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Effect of carbohydrate and nitrogen on hydrogen peroxide formation by wood decay fungi in solid medium

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1. SUMMARY

Hydrogen peroxide (H_2O_2) has been implicated in degradation of wood by both brown-rot and white-rot fungi. This study found that low concentrations of nitrogen and carbohydrates (cellobiose, glucose, xylose and mannose) in an agar medium had little effect on H_2O_2 production by white-rot fungi. However, low concentrations of nitrogen and carbohydrates stimulated H_2O_2 production by brown-rot fungi. Use of the chromogen 2,2'-azino-di(3-ethyl benzthiazoline-6-sulphonic acid) (ABTS) with horseradish peroxidase to detect H_2O_2 by the fungi was slightly better than detection by the chromogen *o*-dianisidine with horseradish peroxidase. An auxiliary test to check the role of H_2O_2 in wood decay found that hydrogen peroxide-negative isolates of the white-rot fungi *Phanerochaete chrysosporium* and *Ganoderma applanatum* were unable to decay sweetgum and southern pine.

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2. INTRODUCTION

The hydroxyl radical ($OH^{\cdot}$) derived from hydrogen peroxide (H_2O_2) has been proposed to be a cellulose-degrading agent in brown-rot decay of wood [1,2]. However, its involvement in cellulose decomposition has been questioned, primarily because of inability to detect its production by several brown-rot fungi [3,4]. Hydrogen peroxide is found in most white-rot fungi where it evidently plays an important role in lignin degradation [5,6]. Glucose oxidase has been shown to be the predominant source of H_2O_2 in lignin-degrading cultures of the white-rotter *Phanerochaete chrysosporium* [7]. Glucose oxidase activity in this fungus appears to be a secondary metabolic event and is triggered in response to nitrogen or carbohydrate starvation [8]. Failure to detect H_2O_2 in certain brown-rot fungi, therefore, could be due to excess nitrogen or carbohydrate in the growth medium if glucose oxidase is the source of H_2O_2.

Another reason for inability to detect H_2O_2 in some brown-rot fungi may be that the amounts of H_2O_2 produced are so low that the chromogens are not sensitive enough to detect it. Muller [9] found that 2,2'-azino-di-(3-ethylbenzthiazole-6-sulphonic acid) (ABTS) used with horseradish peroxidase was 10 times more sensitive than ben-

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zidine in the detection of H_2O_2 in bacteria.

The purpose of this study is to evaluate the influence of nitrogen and carbohydrate source and concentration on H_2O_2 production by brown-rot and white-rot fungi using the highly sensitive chromogen ABTS.

3. MATERIALS AND METHODS

3.1. Fungal strains and cultural conditions

The fungal strains used are listed in Table 1. Two strains of *P. chrysosporium* (ME-446 and ME-461) and *Ganoderma applanatum* (MAD-708 and 7823-S) were used because the originals strains (ME-446 and MAD-708) lost their ability to produce H_2O_2 during the course of the study. Fungal cultures were maintained on 2% (w/v) malt-agar slants at 4° C until used. Tubes containing media for detection of H_2O_2 production were inoculated with a plug from malt-agar tubes and incubated at 27° C and 70% humidity.

3.2. Comparison of chromogens

The basal salts medium contained: NH_4NO_3, 2 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $CaCl_2$, 0.1 g, thiamine, 1 mg; and minor elements [10]; 15 g of agar were added to 900 ml of the basal salts medium containing 10 g of cellobiose. After autoclaving at 121°C for 15 min, the medium was cooled to 50°C. Horseradish peroxidase 20 mg (Sigma, Type II) and 300 mg of ABTS or *o*-dianisidine were dissolved in 100 ml of distilled H_2O, filter-sterilized and added aseptically to the agar medium; 3-ml portions were poured into pre-sterilized test tubes. To determine the effect of wood cell wall polymers on H_2O_2 formation, xylan (Sigma), cellulose (Hercules; cotton type A-600),

Table 1

Comparison of chromogens in detection of H_2O_2 by white-rot and brown-rot fungi grown on a medium of high carbohydrate and nitrogen

Fungus	Coloration of medium using chromogen [a]	
	ABTS	*o*-Dianisidine
Brown-rot fungi		
Leucogyrophana arizonica Ginns, RLG-9902-Sp.	0	0
L. olivascens (Berk. et Curt) Ginns et Weres., FP-104339-Sp.	0	0
Coniophora puteana (Schum.: Fr.) Karst., MAD-515	0	0
Poria placenta (Fr) Cke., MAD-698	0	0
Gloeophyllum trabeum (Pers.: Fr.) Murr., MAD-617	0	0
Lentinus lepideus Fr., MAD-534	0	0
P. carbonica Overh., FP-105585-Sp.	2	0
A. alpina (Litsch.) Gilbn.: Ryv., FP 105523-Sp.	0	0
Antrodia serialis Fr., FP-71951-R	1	0
Fibroporia vaillantii (DC: Fr.) Parm	0	0
Serpula incrassata (Berk. et Curt) Donk, MAD-563	0	0
Laetiporus sulphureus (Bull.: Fr.) Bond. et Sing., Sh-27-R	0	0
Fomitopsis meliae (Underw.) Gilbn., FP-105065-Sp.	0	0
White-rot fungi		
Phellinus contiguus (Pers.: Fr.), HHB-7051-Sp.	4	4
Phanerochaete chrysosporium Burds., ME-446	0	0
Ganoderma applanatum (Pers.: S. F. Gray) Pat. (MAD-708)	1	1
Xylobolus frustulatus (Pers.: Fr.) Boid., FP-24059-S	0	0
Coriolus versicolor (L: Fr.) Quél., MAD-697	3	3
Ph. ferrugineofuscus (Karst.) Bourd, L-14724-Sp.	4	4
Spongipellis unicolor (Schw.) Murr., RGL-7638-Sp.	4	4

[a] 0 = no coloration; 1 = very slight coloration; 2 = slight coloration; 3 = moderate coloration; 4 = heavy coloration.

or hemlock sawdust were layered onto the agar surface.

3.3. Effect of nitrogen and carbohydrate on H_2O_2 production

The brown-rot and white-rot were tested for H_2O_2 production on the above media with 1% (w/v) cellobiose and ABTS with various nitrogen sources: 0.2 and 0.02% NH_4NO_3, 0.02 and 0.2% casein amino acids (Difco), 0.02% yeast nitrogen base (Difco) and 0.2% malt extract. Hydrogen peroxide was also determined using the above basal salts media with 0.02% NH_4NO_3 and the following carbohydrate sources: None, 0.1% cellobiose, 0.1 and 1.0% glucose, 0.1% xylose and 0.1% mannose.

3.4. Decay tests

The standard ASTM D 2017 soil-block method [11] was used to compare the ability of H_2O_2-negative and H_2O_2-positive isolates of *P. chrysosporium* and *G. applanatum* to decay sweetgum and southern pine blocks. Following incubation, test samples were removed from bottles, conditioned at 27°C and 70% humidity, and weighed. Weight loss was used as an estimate of decay susceptibility.

4. RESULTS AND DISCUSSION

With high carbohydrate and nitrogen in the medium, ABTS-peroxidase was only slightly better in detection of H_2O_2 production by the fungi than was *o*-dianisidine-peroxidase (Table 1). How-

Table 2

Effect of nitrogen source and concentration on H_2O_2 [a] formation by white-rot and brown-rot fungi

Fungus	Coloration of medium [b]					
	Nitrogen source [c]					
	1	2	3	4	5	6
Brown-rot fungi						
L. arizonica	0	0	0	0	0	0
L. olivascens	0	0	0	0	0	3
C. puteana	0	0	0	0	0	1
P. placenta	0	0	0	0	0	0
G. trabeum	0	0	0	0	0	0
L. lepideus	0	0	0	0	0	0
P. carbonica	3	2	2	3	3	3
P. xantha	0	1	0	0	2	0
A. serialis	1	3	3	3	3	0
F. vaillantii	0	0	0	0	0	0
S. incrassata	0	0	0	0	0	0
L. surfureus	0	3	0	3	3	2
P. meliae	0	0	0	0	0	0
White-rot fungi						
Ph. contiguus	4	4	4	4	4	4
P. chrysosporium	0	0	1	0	0	1
G. applanatum	2	2	2	2	2	3
X. frustulatus	0	0	0	0	0	0
Ph. ferrugineofuscus	4	4	4	4	4	3
S. unicolor	4	4	4	4	4	3
C. versicolor	3	2	2	2	2	3

[a] H_2O_2 detected with ABTS.

[b] 0 = no coloration; 1 = Very slight discoloration; 2 = slight coloration; 3 = moderate coloration; 4 = heavy coloration.

[c] 1. 0.2% NH_4NO_3; 2. 0.02% NH_4NO_3; 3. 0.2% casein amino acids; 4. 0.02% casein amino acids; 5. 0.02% yeast nitrogen base; 6. 0.2% malt extract.

ever, only 2 out of the 13 brown-rot fungi produced detectable H_2O_2 on the ABTS medium. With the white-rot fungi, H_2O_2 was detected in 5 out of 7 fungi. Addition of xylan, cellulose, or hemlock sawdust to the medium did not change the number of fungi producing detectable H_2O_2 (not shown).

Nitrogen concentration has been found to have a profound effect on H_2O_2 production and ligninolytic activity by the white-rot fungus, *P. chrysosporium* [12]. Cultures of *P. chrysosporium* grown in low nitrogen medium exhibit high levels of H_2O_2 production, whereas cultures grown under identical conditions in high nitrogen medium show little H_2O_2 production [12]. In the present study, low nitrogen in the medium containing 1% cellobiose did not stimulate H_2O_2 production by the white-rot fungi (Table 2). Source of nitrogen also had little effect on H_2O_2 production by the white-rot fungi. *P. chrysosporium* produced slight amounts of H_2O_2 only on the media containing 0.2% casein amino acids or 0.2% yeast extract. *Xylobolus frustulatus* was the only white-rot fungus not producing detectable H_2O_2 on any of the nitrogen sources.

Varying the nitrogen source and concentration in the 1% cellobiose medium gave a vaned response in H_2O_2 production by the brown-rot fungi (Table 2). Seven out of 13 of the brown-rot fungi still did not produce detectable H_2O_2 using different sources of nitrogen.

Table 3 shows H_2O_2 production by the fungi using different concentrations and sources of carbohydrate with 0.02% NH_4NO_3 as the nitrogen source. In this study H_2O_2 production by the white-rot fungi was affected little by carbohydrate

Table 3

Effect of carbohydrate source and concentration on H_2O_2 [a] formation by white-rot and brown-rot fungi

Fungus	Coloration of medium[b]						
	Carbohydrate source						
	None	0.1% cellobiose	0.1% glucose	1.0% glucose	0.1% mannose	0.1% xylose	0.2% malt extract
Brown-rot fungi							
L. arizonica	0	0	0	0	0	0	0
L. olivascens	0	2	0	0	0	3	0
C. puteana	4	4	4	0	4	4	4
G. trabeum	0	0	0	0	0	0	0
L. lepideus	2	2	4	0	1	1	1
P. carbonica	4	4	3	0	4	4	4
P. xantha	4	4	3	0	4	0	1
A. serialis	4	4	4	2	4	4	4
F. vaillantii	1	0	0	0	0	0	0
S. incrassata	0	0	2	0	4	2	2
L. sulfureus	2	2	3	3	3	3	2
F. meliae	0	0	0	0	2	1	0
P. placenta	0	3	3	0	3	3	3
White-rot fungi							
P. chrysosporium (ME-446)	0	1	0	0	0	0	0
P. chrysosporium (ME-461)	3	3	3	3	3	3	3
G. applanatum (MAD-708)	0	0	0	0	0	0	0
G. applanatum (7823-S)	2	4	4	4	4	4	4
X. frustulatus	0	1	0	0	0	0	0
C. versicolor	4	4	4	4	4	3	4

[a] H_2O_2 detected with ABTS.
[b] 0 = no coloration; 1 = very slight coloration; 2 = slight coloration; 3 = moderate coloration; 4 = heavy coloration.

Table 4

Decay of wood by H_2O_2 positive and negative isolates

Fungus	H_2O_2 [a] production	Weight loss (I) of wood[b]	
		Sweet-gum	Pine
G. applanatum (MAD-708)	-	0	0
G. applanatum (7823-S)	+	81.5	56.3
P. chrysosporium (ME-446)	-	0	0
P. chrysosporium (ME-461)	+	55.0	24.1

[a] +, = H_2O_2 detected; - = H_2O_2 not detected.
[b] Blocks removed at 10 weeks.

concentrations even though Kelly and Reddy [7] found that carbohydrate starvation triggered H_2O_2 production in the white-rot fungus *P. chrysosporium* (Table 3). In the present study, by varying the carbohydrate source and concentration, H_2O_2 was detected in all but 2 of the brown-rot fungi, *G. trabeum* and *L. arizonica*. As a whole, H_2O_2 production by most of the brown-rot fungi was stimulated by low sugar concentration. In some cases, H_2O_2 was produced without carbohydrate added to the medium.

Hemlock sawdust was added to the low nitrogen medium containing no other carbon source to see if it would stimulate H_2O_2 formation in *G. trabeum, L. arizonica,* and *X. frustulatus*. The sawdust did not induce production by the above fungi.

Since production of H_2O_2 has been linked to ligninolytic activity of white-rot fungi, the decay abilities of the H_2O_2-negative and H_2O_2-positive isolates of *P. chrysosporium* and *G. applanatum* were compared. The H_2O_2-negative isolates were unable to decay pine and sweetgum, but the H_2O_2-positive isolates readily decayed wood (Table 4). This observation provides circumstantial evidence of H_2O_2 involvement in degradation of wood by white-rot fungi. However, additional degradative studies with H_2O_2-positive and H_2O_2-negative isolates are needed.

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