

SOLUBILIZATION OF LEONARDITE BY WHITE-ROT FUNGI GROWN IN STATIONARY AND SHAKE FLASKS

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INTRODUCTION

Oxidized coals, including a naturally oxidized lignite identified as leonardite, are solubilized and sometimes degraded further by a variety of fungi and bacteria. Evidence for biosolubilization of coal was first presented by Fakoussa (1), and Cohen and Gabriele (2). Subsequent studies concentrated on screening organisms (3), characterization of the product (4), and determination of the biochemical mechanisms. Mechanisms of biosolubilization are poorly known and may vary with the species used and the media. There is evidence for both enzymatic degradation (5,6) and alkaline solubilization (7,8).

The objective of this study was to discover critical factors in solubilization and biosolubilization mechanisms by testing a variety of growth media, growth conditions, and fungi. Lignin-degrading species were emphasized because of similarities between the structures in lignin and in low-rank coals. The results indicate that during idiophase (secondary metabolism), the fungi produce alkaline materials that solubilize leonardite.

METHODS

Phanerochaete chrysosporium BKM-F 1767 was obtained from Dr. T.K. Kirk of the U.S.D.A. Forest Products Laboratory; Trametes versicolor (ATCC 12679), from the American Type Culture collection; and Candida ML-13, from Dr. Bailey Ward of the University of Mississippi. Others were isolated at PETC. All were maintained on Sabouraud maltose agar (SMA) or broth at 35°C and 90%-98% humidity. Malt agar was frequently used to promote spore production in P. chrysosporium. P. chrysosporium was inoculated using filtered conidial suspensions ($A_{650} = 0.5/\text{cm}$); other species were inoculated using fungal mats ground in a blender.

To solubilize leonardite on fungal mats, small (1-2 mm) pieces of sterile leonardite were placed on 1- to 3-week-old agar cultures. The pH of the agar was monitored by the use of puncture and surface pH probes, and the black drops of solubilized coal were sampled for pH and other analyses. Elemental analyses were performed on 6-day-old mats and associated agar to study nutrient depletion.

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Four broths were tested for their characteristics in supporting fungal growth and promoting solubilization: Sabouraud maltose broth (SMB), nutrient broth (NB), tryptic soy broth (TSB), and a defined broth (DB) (9). Leonardite, a naturally oxidized lignite, was provided by American Colloid Co., Reeder, North Dakota.

Fungi were grown in 125-mL flasks (20 mL of broth) and 250-mL flasks (40 mL of broth). Roughly 40 flasks were set out at one time under the same growth conditions. From this set, duplicate samples were selected once or twice per week. They were filtered (1.0 μ -pore glass fiber) and analyzed for fungal dry weight (using tared filter paper), pH, and absorbance (A_{450}). A routine test was developed to assay the activity of cell-free broth for solubilization using both gravimetric and spectroscopic measures. Unless otherwise stated, leonardite [0.3% (wt/vol), <100-mesh] was added to the cell-free broth, and the mixture was shaken on a wrist shaker for 1 hr. The treated coal was filtered with a tared glass fiber filter (1- μ -pore), washed, dried, and weighed to gravimetrically determine the percentage of coal solubilized. The filtrate was analyzed for pH. The optical density at 450 nm (A_{450}) was measured, using diluted samples when appropriate, to obtain the spectrophotometric determination of the relative amount of coal solubilized. When plotted against each other, the two measures were correlated but showed considerable scatter ($R^2=0.820-0.854$).

To determine the effect of shake time on solubilization, replicates of cell-free filtrates were shaken and then filtered, and A_{450} of the filtrates were measured at 15-min intervals over the course of 1 hr. The SMB treated with *P. chrysosporium* was boiled for 1 min or heated at 90°C for 1 hr to determine if denaturation of basic proteins reduced solubilization. Protein concentrations of cell-free extracts were determined with the Bradford test (10).

RESULTS

Solubilization on Agar

Phanerochaete chrysosporium, *Trametes versicolor*, *Candida* SL13, *Rhizopus arrhizus*, and *Geosmithia argulus* all solubilized 1-2 mm leonardite chunks when grown on SMA at pH over 7 (Fig. 1). Solubilization was evidenced by the appearance of black liquid surrounding the coal within 48 hours after the coals were added to 1- to 3-week cultures. Two possible conversion mechanisms were investigated in the case of *P. chrysosporium*: degradation by the ligninase enzymes and solubilization by alkaline products. Ligninase is produced by the organism when nitrogen available for growth is nearly exhausted (9). Neither nitrogen nor two other growth essentials, phosphorus and carbon, were depleted in 6-day cultures (Table 1). Thus, conditions for ligninase production are not evident, even though conversion of coal was observed. Furthermore, ligninase is most active at low pH (9). Solubilization appeared to result from production of alkaline materials. This phenomenon was investigated in more detail using suspension cultures.

Solubilization by Fresh Broth

Results of shaking fresh broth with leonardite for 1 hr are shown in Fig. 2. The SMB and NB were preferred for use as growth media in fungal experiments. The TSB was eliminated because of high levels of solubilization in absence of fungi. Later experiments showed that only low levels of biosolubilization were

observed with extracellular fluid harvested from fungi grown in DB. All biosolubilization data presented below have been corrected for the absorbance due to the medium alone.

Solubilization by Harvested Cell-Free Broth

Particle size was expected to affect solubilization because of the larger surface area presented by the smaller particles. The A_{450} did increase as expected (Table 2) but not greatly within the 60- to 300-mesh range. Results for the standard size used in these studies (<100 mesh) were most similar to those for the 200-300 mesh.

The time needed to solubilize leonardite in cell-free broth harvested from an active fungal growth was studied. The absorbance was determined after shaking and reading A_{450} at 15-min periods for 1 hr (Table 3). The solubilization rate is most rapid during the first 15 min, and there is little increase in A_{450} during the next 45 min or for several hours (data not shown). Thus, the solubilizing agent works quickly, and the 1-hr shake time used under standard conditions is more than adequate.

Leonardite is an acidic material. Addition of leonardite to the harvested growth media reduces its pH and adversely affects its solubilization capacity. As may be seen in Table 4, the percentage of leonardite solubilized as measured by the gravimetric method steadily declines as the leonardite loading is increased. The A_{450} passes through a maximum and begins to decline as the pH is progressively reduced. Thus, the pH ultimately reached by a medium must be taken into account when comparisons of activity are made. The lowest coal loading (0.3 wt%) was chosen to minimize the influence of coal acidity.

A large set of flasks (~40) were set out under the same conditions as described under Methods. Duplicate flasks were selected and their cultures were tested regularly for solubilization of leonardite. The relationship of biomass, pH, percent solubilization, and A_{450} with *P. chrysosporium* in SMB is shown in Fig. 3. In this example, solubilization and A_{450} parallel the change in pH. Solubilization peaked during secondary metabolism and declined during the autolysis stage. Maximum absorbance occurred at 20-25 days in other runs with SMB cultures of *P. chrysosporium*.

The time to reach maximum absorbance could be reduced by continuous gentle agitation of the cultures at 50 rpm (Fig. 4). The highest absorbance occurred before the pH reached a peak. Agitation at 200 rpm considerably reduced solubilizing ability, although the pH of broth was similar to that obtained with more gentle agitation. Absorbance levels similar to those shown in Fig. 3 were found with filtrates of NB cultures of *P. chrysosporium* and SMB cultures of *Trametes versicolor* (data not shown). Absorbance peaked at 20-45 days.

Extracellular proteins are likely agents of biosolubilization. The Bradford test (10) was used to determine protein concentration in harvested broth as a function of growth time over a 35-day period; protein content increased gradually to between 100 and 200 $\mu\text{g}/\text{mL}$. To determine whether denaturing of the proteins could reduce solubilization activity, samples of active broth from *P. chrysosporium* grown in SMB were boiled for 1 min or heated at 90°C for 1 hr. Boiling for 1 min resulted in decreases in A_{450} of 60% in one case and 33% in another. Heating a third broth at 90°C for 1 hr reduced A_{450} from 2.5 to 0.2

(92%). These reductions are evidence that biochemical activity, possibly due to proteins, is lost on heating.

DISCUSSION

Solubilization of untreated leonardite was shown to occur with fungi grown on agar and neat filtrates of suspension cultures. Biosolubilization of leonardite or oxidized coals has been attributed to enzymes (2,5), alkaline materials (7), and basic polypeptides or polyamines (8). Alkaline materials can neutralize acidic functional groups and render the coal soluble. In the present study, biochemical activity was only observed after the agar or broth pH increased above pH ~7. However, elevated pH was not sufficient for observation of peak biochemical activity because the most active broths appeared for various periods after pH had increased to a plateau. The protein content of the broths increases with growth time but with a pattern different from that for the increase in pH. Heating of the active broths decreased activity, as would be expected if an agent of biosolubilization was protein subject to loss of activity through denaturation. However, the operation of the agent at elevated pH, and the appearance of bioactivity before nutrient nitrogen starvation, are facts inconsistent with ligninases, such as described by Tien and Kirk (9), being responsible agents. Apparently the elevated pH is a condition that enables peak biosolubilization to be observed; this activity is apparently due to heat-sensitive proteins that may act on leonardite within 15 minutes to bring about solubilization in a standard activity test.

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Table 1. Elemental Composition (mg) of Phanerochaete chrysosporium at 6-Days Old, and Decrease of elements in Sabouraud Maltose Agar Growth Medium

	<u>Fungus</u>	<u>Elements in agar, day 0</u>	<u>Elements in agar, day 6</u>	<u>Decrease of elements in agar</u>
C	248 mg	1150 mg	854 mg	296 mg
H	36	102	102	0
O	162	749	695	54
N	28	63	46	17
S	3	12	11	1
P	5	<=1	1	0
Dry Weight	482 mg	2077 mg	1709 mg	

Table 2. Effect of Particle Size on Solubilization of Leonardite by Filtrates from SMB Cultures of Phanerochaete chrysosporium.

<u>Mesh Size</u>	<u>A₄₅₀</u>
60 - 100	0.834
100 - 200	1.110
200 - 300	1.122
<100	1.154

Table 3. Effects of Shake Time on Rate of Solubilization (A_{450}) of Leonardite per 15 min and 1 hr by Fungus-Treated Broths.

pH	Absorbance (A_{450})		Species
	15 min	60 min	
7.9	1.80	2.40	TV
7.4	4.00	4.60	PC
7.4	5.10	5.10	TV
5.9	0.25	0.37	TV

Note: TV = Trametes versicolor
 PC = Phanerochaete chrysosporium

Table 4. Effect of Leonardite Concentration on Solubilization with P. chrysosporium Culture at Initial pH 7.9 and Initial $A_{450} = 1.3$.

Concentration % (wt/vol)	A_{450}	% Solubilization	Final pH
0.3	4.0	43	7.9
1.0	5.1	11	7.5
3.0	3.9	2	6.2
10.0	1.3	1	4.6

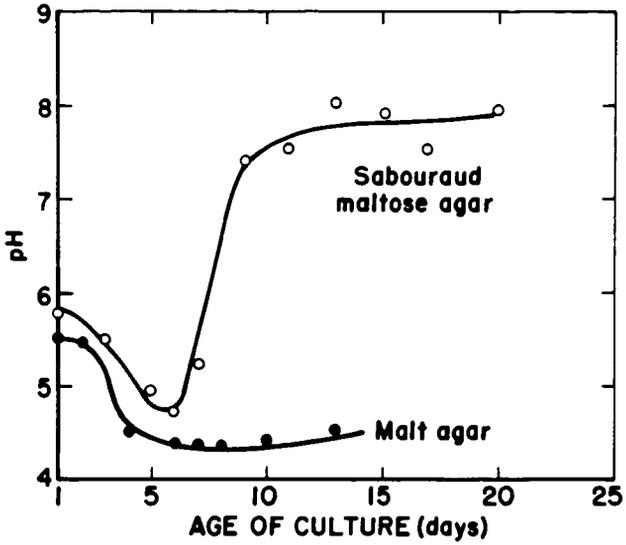


Figure 1. Change in pH of agar associated with growth *P. chrysosporium*.

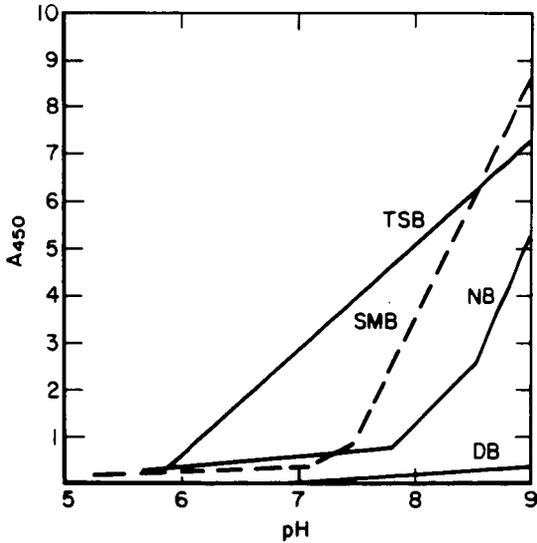


Figure 2. Solubilization of 0.3% leonardite after shaking for one hour with fresh broths.

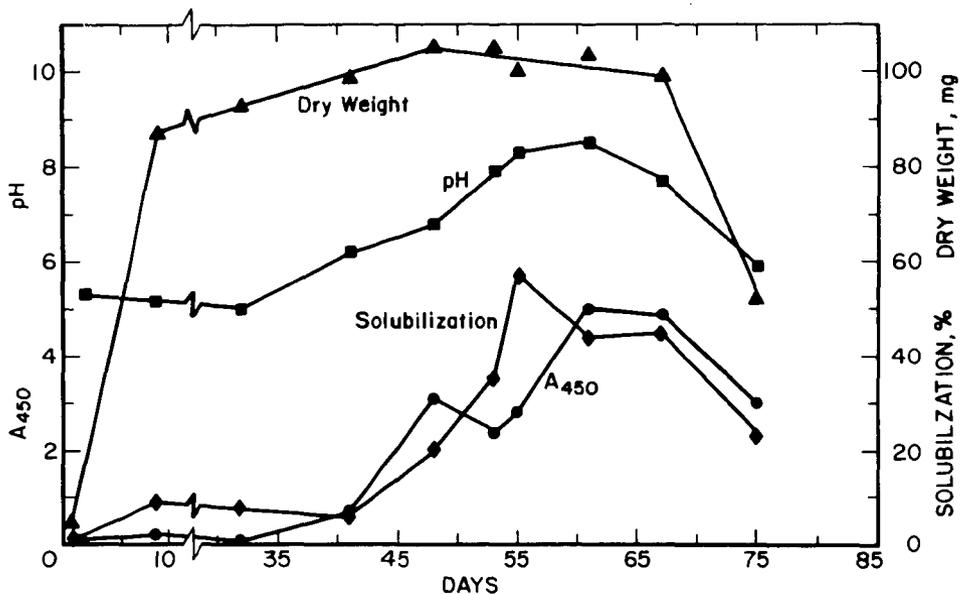


Figure 3. Relationship of dry weight, pH, percent solubilization and A450 from solubilization of leonardite by filtrates from SMB cultures of *P. chrysosporium*.

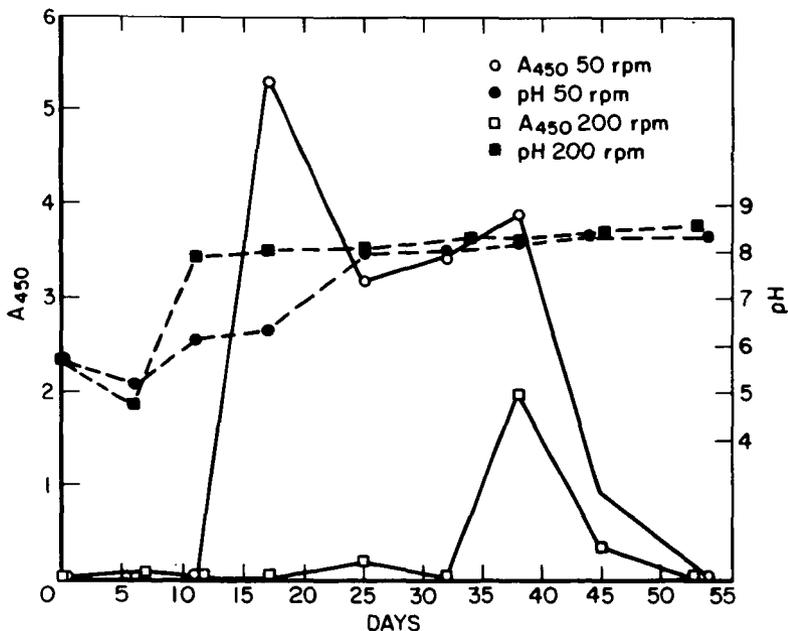


Figure 4. Solubilization of leonardite by filtrates from SMB cultures of *P. chrysosporium* at 50 rpm and 200 rpm.