

The Biological Degradation of Lignin¹

IV. The Inability of *Polyporus Versicolor* to Metabolize Sodium Lignosulfonate

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INTRODUCTION

Studies made in the field of microbiological degradation of lignin have included investigations with sodium and calcium lignosulfonates.

Kazanskii and Mikhailova (1936) were unable to grow several wood-rotting fungi on neutralized waste sulfite liquor, but found that infusorial earth mixed cultures could grow albeit slowly (in 50–60 days) if several alterations were made in the composition of the waste sulfite liquor.

Ledingham and Adams (1942) investigated *Polyporus versicolor*, other wood-rotting fungi, and several soil fungi for their ability to metabolize calcium lignosulfonate in a glucose-mineral salts medium. They reported substantial degradation of the calcium lignosulfonate when a beta-naphthylamine precipitation method of analysis was used, but much less evidence of degradation was found where an ultraviolet method was used (Adams and Ledingham, 1942). Their conclusions were based on the disappearance of the lignosulfonate from liquid cultures in which the fungi were growing.

In previous papers it was reported by Pelczar *et al* (1950) and Gottlieb *et al* (1950), that the wood-rotting (white rot) fungus *Polyporus versicolor* could utilize native lignin to a limited degree as a sole source of carbon.

It was the purpose of this investigation to determine whether this organism could utilize sodium lignosulfonate. Using a system of analyses for both mycelial substance and culture fluid it has been shown that although the lignosulfonate disappears from solution it can be accounted for in or on the mycelia.

METHODS AND MATERIALS

Cultures

The wood destroying (white rot) fungus *Polyporus versicolor* was the same culture employed in previous investigations (Pelczar *et al* 1950 and Gottlieb *et al* 1950). Stock cultures of this organism were maintained on potato dextrose agar, subcultured at monthly intervals and stored at 10 C during the interim.

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Preparation of Sodium Lignosulfonate

Sodium lignosulfonate was prepared from stripped sulphite waste liquor obtained from the Rhinelander Paper Company, Rhinelander, Wisconsin. Twelve L of this liquor were filtered and then saturated with sodium chloride. The precipitate of impure sodium lignosulfonate was centrifuged down and thoroughly washed with a saturated solution of sodium chloride. The washed precipitate was redissolved in water, precipitated with sodium chloride and washed. The twice precipitated sodium lignosulfonate was suspended in 1 L of water and dialyzed against running tap water until no chloride ion could be detected in the dialyzing solution. Finally the solution was dialyzed against distilled water. The dialyzed solution was evaporated to near dryness at reduced pressure and absolute alcohol was used to take out the remainder of the water azeotropically. The anhydrous material was ball milled with absolute alcohol for 1.2 hr, and finally centrifuged and dried. The yield was 250 g of a light cream-colored product. The analysis was as follows: methoxyl, 13.05 per cent; sulfur, 5.28 per cent; and ash, 8.30 per cent.

Preparation of Test Media

The substrate sodium lignosulfonate was very soluble in water and was added in 0.5 per cent concentration to the following basal medium: (NH₄)₂HPO₄, 5.0 g; KCl, 1.0 g; MgSO₄·7H₂O, 0.5 g; thiamine, 1000 μg; FeSO₄, 0.02 g; and distilled water to make 1 L of medium. In most of the experiments 2 per cent glucose was also added as an additional carbon source to the above medium. After adjusting the medium to the desired pH, it was pipetted into 250 ml Erlenmeyer flasks. These flasks were then capped with aluminum foil and the contents sterilized with ethylene oxide according to the method described by Wilson and Bruno (1950). The final concentration of sodium lignosulfonate in each test flask was 200 mg and the methoxyl percentage was 13.

Preparation of Inoculums

Inoculum consistent as to dry weight and metabolic activity was prepared in the following manner: Five 250 ml Erlenmeyer flasks containing 40 ml of basal

medium plus 2 per cent glucose were inoculated with a portion of mycelial mat approximately 0.5 cm square. These flasks were then incubated for seven days in still-culture at 28 C, at which time the entire liquid surface was covered with a mycelial mat. These mats were removed aseptically and washed with sterile distilled water and transferred to a sterile Monel metal jar (semi-micro size) together with 50 ml of sterile distilled water. The mycelia and water were blended for 2 min and transferred to a sterile flask. One ml of this fresh inoculum was added to each culture flask (approximately 1 mg dry weight).

In the latter part of this study the design of experiments required a large amount of inoculum for each culture flask. These inoculums were designated as (1) normal, (2) starved and (3) killed and prepared as described below.

Normal inoculum. Culture flasks (containing 40 ml of basal medium plus 2 per cent glucose) were each inoculated with 1 ml of ground mycelia as prepared above. These flasks were then cultivated under shake culture conditions for 12 days at 28 C. The contents of the flask were harvested aseptically and washed with sterile distilled water and then transferred to test media. The dry weight of mycelia in each flask was approximately 250 mg.

Starved inoculum. This inoculum was prepared in the same way as the "normal inoculum" except that after the first incubation period, the inoculum was washed and reinoculated into flasks of fresh basal medium minus glucose. These flasks were shaken for 7 days at 28 C after which the mycelia were washed and inoculated into test media.

Killed inoculum. This inoculum was also prepared in the same manner as the "normal inoculum" except for the second incubation period the inoculum was transferred to flasks of basal media containing 5 ppm of Copper-8-Quinolinolate, a known fungicidal compound. These flasks were then incubated as shake-culture flasks at 28 C for 7 days, after which the mycelia were transferred to test media. The mycelia, thus prepared, failed to grow on potato dextrose agar.

Incubation of Cultures

Liquid media for all experiments were dispensed in 250 ml flasks and placed on a shaking apparatus of a reciprocating type with a stroke of 1½ inches and 100 three-inch excursions per min at a temperature of 28 C.

Analytical Methods Employed to Determine Utilization or Disappearance of Sodium Lignosulfonate from the Test Media

The analytical methods employed were performed as follows: (1) Weighing of dried mycelia; (2) determination of methoxyl content of the media and mycelia by the Viebock-Schwappach method as described by

Clark (1932); (3) measurement of residual lignosulfonate in the culture filtrate by an ultraviolet absorption method described by Adams and Ledingham (1942); (4) determination of mycelial nitrogen by the semi-micro Kjeldahl method of Umbreit and Bond (1936), which was modified by application of the titration technique of Sobel *et al.* (1937) and (5) measurement of residual glucose in the culture filtrate as described by Somogyi (1945).

RESULTS

From initial experiments, the results not reported herein, it was found that the test organism could not utilize sodium lignosulfonate as a sole source of carbon when incorporated into the mineral-salt basal medium. This was ascertained visually as well as by quantitative determinations which indicated no change in lignosulfonate and no increase in mycelial nitrogen. Therefore, 2 per cent glucose which supports luxuriant growth of the fungus was added to the medium along with 0.5 per cent lignosulfonate. This experimental procedure was similar to that employed by Ledingham and Adams (1942).

Recordings of pH observed from preliminary experiments of the above type, revealed that a rapid drop of pH from 7 to 2.5 occurred after 4 days' incubation. This drop in pH took place in each of the above described media and remained thus throughout the 12-day incubation period. Methoxyl values obtained at the same time indicated a rapid disappearance of methoxyl from the medium, which at first seemed to indicate a rapid utilization of lignosulfonate. However, methoxyl analyses made simultaneously with the mycelia from the same culture flasks showed that the methoxyl disappearing from the medium reappeared quantitatively in or on the mycelia. The total methoxyl content consisting of methoxyl from the medium plus methoxyl from the mycelia remained essentially unchanged when compared simultaneously with the methoxyl content of the control flask, indicating probable "adsorption"² of the lignosulfonate or methoxyl by the mycelia. Figure 1 illustrates graphically this relationship and also shows that the mycelia grown in the medium containing glucose as the sole carbon source contained an insignificant amount of methoxyl. This observation (negligible methoxyl in glucose grown mycelium) suggests that the large amount of methoxyl occurring with the lignosulfonate-glucose grown mycelia did not represent a normal growth picture.

The possibility was considered that the concentration of lignosulfonate or methoxyl on the surface of the mycelium was a function of the acidity of the solution, a situation related to the tanning reaction, since cer-

² The term adsorption is employed here to indicate removal of lignosulfonate from the media by being deposited either on or in the mycelia.

tain preparations of lignosulfonate have been employed as tanning agents (Brauns, 1948). Therefore, an experiment such as recorded in table 1 was performed with the addition of 0.5 g of calcium carbonate per culture flask. This held the pH to a much narrower range than previously mentioned. From residual glucose determinations recorded in table 1, it can be seen that the glucose was completely utilized on the sixth day and that after this time a measureable increase in methoxyl

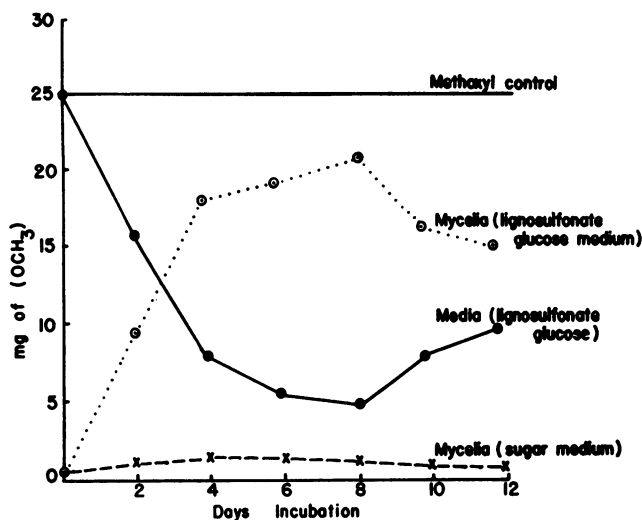


FIG. 1. Methoxyl balance for growth of *Polyporus versicolor* on lignosulfonate plus glucose.

TABLE 1. Analytical results from *Polyporus versicolor* culture grown in sodium lignosulfonate-glucose medium plus calcium carbonate

GROWTH PERIOD	MEDIA						
	Basal + Sodium lignosulfonate (0.5%) and glucose (2%)						Basal + Glucose (2%)
	pH	Residual Glucose	Methoxyl (OCH ₃) Content				(OCH ₃) Content
			Media	Mycelia	Media + Mycelia	Control (Uninoculated)	
days		mg	mg	mg	mg	mg	mg
0	6.25	800.0	24.40	00.00	24.40	24.40	0.00
2	4.90	365.0	15.00	10.76	25.76	25.85	1.66
6	7.20	0.0	7.60	17.89	25.49	25.10	2.49
12	7.71	0.0	8.50	15.75	24.20	24.00	1.10

content of the mycelia could not be demonstrated. From these results it would seem that the apparent adsorption of lignosulfonate or methoxyl was not necessarily a function of pH *per se*.

Mycelial nitrogen and dry weight determinations plus ultraviolet absorption results are recorded in table 2.

Comparison of mycelial nitrogen values revealed insignificant differences in amount of nitrogen produced between the two media for the same incubation period. However, the nitrogen values for both media showed a

tendency to decrease after the sixth day, which might be attributed to autolysis of the mycelia. The dry weight values obtained from the lignosulfonate-glucose medium were consistently higher than the values from the glucose medium on any one day. Upon closer examination of these values it can be seen that the mycelial yield from the lignosulfonate-glucose medium varied from the glucose yield only in an amount approximately equal to the lignosulfonate removed simultaneously from the test medium. From the nature of this observation plus the fact that the nitrogen values were approximately equal, it was apparent that the increase in mycelial yield from the lignosulfonate-glucose media was due to the presence of lignosulfonate on the mycelia.

TABLE 2. Results of mycelial nitrogen and dry weight determinations and their relationship to lignosulfonate disappearance from solution*

GROWTH PERIOD	MEDIA				
	Basal medium plus glucose (2%)		Basal medium plus ligno-S (0.5%) and glucose (2%)		
	Mycelial N	Mycelial Wt	Ligno-S† Removed	Mycelial Wt	Mycelial N
days	mg	mg	mg	mg	mg
0	.045	1.0	00.0	1.0	.045
2	8.80	175.0	65.0	243.5	8.90
4	15.30	305.8	123.1	435.9	16.80
6	18.10	360.5	142.5	521.0	20.60
8	17.80	355.5	143.8	510.0	20.40
10	15.70	305.4	125.5	430.0	18.50
12	12.80	275.6	110.5	390.1	14.90

* All analytical values were based on the entire contents of the culture flask.

† Lignosulfonate determined by ultraviolet absorption method.

To examine further the effect of pH on lignosulfonate disappearance from the culture media an experiment was conducted at three pH levels and simultaneously the effect of the addition or subtraction of glucose from the test medium was examined. In this experiment a large amount of mycelia was used (250 mg) as inoculum. This inoculum is described under Methods and Materials as "normal inoculum." The mycelial inoculum was transferred to two different test media, one containing lignosulfonate and glucose as carbon sources and the other lignosulfonate. These media were incubated in the usual procedure for 12 days. Ultraviolet absorption and pH results of this experiment are recorded in table 3. It can be seen that when two carbon sources were present in the medium approximately twice as much lignosulfonate was removed or "adsorbed" from the media as when lignosulfonate was present alone. It can also be noted that pH did not have an appreciable effect on the "adsorption" phenomena. These results revealed that the "adsorption" process definitely proceeded at a more efficient rate when a readily available carbon source was present.

The mycelia under these conditions were actively metabolizing and it appeared that the amount of "adsorption" depended on the metabolic state of the mycelial cells. In order to prove this assumption, an experiment was set up in which normal, starved and killed inoculums were employed. The amount of mycelial inoculum per culture flask was the same as used in a previous experiment (table 3). The three different types were inoculated into the same media as shown in table 3 and under the same conditions, except that the initial pH of all media was 7.0.

The results of this experiment are recorded in table 4. It can be seen that the starved mycelia "adsorbed" as

TABLE 3. Effect of different pH levels and glucose on lignosulfonate disappearance from solution

INITIAL pH	MEDIA			
	Basal Medium plus Ligno-S (0.5%) & glucose (2%)		Basal Medium plus Ligno-S (0.5%)	
	Per Cent Ligno-S Removed*	Final pH	Per Cent Ligno-S Removed	Final pH
7.20	55.0	2.20	25.0	7.00
3.00	54.5	2.00	24.0	3.00
1.50	52.0	1.60	24.8	1.68

* Analyses made after twelve days incubation by the ultraviolet absorption method.

TABLE 4. Effect of the condition of mycelium on lignosulfonate disappearance from solution

TYPE OF INOCULUMS	MEDIA			
	Basal Medium plus Ligno-S (0.5%) & Glucose (2%)		Basal Medium plus Ligno-S (0.5%)	
	Per Cent Ligno-S Removed*	Final pH	Per Cent Ligno-S Removed	Final pH
Normal.....	54.2	2.50	24.5	6.50
Starved.....	50.1	2.80	20.0	6.60
Killed.....	3.5	6.75	3.0	6.85

* Analyses made after twelve days incubation by the ultraviolet absorption method.

much lignosulfonate as the normal in either medium. The killed cells, for practical purposes, did not remove any lignosulfonate in either media. These results seem to indicate that the "adsorption" of sodium lignosulfonate from the media was not a mechanical process, but was a reaction directly connected with actively metabolizing mycelial cells. It would seem, therefore, that in the experiments previously described, lignosulfonate was not metabolized in the sense of a degradation of the molecule but was either "adsorbed" or incorporated into the mycelium in an intact form. In this study it may be said from comparative results not shown here, that the amount of methoxyl in solution compared with ultraviolet data on lignosulfonate removal was practically the same. It is believed by the authors that

these two methods should be used to examine both the medium and mycelial yield in order to obtain valid analyses, when using soluble lignosulfonate substrates as carbon sources.

SUMMARY

The wood-rotting fungus *Polyporus versicolor* was tested for its ability to utilize sodium lignosulfonate either as a sole source of carbon or combined with glucose in a mineral salt basal medium. It was found by application of dry weight measurements, mycelial nitrogen determinations, methoxyl determinations, ultraviolet absorption recordings and glucose determinations that this fungus apparently did not utilize lignosulfonate as a sole carbon source. However, this fungus did remove the lignosulfonate from solution. Proof of this was revealed by methoxyl and ultraviolet absorption results which showed the lignosulfonate to be present in or on the mycelia.

It was also noted that glucose enhanced the disappearance of lignosulfonate from solution and that viable cells were required for this phenomenon to occur.

The analytical procedures employed in this study presented a rational approach to the evaluation of microbial attack on lignin and related substances.

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