)	1.15	Complete decolorization in 1.5 min
Dextran blue (0.02%)	2.3	Complete decolorization in 1.5 min
Remazol blue (0.000038%)	1.84	Complete decolorization in 1 min
Coomasie blue (0.002%)	2.3	Complete decolorization in 2 min
Horseradish peroxidase (Boehringer Mannheim)		
Methylene blue (0.004%)	160	No decolorization
Dextran blue (0.02%)	40	Complete decolorization in 30 min
Remazol blue (0.000038%)	20	The color changed first to light purple and then to light green in 5 min
Coomasie blue (0.002%)	160	The color changed to yellowish green in 1 min
LiP (Laplante, 1994; produced in our laboratory)		
Methylene blue (0.004%)	0.3	Color changed to bluish green
Dextran blue (0.02%)	0.3	Color changed to bluish green
Remazol blue (0.000038%)	0.3	Color changed to yellow within 1 min and then to light pink after 3 min
Coomasie blue (0.002%)	0.3	Color changed to light yellow and remained yellow

[a]Visual examination.

The unidentified actinomycete showed decolorization of methylene blue under all the nutritional conditions of the medium. It did not show any decolorization or change in color of the other dyes, i.e., dextran blue and remazol blue. This test clearly indicated that all the test actinomycetes have ligninolytic or LiP activities. Wild-type *S. lividans* was producing almost as much ligninolytic activity as that of its clones, indicating that no activity was coded by the insert in the multicopy plasmids.

Table 3
Effect of Test Actinomycetes on Various Dyes (14 d)

Actinomycete/dye	Carbon source	*N*-limited medium		*N*-complete medium	
		Colony diameter, mm	Width of halo, mm	Colony diameter, mm	Width of halo, mm
S. lividans parent strain 1326					
Methylene blue	Glucose	6	3	6	5
	Xylan	6	7	4	8
Dextran blue	Glucose	26	0	12	3
	Xylan	17	0	15	0
Remazol	Glucose	15	14	16	14
	Xylan	16	15	15	14
S. lividans IAF 18					
Methylene blue	Glucose	7	4	11	5
	Xylan	5	15	5	12
Dextran	Glucose	16	0	19	0
	Xylan	17	0	12	0
Remazol	Glucose	22	7	24	6
	Xylan	12	10	13	12
S. lividans IAF 42					
Methylene blue	Glucose	5	2	5	2
	Xylan	5	1	5	2
Dextran blue	Glucose	10	0	12	0
	Xylan	12	0	10	0
Remazol blue	Glucose	11	10	16	10
	Xylan	11	1	7	9
Unidentified actinomycete					
Methylene blue	Glucose	25	5	43	3
	Xylan	5	3	5	3
Dextran blue	Glucose	44	0	75	0
	Xylan	30	0	40	0
Remazol blue	Glucose	50	0	62	0
	Xylan	30	0	42	0

Usually the gene dosage effect produced over 60 times more enzymes than the wild-type strain (*13*). The unidentified actinomycete seems to be very specific because it indicated the ligninolytic activity only on methylene blue. However, the unidentified actinomycete was fast growing and also showed ligninolytic activity within first 24 h of growth as compared to the other test actinomycetes, where the activity started to appear after 3rd d of incubation.

CONCLUSIONS

The use of dyes in solid medium proved to be an accurate, quick, and easy method for screening of ligninolytic activity of a large number of microorganisms, their mutants, or clones. The decolorization of dyes indicated the presence of strong ligninolytic or LiP activities as shown by *P. chrysosporium*. Change of blue color of the dyes to other hues indicated the presence of weak ligninolytic or LiP activities as shown by *P. sajor-caju*. No decolorization of the dyes indicated the absence of ligninolytic or LiP activities as shown by nonligninolytic test organisms. Since there was change of blue color of dyes into various hues, the colorimetric method may be difficult to develop.

The ligninolytic activities of actinomycetes test organisms were almost equally good on *N*-limited and on *N*-complete medium irrespective of carbon source. Therefore, we see good potential in using such actinomycetes for production of ligninolytic enzyme system to achieve high yields because there will not be any limitation of nitrogen in the medium for synthesis of extracellular enzyme proteins. The ligninolytic activity was also noticed when the test actinomycetes were actively growing, i.e., ligninolytic enzymes were being produced during the primary metabolism.

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