

**A QUANTITATIVE STUDY OF THE ABILITY OF WOOD-ROTTING FUNGI
TO UTILIZE NATIVE LIGNIN AND SODIUM LIGNOSULPHONATE**

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I

INTRODUCTION

During the past decade numerous reports pertaining to the action of bacteria, fungi and enzymes on isolated lignin have been published. Some of these data have undoubtedly advanced our knowledge of the microbial degradation of lignin. However, due to the use of chemically distorted substrates, unidentified mixed cultures and questionable analytical techniques, the majority of experimental data reported in the past have been difficult to reproduce and comprehend.

Even though the above criticisms may apply in a small degree to work done on in situ lignin, ample and adequate evidence has been presented to establish the fact that in situ lignin can be degraded by microorganisms, chief among these being the wood-rotting (white-rot) fungi, belonging to the Basidiomycete group.

The main difficulty incurred in the past with investigations involving lignin degradation, has been the unavailability of an unaltered substrate of consistent purity, which could be employed for accurate quantitative study. Recent investigations, however, were performed on the fungal utilization of a lignin isolated by mild solvents (native lignin). These studies have made the quantitative study of lignin degradation a more feasible one, under laboratory conditions. Therefore, further investigation was undertaken to study

quantitatively the ability of specially selected species of white-rot fungi to utilize the substrates native lignin and sodium lignosulphonate when incorporated into synthetic liquid media as carbon sources.

II

HISTORICAL

A survey of the literature relative to the microbial utilization of lignin has revealed several excellent reviews on this subject, namely, those of Phillips (1934), Norman (1936), Waksman (1944) and Gottlieb and Pelczar (1951). However, since the work reported herein was mainly concerned with the quantitative study of the microbial degradation of isolated lignins, only those reports which appeared pertinent to this subject are discussed.

Since the evidence to date indicates that bacteria play the lesser role in the degradation of isolated lignins, they shall be discussed first.

Pringsheim and Fuchs (1923) reported that part of the alkali lignin exposed to mixed cultures of bacteria from garden soil was rendered alcohol-soluble and the alcohol-soluble portions had a lower methoxyl than the original material. In a study of the lignin materials in sulphite waste liquors discharged from pulp mills Benson and Partansky (1934) reported that these materials were slowly fermented by mixed cultures of bacteria from sea water and marine mud. Experiments performed by Boruff and Buswell (1934) indicated no fermentation of alkali lignin with mixed cultures of anaerobic gas-forming bacteria. They also reported the bacteriostatic effect of Willstatter hydrochloric acid lignin. Unsuccessful

attempts were made by Levine et al., (1935) in developing a specific lignin-decomposing flora to utilize alkali lignin. Unidentified species of bacteria were reported by Waksman and Hutchings (1936) to be able to make slow growth on phenol lignin (isolated from wood by action of phenol and hydrochloric acid). The conversion of lignin to mycelium and CO₂ was described as practically quantitative. Other types of isolated lignin used did not support growth. A few studies made by Berl and Koerber (1938) with hydrochloric acid lignin indicated that aerobic and anaerobic species of the cellulose fermenter Amylobacter navicula were not able to utilize this type of substrate. Zobell and Stadler (1940) reported that mixed cultures of bacteria from seven American lakes were able to utilize between 4 to 15 per cent sulfuric acid lignin after 30 days incubation. Measurement of utilization was based on oxygen consumption and decrease in lignin content. This same observation was reported on alkali lignin. It has also been reported by Erickson (1941) that most of the strains of Micromonospora isolated from lake mud could attack lignin isolated from spruce wood. Recent studies by Konetzka et al., (1952) revealed that a species of Flavobacterium isolated from river mud could utilize α -conidendrin (related structurally to lignin) as a sole source of carbon in a mineral-salt basal medium. They reported that this organism could utilize approximately 200 mg of α -conidendrin during a 12-day incubation period.

The main criticisms which can be directed at the majority

of the above-mentioned works were, that primarily the type of lignins used were of questionable purity and secondarily, the organisms in most cases were unidentified and were used as mixed cultures for inoculum.

Fungi, in light of the claims made in the literature, have undoubtedly played the major role in the field of microbial degradation of lignin.

Smith and Brown (1935) were unable to demonstrate decomposition of sulfuric acid lignin (mixed with sterile sand, soil or liquid cultures) by the fungi Trichoderma lignorum, Aspergillus terreus or Penicillium vinaceum. However, these same investigators reported that Stereum purpureum was able to attack the sulfuric acid lignin after it had been oxidized with H_2O_2 . Species of soil fungi of the genera Alternaria and Fusarium were reported by Waksman and Hutchings (1936) to slowly utilize phenol lignin.

Garren (1938) was able to grow the white-rot fungus Polyporus abietinus on mineral-salt agar containing 2.5 per cent sulfuric acid lignin as the sole source of carbon. He reported that the fungus produced a very sparse giant colony (23 mm in diameter) after 30 days incubation.

Several studies have also been reported, which were concerned mainly with the utilization of the isolated lignin portion of sulphite waste liquor, i.e. lignosulphonic acid and calcium lignosulphonate as indicated below.

Kazanskii and Mikhailova (1936) were unable to grow the fungi Fomes pini, Fomes annosus and Stereum hirsutum on

neutralized sulphite waste lignin but found that infusional earth-mixed cultures would grow albeit slowly (in 50-60 days) when minor alterations were made in the composition of the sulphite liquor. The results reported from this experiment indicated that part of the alcohol-insoluble sulphonic acids were transformed into alcohol-soluble ones, which might be interpreted as partial desulphonation.

Working with the wood-staining fungus Endoconidiophora adiposa Adams and Ledingham (1942a) found that this organism could utilize 10 per cent of the lignosulphonic acids in sulphite waste liquor after 20 days incubation. These same investigators (1942b) inoculated calcium lignosulphonate (mineral salts and 2 per cent glucose) separately with Polyporus versicolor and other wood-rotting fungi, plus several soil fungi. They reported utilization to range as high as 23 per cent for some of these organisms when a beta-naphthylamine precipitation method of analysis was employed. However, approximately 7 per cent utilization was reported later (1942c) when an ultraviolet method was employed.

All of the studies mentioned heretofore have dealt with lignin isolated by standard methods, namely, treatment with sulfuric and hydrochloric acids, sodium hydroxide, alkaline sulphite and phenol. According to Gottlieb and Pelczar (1951) lignin prepared by the above methods should not be used as a substrate representing the natural material in nutritional studies.

Recent studies reported by Day et al., (1949) were performed with native lignin, extracted as proposed by Brauns

(1939) from spruce wood with the inert solvents ethyl alcohol and dioxane. These studies indicated that the white-rot fungi Poria subacida and Polyporus abietinus could be adapted to the utilization of native lignin as a sole energy source, after glucose had been removed completely from the culture medium. In a more detailed study by Gottlieb et al., (1950) it was found that the above-mentioned fungi had become simultaneously adapted to the utilization of nearly all of 16 lignin materials isolated by various laboratory and commercial techniques. It was later reported by Pelczar et al., (1950) that several strains of the white-rot fungus Polyporus versicolor made fair growth in liquid medium containing native lignin as the sole source of carbon without the necessity of prior adaptation.

There have been numerous reports made in the past concerning the ability of the fungus Polyporus versicolor, to utilize in situ lignin, to elaborate lignin degrading enzymes, and to utilize phenolic compounds, supposedly similar to lignin in structure.

In a study of the enzymes elaborated by Polyporus versicolor Bayliss (1908) found with the aid of the Weisner reagent (phloroglucinol and hydrochloric acid) that wood was entirely devoid of lignin after being exposed to enzymes extracted from the mycelia of this fungus. Smith (1924) made a "proximate analysis" of apple wood rotted by Polyporus versicolor and indicated that the lignin portion was not attacked. Using the same organism Campbell (1930) reported that this fungus

could decompose 35 per cent of the lignin in beechwood after 10 weeks incubation. He also reported that pentoses were removed first, then lignin was attacked and finally in the presence of increased acidity, cellulose. In an extensive study of Polyporus versicolor on red gum sapwood Scheffer (1936) found lignin to be utilized but the relative proportion of the principal components were not materially altered. Lindeberg (1946) reported that this same fungus could utilize 73.7 per cent of lignin as found in beech leaves.

Bose and Sarkar (1937), La Fuze (1937), Fahraeus et al., (1949) and Law (1950) have all reported the ability of this organism to elaborate phenolic oxidases as well as to utilize phenolic-like compounds such as guaiacol, resorcinol and pyrogallol, which are similar in structure to the proposed phenylpropane building unit of native lignin (Brauns, 1951). Cartwright and Findlay (1943), in a comprehensive study of fungi which caused timber decay, demonstrated that the fungus Polyporus versicolor had a large host range and could grow on almost any variety of timber.

In view of the afore-mentioned studies plus preliminary work done in this laboratory with the same fungus an investigation was undertaken to quantitatively study the ability of Polyporus versicolor to utilize isolated lignin substrates such as native lignin and sodium lignosulphonate.

III

MATERIALS AND METHODS

CULTURES.

The wood-destroying (white-rot) fungus Polyporus versicolor employed in the majority of experiments in this investigation was the same as that employed in previous investigations by Pelczar et al., (1950) and Gottlieb et al., (1950). Other cultures of white-rot fungi tested in only a few experiments, were the same as those employed by Day et al., (1949) and Gottlieb et al., (1950). These cultures were as follows: Polyporus abietinus, Poria subacida, Ustulina vulgaris, Fomes geotropus and Daedala unicolor.

Stock cultures of all the above organisms were maintained on potato dextrose agar by subculturing at monthly intervals and storing at 10 C during the interim.

SOURCE AND PREPARATION OF LIGNIN SUBSTRATES.

A. Preparation of native lignin. The native lignin employed throughout this study was isolated and prepared as previously described by Day et al., (1949) and Brauns (1939). This product contained 63.8 per cent carbon, 14.8 per cent methoxyl, and 6.2 per cent hydrogen and was soluble in dioxane, alcohol and methyl cellusolve but insoluble in diethyl ether and water.

B. Preparation of sodium lignosulphonate. The sodium

lignosulphonate used in the latter part of this study was isolated by Dr. Sidney Gottlieb of the Department of Chemistry, University of Maryland. This product was prepared from stripped sulphite waste liquor obtained from the Rhineland Paper Company, Rhineland, Wisconsin. Twelve L of this liquor were filtered and then saturated with sodium chloride. The precipitate of impure sodium lignosulphonate 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 lignosulphonate 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 then evaporated to near dryness at reduced pressure and absolute ethyl alcohol was used to take out the remainder of the water azeotropically. The anhydrous material was ball-milled with absolute ethyl 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. This product was highly soluble in water.

STERILIZATION OF LIGNIN SUBSTRATES.

A. Sterilization of native lignin. Because of thermo-plastic changes which occurred during the autoclaving of native lignin, it was decided that this method of sterilization was undesirable. It was found after experimentation with various

solvents such as chloroform, dioxane, alcohol and diethyl ether, that ether was the most satisfactory sterilization agent. Thus for the majority of experiments reported herein, native lignin was sterilized with diethyl ether by the following method. Fifty ml portions of freshly distilled anhydrous diethyl ether were added to 250 ml Erlenmeyer flasks containing either 100 or 200 mg of lignin. The flasks were capped with aluminum foil and placed at 10 C for 24 hr. The flasks were then placed under a drying hood and the ether allowed to evaporate at room temperature over a period of 3 days. Sterile basal media was then added to the ether-sterilized lignin in the flasks.

Native lignin used in a few of the final experiments was sterilized with ethylene oxide. One ml of ethylene oxide was added to each 250 ml test flask containing lignin and 40 ml of basal medium. These flasks were incubated at 10 C for 2 hr and then placed under a drying hood, where the ethylene oxide was allowed to evaporate at room temperature over a period of 48 hr. After this procedure the flasks were ready for inoculation. This procedure for sterilization of materials with ethylene oxide has been described by Wilson and Bruno (1950).

B. Sterilization of sodium lignosulphonate. All sodium lignosulphonate medium was sterilized by the ethylene oxide method described above.

PREPARATION OF TEST MEDIA.

A. Preparation of media for native lignin experiments.

The basal medium employed initially in native lignin studies

was a slight modification of that used by Perlman (1949) in his study of Polyporus anceps in submerged culture. This modified medium had the following composition: NH_4NO_3 , 2.5 g; KH_2PO_4 , 1.5 g; K_2HPO_4 , 1.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mg; thiamine, 1000 μg and enough glass-distilled water to make 1 L of medium. This medium was adjusted to pH 5.5 with a Beckman pH meter. When native lignin was sterilized with ether, the above-mentioned medium was pipetted in 40 ml amounts into 250 Erlenmeyer flasks, capped with aluminum foil, autoclaved at 15 lbs pressure (121 C) for 15 min and then aseptically added to the ether-sterilized lignin flasks. These flasks contained enough lignin to make a final concentration, depending on the experiment, of either 0.25 or 0.5 per cent. When ethylene oxide was employed as the sterilization agent, autoclaving of the basal medium was abandoned, and the basal medium plus lignin were added to flasks which were subsequently treated with ethylene oxide.

In some of the experiments, the original basal medium was altered by either substituting different inorganic and/or organic nitrogen sources or supplemented by adding materials such as spawn extract, wood extract, minerals, glucose and vitamins. All of these factors were sterilized separately by filtering through a sterile bacteriological sintered glass filter and then added aseptically to the sterilized basal media according to the concentration desired.

Spawn extract was obtained by extracting an intimate mixture of horse manure and mycelia of the commercial mushroom Agaricus campestris. This extract was prepared by the method

described by Gottlieb and Geller (1949).

Wood extract was prepared by Dr. Sidney Gottlieb, Department of Chemistry, University of Maryland, and was obtained by extracting spruce sawdust with boiling water.

The mineral supplement mixture was a slight modification of Hoagland's A to Z mixture as employed by Robbins and Kavanagh (1938). This mineral supplement consisted of LiCl, 0.5 g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1 g; FeSO_4 , 1 g; H_3BO_3 , 11 g; $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$, 1 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 7.0 g; KI, 0.5 g; and NaBr, 0.5 g. The supplement was prepared according to the procedure employed by Day et al., (1949).

A vitamin mixture was prepared in which approximately 10 mg of each compound was added to 100 ml of distilled water and then sterilized by filtration. This mixture consisted of the following compounds: inositol, biotin, pyridoxine, pyridoxamine, pyridoxal, thiamine, indol-3-acetic acid, folic acid, Ca-pantothenate, pimelic acid, riboflavin, niacin, B-alanine, nucleic acid, adenylic acid, nicotinamide, cozymase, para-amino-benzoic acid, glutathione, adenine, adenine triphosphate and crystalline vitamin A (cytoline).

All vitamin mixtures, minerals, and extracts were added aseptically in 1 ml amounts to each flask of test media and controls were ran simultaneously.

Buffered media were made by adding the constituents of the basal medium to the buffer mixture and diluted to 1 L volume with glass-distilled water. The buffer mixtures,

according to the pH desired, were prepared as listed below.

pH 1.5	26.3 ml of N HCl + 50 ml N KCl
pH 3.0	20.3 ml of N HCl + 50 ml M $\text{KHC}_8\text{H}_4\text{O}_4$
pH 4.5	12.2 ml of N NaOH + 50 ml M $\text{KHC}_8\text{H}_4\text{O}_4$
pH 6.0	5.7 ml of N NaOH + 50 ml M KH_2PO_4
pH 7.5	42.8 ml of N NaOH + 50 ml M KH_2PO_4
pH 9.0	21.3 ml of N NaOH + 50 ml of M H_3BO_3

Where phosphates were employed as buffers these compounds were eliminated from the basal medium.

Baker's C.P. analytical grade reagents were employed throughout this study. Pyrex glassware was used in all experiments and was rinsed 3 times with glass-distilled water after washing.

B. Preparation of media for sodium lignosulphonate experiments. The basal medium employed in the majority of sodium lignosulphonate studies was made by adding this water-soluble substrate in 0.5 per cent concentration to the following constituents of the basal medium: $(\text{NH}_4)_2\text{HPO}_4$, 5 g; KCl, 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; thiamine, 1000 μg ; FeSO_4 , 0.02 g; and enough glass-distilled water to make 1 L. This medium was adjusted to the desired pH with a Beckman pH meter and was then pipetted into 250 ml Erlenmeyer flasks and capped with aluminum foil. When glucose or substituted nitrogen sources were employed they were filtered through separate sterile bacteriological sintered glass filters and aseptically transferred to test media. All other components of the basal media were sterilized with ethylene oxide, except when CaCO_3 was

employed. This compound was sterilized separately in the autoclave in 250 ml flasks and the sterile test media added aseptically to them.

The final concentration of sodium lignosulphonate in each test flask was 200 mg (0.5 per cent) and the methoxyl percentage was 13.0.

PREPARATION OF INOCULA.

A. Preparation of inocula for native lignin experiments.

The inocula employed in native lignin experiments, consistent as to dry weight and metabolic activity was prepared in the following manner. Five 250 ml Erlenmeyer flasks, each containing 40 ml of basal medium plus 0.5 per cent glucose, were inoculated separately either with portions of mycelial mat approximately 0.5 cm sq taken from potato dextrose agar, or 1 ml of homogenized mycelium prepared from a mycelial mat. These inoculated flasks were incubated at 28 C for 7 days under still-culture conditions. After this time the entire liquid surface of the medium in the flasks was covered with a mycelial mat approximately 5 mm thick. These mats were removed aseptically and washed with sterile glass-distilled water and aseptically transferred to a sterile Monel metal Waring blender jar (semi-micro capacity) together with 50 ml of sterile glass-distilled water. The mycelia and water were then blended for 2 min in the Waring blender. One ml of this fresh inoculum was added to each test flask and consisted of approximately 0.5 mg of mycelia by dry weight. This quantity of inoculum was decided on by results obtained

from a preliminary experiment to determine the viability and metabolic activity of mycelia, when inoculated in varying amounts, at different ages. The results of this experiment are recorded in table 1.

In experiments where the term "rehomogenized mycelia" is employed, 1 ml of inoculum was incubated in test media under shake-culture conditions for 5 days. After this time the entire contents of the test flask were removed aseptically, homogenized and reinoculated into the same test flask. Where the term "rehomogenized inoculum" was used, 1 ml of inoculum was allowed to incubate for 12 days in test medium. The same treatment as described above was then employed except that only 1 ml of the rehomogenized mycelial yield was reinoculated into separate flasks of fresh test media. These flasks contained either fresh lignin or lignin which had previously been exposed to fungal attack for a period of 12 days.

B. Preparation of inocula for sodium lignosulphonate experiments. Most of the inocula employed in sodium lignosulphonate experiments were prepared the same as described previously for native lignin experiments.

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

(1) Normal inoculum. Culture flasks (containing 40 ml

TABLE 1

Results of experiments to demonstrate the effect of age and size of inoculum on the growth of Polyporus versicolor in a synthetic medium

Age of inoculum (hr)	Amount of inoculum added to test flask ¹			
	1 ml ²	2 ml	3 ml	4 ml
1	65.0 ³	63.5	60.2	55.4
24	64.8	64.0	61.5	56.0
48	56.4	62.8	55.5	52.1
72	57.0	56.4	56.0	53.0
96	51.2	49.6	50.1	45.8
120	53.0	45.4	46.5	40.4
144	48.5	44.5	46.0	39.7

¹Inoculum stored at 10 C between inoculations.

²Average dry weight of inoculum was 0.5 mg per ml, and grown in basal medium as described under Materials and Methods plus 0.5 per cent glucose as a carbon source.

³Numbers refer to mycelial dry wt in mg harvested after 14 days incubation and are an average of 3 test flasks.

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

(2) 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.

(3) 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 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.

All culture flasks throughout the testing period of this investigation were incubated at 28 C. All experiments were performed with liquid media dispensed in 40 ml quantities in 250 ml Erlenmeyer flasks capped with aluminum foil. These flasks were all incubated by shake-culture technique. The

shaking apparatus was of a reciprocating type and was adjusted to a stroke of 1.5 in and made 100 complete excursions per min.

ANALYTICAL PROCEDURES EMPLOYED TO DETERMINE UTILIZATION OR DISAPPEARANCE OF LIGNIN SUBSTRATES FROM THE TEST MEDIA.

A. Examination of cultures grown in native lignin media.

The routine experimental procedure employed with native lignin was performed in the following manner. After a prescribed incubation period, test flasks were removed from the shaking apparatus and examined visually for growth and possible contamination. The pH of the culture medium was then determined with a Beckman pH meter, after which the contents of each flask were acidified with 2 ml of 0.1N H₂SO₄. This was done in order to facilitate the precipitation and quantitative recovery of residual lignin from the culture medium. After this step the components of each flask were transferred to separate 50 ml round-bottom centrifuge tubes. These tubes were placed in an International centrifuge and centrifuged for 15 min at 5000 rpm. The supernatant portion of the test medium was filtered through Whatman no. 1 paper into 50 ml volumetric flasks, and diluted to volume with glass-distilled water. Ten ml aliquots of this liquid were set aside for future determination of soluble carbon.

The precipitate remaining in the centrifuge tubes (lignin and mycelial pellets) was then washed 3 times by centrifugation and resuspension with separate 50 ml portions of glass-distilled water. The washed precipitate was then ready for

dioxane extraction of residual lignin. This procedure was necessary due to the fact that practically all the lignin in the test flasks adhered to the mycelial pellets during shake-culture incubation. This condition caused a clearing of the medium which on visual inspection might be interpreted as complete degradation of lignin but this was not the case, since the majority of lignin removed from the mycelia pellets by dioxane extraction was apparently unchanged.

To the washed precipitate in the centrifuge tube was added 20 ml of hot dioxane (50 C). This mixture was then transferred to a Monel metal jar (semi-micro size), blended for 1 min and poured into a 125 ml flask which contained 5 g of glass beads (size 5 mm). An additional 20 ml of dioxane was added to the blender in order to remove any remaining particles of lignin or mycelia. This portion of dioxane was then added to the original 20 ml to make a volume of 40 ml. The dioxane plus beads, mycelia and lignin contained in a 125 ml flask was placed on a shaking machine for 18 hr at 28 C. After the shaking period had elapsed, the contents of the flask were transferred to a tared 50 ml centrifuge tube and centrifuged at 5000 rpm for 15 min. The supernatant fluid consisting of dioxane and lignin was transferred to a 125 ml flask, which was sealed with a rubber stopper covered with aluminum foil. In order to remove any lignin or mycelia adhering to the walls of the shake-culture flask, the walls of the flask were washed twice with 10 ml portions of dioxane. These portions were added separately to the mycelia in the

centrifuge tube which were centrifuged after each addition. This 20 ml of dioxane was then added to the 40 ml contained in the original 125 ml flask which was set aside to be examined later for residual lignin.

The extracted mycelium in the tared centrifuge tube was then dried in a vacuum oven at 40 C for 3 hr and weighed on an analytical balance.

In order to determine the efficiency of the dioxane extracting procedure, an experiment was conducted to determine the amount of methoxyl in mycelia utilizing glucose (0.25 per cent) or native lignin (0.25 per cent) as a sole carbon source. The results of this experiment are recorded in table 2. If one wishes to interpret the excess methoxyl demonstrated in the lignin mycelia as unextracted residual methoxyl, when compared with the glucose-grown mycelia, the results would show in most instances, that the lignin-grown mycelia consistently contained approximately 1 mg of methoxyl in excess of the glucose mycelia. This condition could theoretically be interpreted as an indication that the lignin mycelia contained between 6 and 7 mg of unextracted residual lignin, since 1 mg of methoxyl represents approximately 6.8 mg of lignin (native lignin contains 14.8 mg of methoxyl per 100 mg).

The correction factor of 6.8 mg was not applied to the results obtained in this study, since it was impossible to determine whether the excess methoxyl on the lignin-grown mycelia represented residual lignin or the normal methoxyl

TABLE 2

Results of experiments demonstrating the methoxyl
content of glucose and lignin-grown mycelia
of *Polyporus versicolor*¹

Incubation period (days)	Methoxyl content of extracted mycelia ²					
	Lignin-grown ³ mycelia		Glucose-grown mycelia		Excess methoxyl in lignin-grown mycelia	
	(mg)	(%)	(mg)	(%)	(mg)	(%)
3	1.26	2.52	0.28	0.57	0.98	1.95
6	1.55	3.10	0.48	0.96	1.07	2.05
9	1.74	3.48	0.72	1.44	1.02	2.04
12	2.35	4.70	1.32	2.64	1.03	2.06
15	2.34	4.68	1.28	2.56	1.06	2.12
18	1.84	3.68	0.98	1.98	0.86	1.72
21	1.70	3.40	0.78	1.56	0.92	1.84

¹ Basal medium same as described under Materials and Methods plus 0.25 per cent native lignin or 0.25 per cent glucose.

² All mycelia were extracted with dioxane.

³ Methoxyl analyses were made in duplicate on 50 mg portions of dried mycelia obtained by pooling the yields of 3 test flasks at 3-day intervals.

value of mycelia when grown in the presence of lignin as the sole carbon source of the test medium.

After the routine extracting procedure had been completed, the analytical techniques as described below were performed on mycelia, culture filtrate and residual lignin harvested from triplicate flasks of which an average was taken.

(1) Colorimetric determination of residual lignin. The dioxane-lignin solution was poured into a 100 ml graduate and enough dioxane added to make a final volume of 60 ml. This solution gave a clear amber color of varying intensity, depending on the concentration of lignin.

A standard linear graph as shown in figure 1 was obtained by adding weighed amounts of lignin (ranging from 10 to 100 mg) to 60 ml of dioxane. The intensity of amber color which developed was measured in the Klett-Summerson photoelectric colorimeter using a no.42 blue filter (400-465 m μ). The colorimeter was standardized initially with pure dioxane.

(2) Soluble carbon determination. The amount of soluble carbon remaining in the liquid culture medium after incubation was determined by the wet combustion method as described by Clark and Ogg (1942). Five ml of the original 50 ml of culture filtrate were boiled in the presence of potassium dichromate (1 gm) and 25 ml of a 3:2 mixture of concentrated sulfuric acid and 85 per cent phosphoric acid, until all material had been oxidized to CO₂ in a CO₂-free system. The CO₂ produced was drawn into an evacuated flask containing 25 ml of 0.2N Ba(OH)₂. The remaining Ba(OH)₂ was then

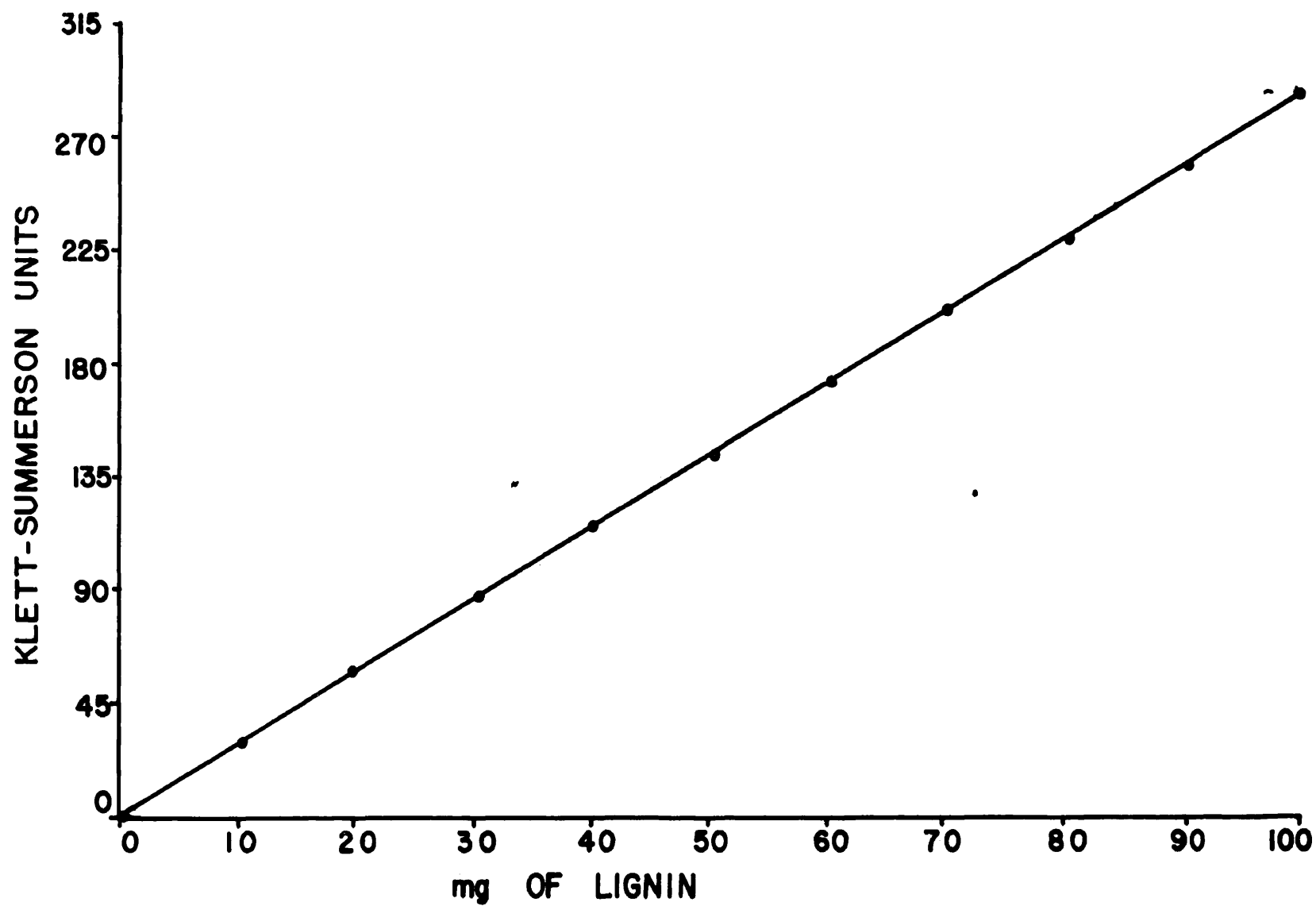


Figure 1. Relation between intensity developed and amount of lignin present in 60 ml of dioxane.

titrated with 0.2N HCl, using thymol blue as an indicator. The data from this titration was used to calculate the entire amount of soluble carbon present in the test culture filtrate. Uninoculated media were also tested as controls and were found to contain no soluble carbon. The formula used for calculation of soluble carbon was as follows:

$$\left[(\text{ml Ba(OH)}_2 \times N \text{ Ba(OH)}_2) - (\text{ml HCl} \times N \text{ HCl}) \right] \times 6 = \text{mg of Carbon}$$

(3) Mycelial nitrogen determinations. Mycelial nitrogen was determined by the semi-micro Kjeldahl method of Umbreit and Bond (1936) slightly modified to incorporate the titration technique employed by Sobel et al., (1937). The procedure employed was as follows: Either weighed samples of dried mycelia were transferred to digestion flasks or washed contents of the entire culture flask were left in centrifuge tubes to which was added 5 ml of digestion mixture (CuSO₄, 2.0 g; H₂SeO₃, 2.0 g; Na₂SO₄, 100 g; H₂SO₄, 500 ml and enough glass-distilled water to make 1 L). The mycelial samples were digested for 2 hr by heating over a bunsen burner and then transferred with the aid of 30 ml of glass-distilled water to 250 x 35 mm pyrex test tubes. In order to liberate the NH₃ gas, 10 ml of 40 per cent NaOH was added to each test tube. The test tubes were immediately stoppered and aerated thru a boric acid trap (250 x 35 mm test tube containing 20 ml of 3 per cent boric acid). Six samples and 1 control were aerated at a rate of 1500 ml of air per min, for a period of 3 hr. After aeration the boric acid was transferred to 125 ml

flasks with the aid of 20 ml of glass-distilled water. To each test tube was then added 10 drops of M.E.P. indicator (contained 1.250 g of methyl red and 0.825 g of methylene blue dissolved in 1 L of 90 per cent ethyl alcohol) which caused the aerated test boric acid to turn green and the control boric acid purple. At pH 4.1 the test boric acid was purple, pH 4.2-4.3, gray and pH 4.4, green. After addition of indicator the test flasks were back-titrated with .0185N H_2SO_4 to match the color of the control tube of boric acid. Data from this titration was then used to calculate the nitrogen content of the mycelia harvested from the test flask. Ammonium sulphate controls were also ran simultaneously to correct for experimental error. The formula employed for calculation of mycelial nitrogen was as follows:

$$N H_2SO_4 \times \text{mol wt of N} \times \text{ml } H_2SO_4 = \text{mg Nitrogen}$$

(4) Methoxyl determinations. The native lignin employed in this study consistently contained a methoxyl of 14.8 per cent under the experimental testing conditions employed. Therefore, methoxyl analyses were used to measure disappearance of native lignin from the test media.

Methoxyl determinations were carried out by the Viebock-Schwappach method as described by Clark (1932). Duplicate determinations were performed on ground mixtures of lignin and mycelia from triplicate flasks which had been previously dried in vacuum and weighed. Determinations were also made simultaneously on glucose-grown mycelia and residual media.

In this procedure the methoxyl-containing material was refluxed in the presence of hydriodic acid (5 ml) and phenol (2.5 ml) for 1 hr. During this reaction it was assumed that volatile methyl iodide was carried off under a constant stream of nitrogen and was trapped in a solution of potassium acetate and bromine. In the presence of this solution methyl iodide was converted to iodic acid. The iodic acid with the aid of distilled water was transferred to a 250 ml flask containing 5 ml of sodium acetate. The excess bromine was removed with formic acid, potassium iodide added, the solution acidified with sulfuric acid and the liberated iodine titrated with sodium thiosulfate (OCH_3 factor 0.4168) using starch as an indicator. Data from this titration was used to calculate the entire methoxyl content of the test flask. The formula used to calculate percentage of methoxyl was as follows:

$$\frac{\text{ml thiosulfate} \times \text{methoxyl factor} \times 100}{\text{Sample wt (mg)}} = \text{percent Methoxyl (OCH}_3\text{)}$$

B. Examination of cultures grown in sodium lignosulphonate media. The initial experimental routine employed with this substrate was performed as described below.

After a prescribed incubation period the pH of the liquid media in the shake-culture flasks was measured with a Beckman pH meter. The entire contents of each flask were then centrifuged in 50 ml round-bottom centrifuge tubes at 5000 rpm for 15 min. Following this, the supernatant was filtered through Whatman no.1 filter paper into 50 ml volumetric flasks and diluted to volume with glass-distilled water. The remaining

contents of the centrifuge tube were washed 3 times with separate 50 ml portions of glass-distilled water and then dried under vacuum at room temperature, until constant weight was obtained.

After the completion of the previously mentioned procedures, special analytical tests were performed as described below on mycelia, culture filtrate and residual sodium ligno-sulphonate, harvested from triplicate flasks, of which an average was taken.

(1) Methoxyl analysis. Since the substrate sodium ligno-sulphonate also contained a constant amount of methoxyl (13 per cent) under the experimental conditions employed, the same methoxyl analysis was used here as with native lignin.

The culture medium and dried mycelial pellets from triplicate flasks were examined for methoxyl content in duplicate. Five ml of the supernatant culture medium plus 10 drops of octyl alcohol (to prevent foaming) were evaporated under vacuum at 50 C in specially-made glass reflux flasks. The contents of these flasks were then assayed for methoxyl content. A weighed sample of dried mycelia from the same culture flask was also examined in the same fashion for methoxyl content. This analysis was also made on uninoculated control flasks and on glucose-grown mycelia.

(2) Ultraviolet absorption determinations. The sodium lignosulphonate, when incorporated into basal medium, was the only component of the medium which had a strong absorption peak at 2800A. On the basis of this characteristic an analytical procedure was designed for the analysis of the

soluble residual sodium lignosulphonate in the test medium.

One ml of the original 50 ml of diluted test medium was added to a 100 ml volumetric flask and diluted to volume with glass-distilled water. The diluted medium to be analyzed for ultraviolet absorption was then compared with glass-distilled water in matched cells. The ultraviolet absorption reading was obtained by the use of a Beckman model DU spectrophotometer, with a hydrogen discharge lamp as the light source.

The ultraviolet reading recorded as percentage of light transmission was converted by use of tables to optical density, which was in turn used to calculate the amount of lignosulphonate remaining in the test medium after a prescribed incubation period. The proportion employed for this calculation was as follows:

$$\frac{\text{Optical density of control media}}{200 \text{ mg of lignosulphonate}} = \frac{\text{Optical density of test media}}{\text{mg of residual lignosulphonate}}$$

(4) Residual glucose. Residual glucose was determined by the method described by Somogyi (1945). This test was performed on 1 ml of culture filtrate taken from separate flasks at 3-day intervals over a 12-day incubation period. The analytical procedure was carried out as follows: 5 ml of the testing reagent (1 L of reagent contained 28 g of anhydrous disodium phosphate, 100 ml of normal sodium hydroxide, 40 g of Rochelle salt, 8 g cupric sulphate, 180 g of anhydrous sodium sulfate and 25 ml of normal potassium iodate) and 1 ml of culture filtrate diluted to 5 ml with glass-distilled water were mixed in a 25 x 200 mm pyrex test tube covered with a

glass bulb, and heated by immersion in a vigorously boiling water bath for 10 min. After cooling, 2 ml of a 2.5 per cent solution of potassium iodide were added, and then 1.5 ml of 2N H_2SO_4 . This mixture was titrated with 0.005N $Na_2S_2O_3$ employing starch as an indicator.

Since a direct proportionality prevails between the amounts of glucose present and the amounts of copper reduced, the reduction equivalent has been found to be 1.0 mg of glucose per 7.40 ml of 0.005N $Na_2S_2O_3$; hence each ml of the titration value corresponds to 0.135 mg of glucose. Multiplication of this factor by the titration value gave the amount of glucose that was present in 1 ml of culture filtrate.

IV

RESULTS

A. Native lignin

Screening experiments to determine optimal concentration of native lignin in the medium. Results from experiments performed during the early phases of this study showed that the wood-rotting fungus P. versicolor would grow in synthetic medium with native lignin as the sole carbon source. Since previous experiments with the wood-rotting fungi Polyporus abietinus and Poria subacida indicated that these organisms could be adapted to lignin utilization (Day et al., 1949), it was deemed feasible to perform an experiment to compare the ability of the 3 above-mentioned fungi to utilize native lignin, and also to determine the optimal lignin concentration for incorporation in the medium at the same time. Results from this experiment, recorded in table 3, indicated that P. versicolor was able to utilize lignin as a sole source of carbon to a much greater extent than either Polyporus abietinus or Poria subacida. Also the optimal concentration of lignin was shown to be between 0.25 and 0.5 per cent. It should be emphasized at this point that all values reported in this table as well as in all other tables represent an average of the determinations from at least 3 test flasks.

TABLE 3

The effect of native lignin concentration on the growth of 3 species of white rot fungi

Organism	Concentration of Lignin ¹ (per cent)			
	0.25	0.5	0.75	1.0
<u>Polyporus versicolor</u>	58.7 ²	60.5	50.1	49.8
<u>Polyporus abietinus</u>	31.5	32.4	31.4	28.9
<u>Poria subacida</u>	35.0	36.7	34.4	33.6

¹ Basal medium added to native lignin was same as described under Materials and Methods.

² Weight of dried dioxane-extracted mycelia in mg harvested after 14 days incubation.

Determination of the effect of various nitrogen sources on the ability of *P. versicolor* to utilize native lignin.

Several experiments were performed in an effort to increase growth of the fungus *P. versicolor* in native lignin media, by providing additional nitrogen sources at various concentrations. Results of mycelial dry weight measurements from these experiments recorded in table 4 showed that L-glutamic and L-aspartic acid served as the best sources of nitrogen regardless of concentration; however, since these organic compounds contained available carbon, high dry weight figures are not necessarily indicative of native lignin utilization. Among the inorganic nitrogen sources, ammonium nitrate at a concentration of 0.025 per cent resulted in better growth than any of the other inorganic nitrogen sources employed. Sodium nitrate was shown to be the poorest nitrogen source.

Mycelial nitrogen results from this same experiment (table 5) could be correlated exactly with mycelial dry weights in table 4.

In order to follow the formation of mycelial nitrogen more closely as a measurement of growth response, mycelial nitrogen determinations of cultures grown in glucose and native lignin media were made at 3-day intervals from triplicate test flasks over an incubation period of 30 days. Results of this experiment illustrated graphically in figure 2 indicated that mycelia produced in lignin media contained a larger amount of nitrogen throughout the entire incubation period than that produced in glucose. In native lignin

TABLE 4

The effect of different sources and concentrations of nitrogen on the growth of *Polyporus versicolor*

Nitrogen source	Basal medium ¹ and lignin (0.25 per cent) plus nitrogen source calculated on the basis of per cent nitrogen			
	0.0125	0.025	0.05	0.1
Ammonium nitrate	45.1 ²	63.0	58.0	35.2
Diammonium hydrogen phosphate	42.0	58.2	57.8	31.6
Ammonium sulphate	25.0	49.5	38.0	22.4
Ammonium chloride	37.8	51.4	41.0	30.3
Sodium nitrate	1.5	3.8	2.1	1.0
L-Glutamic acid	64.5	65.9	70.8	78.9
L-Aspartic acid	74.7	75.5	82.8	85.4

¹ Basal medium same as described under Materials and Methods except for different nitrogen sources.

² Weight in mg of dried dioxane-extracted mycelia harvested after 14 days incubation.

TABLE 5

The effect of different sources and concentrations of nitrogen on the mycelial nitrogen content of *Polyporus versicolor*

Nitrogen source	Basal medium ¹ and lignin (0.25 per cent) plus nitrogen source calculated on the basis of per cent nitrogen			
	0.0125	0.025	0.05	0.1
Ammonium nitrate	2.30 ²	3.25	3.10	1.85
Diammonium hydrogen phosphate	2.10	3.10	3.00	1.55
Ammonium sulphate	1.35	2.31	1.95	1.20
Ammonium chloride	1.95	2.60	2.35	1.65
Sodium nitrate	0.08	0.19	0.15	0.05
L-Glutamic acid	3.45	3.55	3.75	4.25
L-Aspartic acid	3.85	3.95	4.25	4.45

¹ Basal medium same as employed in table 4.

² Weight in mg of mycelial nitrogen determined from mycelia harvested after 14 days incubation.

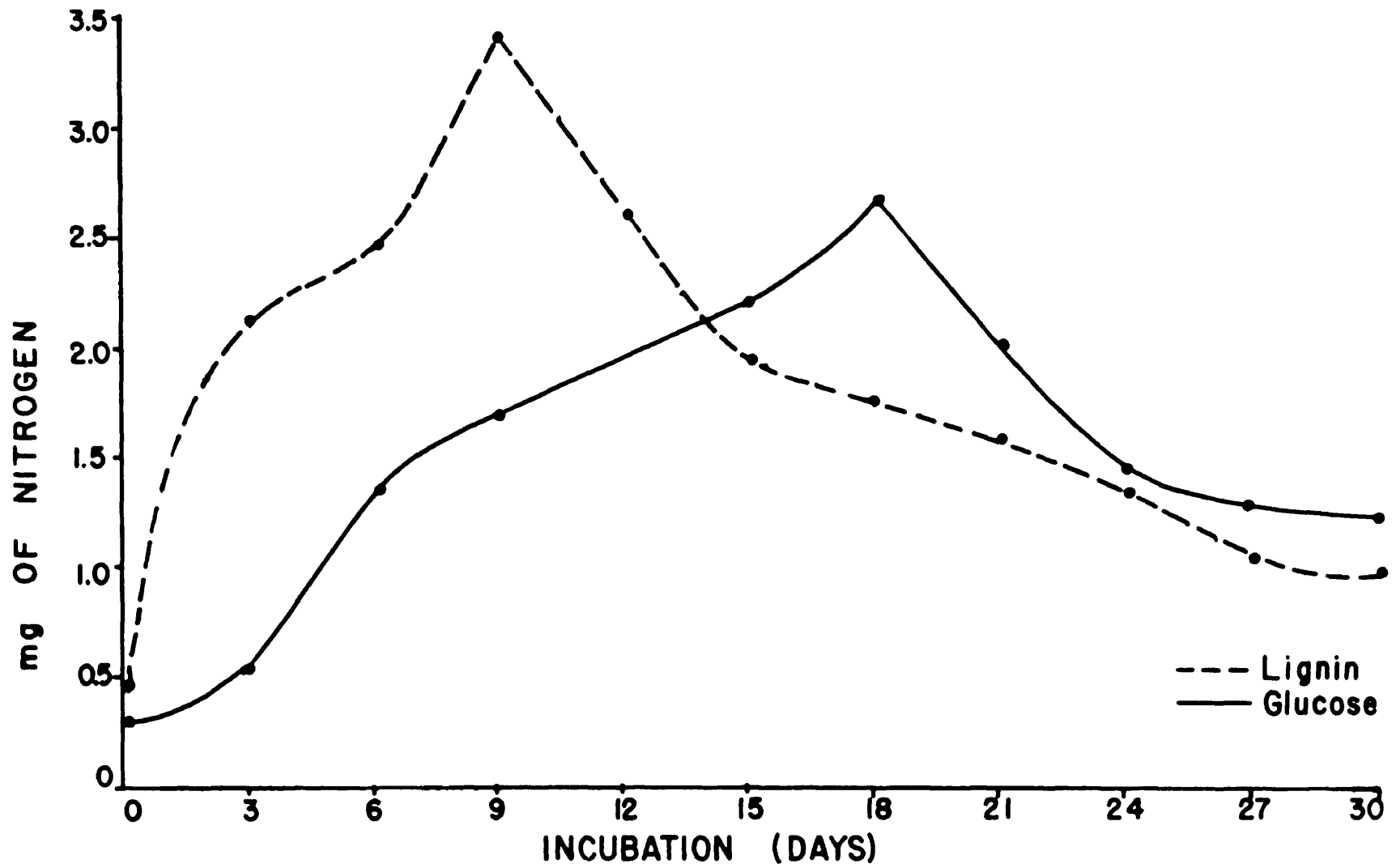


Figure 2. Comparison of mycelial nitrogen produced by Polyporus versicolor employing glucose and lignin as separate carbon sources.

medium the nitrogen curve reached a peak in 9 days where as this did not occur until after 18 days in the glucose medium. The mycelial nitrogen content declined gradually in both media after reaching their respective peaks, as the incubation period progressed. This condition would seem to indicate that autolysis may have taken place midway through the incubation period.

Effect of pH on growth of *P. versicolor*. The effect on growth response was determined when test media were buffered at various pH levels and different sources of nitrogen were employed. Results of this experiment recorded in table 6 revealed that the most ideal pH level was between 6.0 and 7.5 and that ammonium nitrate served as the best nitrogen source at these levels. It was also evident that regardless of the pH level, sodium nitrate was a poor nitrogen source. From these results it could be seen that the fungus grew much better at the highest acid level than at the highest alkaline level.

In order to determine the pH curve produced by this fungus, an experiment was performed whereby the pH was recorded at 2-day intervals over a 20-day incubation period. Two media were tested, one containing glucose (0.5 per cent) and the other native lignin (0.5 per cent) as sole carbon sources. Two hundred fifty ml quantities of these media were incubated in separate 500 ml flasks and 3 ml were withdrawn from each flask and tested at designated time intervals. Results from this experiment are graphically illustrated in

TABLE 6

The effect of buffered media on
the growth of Polyporus versicolor

pH of media	Basal medium ¹ and lignin (0.5 per cent) plus			
	NH ₄ NO ₃	NH ₄ Cl	NaNO ₃	(NH ₄) ₂ SO ₄
1.50	25.0 ²	15.5	5.1	13.8
3.00	31.5	24.2	3.4	18.5
4.50	45.4	34.8	2.1	28.5
6.00	64.5	53.2	2.2	39.5
7.50	63.0	52.5	1.8	40.1
9.00	5.5	3.2	0.6	2.4

¹Basal media same as described under Materials and Methods except for different nitrogen sources (0.025 per cent as nitrogen).

²Weight in mg of dried dioxane-extracted mycelia harvested after 14 days incubation.

figure 3. Observation of pH levels in native lignin media indicated that the pH curve reached a minimum level after 8 days and then rose gradually to a higher level more rapidly, than displayed by the glucose curve, which did not reach its minimum level until the eighteenth day.

Determination of the effect of additional supplements on growth of *P. versicolor*. Experiments were designed in which the basal medium was supplemented with a variety of vitamins, extracts, minerals etc., and the resulting effect on growth was measured after suitable incubation periods. Results of these experiments are recorded in tables 7 and 8. Observation of some of these results (table 7) indicated that when glucose and native lignin were present in the same medium more mycelia was produced than in any other instance. However, this condition did not significantly influence the removal of lignin from the test medium. Spawn and wood extract appeared to stimulate growth and at the same time stimulate the utilization of native lignin to a greater extent than any of the other factors employed. The introduction of supplements such as minerals, homogenizing of mycelia and vitamin mixtures did not appear to stimulate growth and in some instances seemed to produce a slight inhibition. The continuation of the incubation period from 2 to 4 weeks also appeared to be unnecessary, since less growth was evidenced after the longer incubation period, which was probably due to autolysis. The maximum amount of lignin removed as determined colorimetrically ranged between 20 to 30 mg.

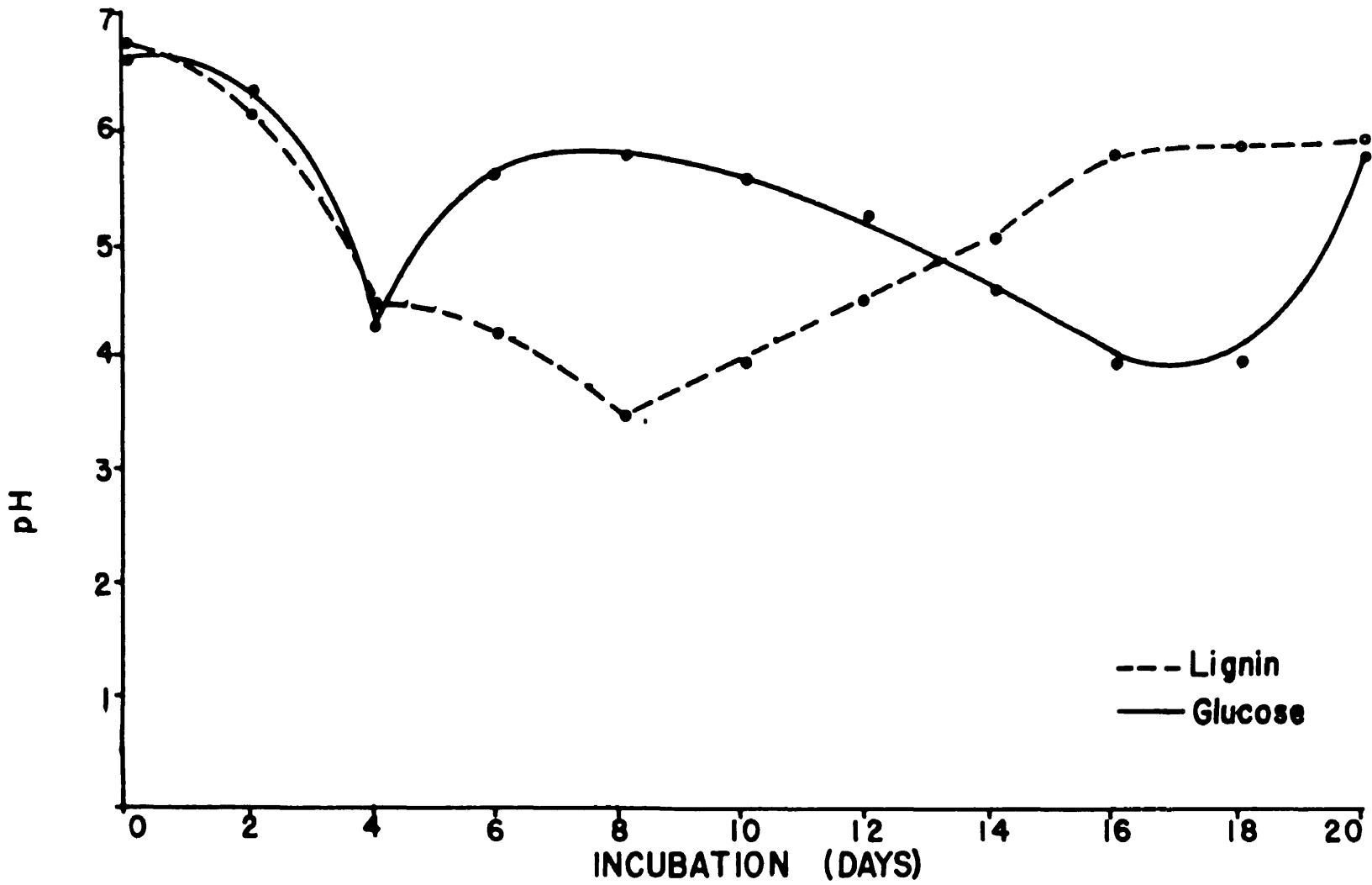


Figure 3. pH levels produced by Polyporus versicolor grown in media with glucose and lignin as separate carbon sources.

TABLE 7

The effect of added vitamins, extracts, etc. on the growth and utilization of native lignin by *Polyporus versicolor*

Basal medium ¹ and lignin (0.25%) plus supplements ²	Two weeks incubation		Four weeks incubation	
	Mycelial ³ wt (mg)	Lignin ⁴ removed (mg)	Mycelial wt (mg)	Lignin removed (mg)
None	55.8	30.0	45.0	22.2
Dextrose (0.25%)	80.1	25.0	65.8	16.5
Vitamin mixture	55.6	28.7	42.0	20.4
Wood extract	68.9	30.4	53.6	21.5
Spawn extract	70.3	31.8	35.0	28.9
Mineral supplement	54.5	27.5	39.3	21.0
Rehomogenized mycelia	35.5	15.8	25.5	10.0

¹ Nitrogen source of basal media was ammonium nitrate (0.025 per cent as nitrogen); otherwise medium same as described under Materials and Methods.

² The supplements used and the concentrations employed are described under Materials and Methods.

³ Mycelia was extracted by dioxane and dried under vacuum.

⁴ Native lignin determined with Klett-Summerson colorimeter and was obtained by extracting the mycelia with dioxane.

However, depending on the interpretation one wishes to give to results in table 2 these figures may or may not appear to be entirely accurate. Additional results of this experiment are recorded in table 8. Mycelial nitrogen and methoxyl values showed agreement with results in table 7, except that methoxyl results were somewhat lower as compared with results of native lignin removal.

Comparison of analytical results. In order to acquire a comparative picture of all analytical results, a long range experiment was performed. Results of this experiment recorded in table 9 indicated clearly that the fungus P. versicolor attained maximal growth under the experimental conditions employed, after 9 days incubation. Growth after this time was of a variable nature. During the same incubation period the maximal amount of native lignin and methoxyl were removed, and the largest mycelial nitrogen yields were measured. Soluble carbon results were extremely low and believed to be insignificant. There was, however, a slight increase in soluble carbon as the incubation period progressed, which indicated that a small amount of native lignin was converted to a soluble form during incubation. The pH recordings also showed that as the fungus reached its maximal growth acid was produced, which coincided with the lignin pH curve shown in figure 3.

Comparison of 3 strains of P. versicolor. In the latter part of this study it was decided to compare the fungus P. versicolor (strain no.37) used throughout this study with

TABLE 8

The effect of added vitamins, extracts, etc. on the utilization of methoxyl by *Polyporus versicolor*¹

Basal medium ² and lignin (0.25%) plus supplements ³	Mycelial N (mg)	Methoxyl analysis of culture contents		
		Methoxyl Removed (mg)	(%)	Residual Methoxyl (mg)
None	2.98	3.55	20.0	11.80
Dextrose (0.25%)	4.16	3.15	21.0	10.65
Vitamin mixture	2.80	2.22	15.0	12.58
Wood extract	3.45	3.76	25.4	10.98
Spawn extract	3.68	4.28	29.6	10.55
Mineral sup- plement	2.40	1.84	12.4	12.86

¹ Mycelia were harvested after a 2-week incubation period.

² Basal media same as employed in table 7.

³ Supplements same as those described in table 7.

TABLE 9

Comparison of analytical results from experiments employed to determine the ability of *Polyporus versicolor* to utilize native lignin

Incubation period (days)	pH	Mycelial Yield (mg)	Lignin ¹ Removed (mg)	Soluble Carbon (mg)	Mycelial ² N (mg)	Methoxyl Removed (mg)
0	6.00	0.45	0.00	0.00	0.023	0.00
3	4.05	22.0	15.5	2.60	1.05	1.50
6	3.90	41.0	21.7	2.95	2.35	3.97
9	4.80	59.5	31.6	3.15	3.26	4.43
12	5.40	55.4	29.2	3.00	2.95	3.63
15	5.80	53.3	29.5	3.20	2.65	3.75
18	6.00	52.8	31.4	3.50	2.58	3.85
21	5.90	49.6	30.5	3.10	2.41	3.81
24	5.90	50.2	28.2	2.50	2.45	3.45
27	6.10	49.5	25.4	2.45	2.47	3.04
30	6.20	49.6	22.5	2.05	2.38	2.81

¹Native lignin measured by Klett-Summerson colorimeter on dioxane-extracted mycelia. Original concentration of lignin was 0.25 per cent.

²Basal media same as described under Materials and Methods except ammonium nitrate concentration as per cent nitrogen was 0.025.

2 other strains (no.37A and no.37B) as to their ability to utilize native lignin. Results of this experiment recorded in table 10 indicated that there was comparatively little difference between the 3 strains as to their ability to utilize native lignin. Strain no.37 appeared to be able to utilize slightly more native lignin than the other 2 strains. However, experimental error could easily account for the small difference.

Comparison of various inocula with respect to utilization of fresh and residual lignin. Results of experiments reported thus far, indicated the inability of the fungus to grow or utilize native lignin after the eighth or ninth day of incubation. In order to determine the reason for this behavior, an experiment was performed with 3 different types of inocula (described under Materials and Methods), and 2 types of native lignin, i.e., (1) the original native lignin, or (2) lignin which had been previously exposed in culture to P. versicolor and subsequently recovered (residual lignin). The 3 inocula were used to inoculate a duplicate series of flasks, one set containing native lignin and the other residual lignin. Results of this experiment recorded in table 11 demonstrated that none of the inocula were able to grow to a significant extent in residual lignin which had previously been employed for one testing period. Also mycelial pellets employed as inoculum, which had been incubated (shake-culture) previously for 12 days in a native lignin medium, did not show a significant growth response in any of the lignin media.

TABLE 10

Comparison of the ability of different strains of
Polyporus versicolor to utilize native lignin¹

Analytical results ²	Strains of <u>Polyporus versicolor</u> ³		
	no.37	no.37A	no.37B
Final pH	5.85	5.90	5.80
Mycelial yield	56.80 mg	54.90 mg	50.60 mg
Mycelial N	2.87 "	2.35 "	2.22 "
Lignin removed	28.60 "	25.80 "	23.60 "
Methoxyl	3.50 "	3.15 "	2.96 "

¹Results obtained after 14 days incubation.

²Initial pH of basal medium was 6.0 and was same as described under Materials and Methods except ammonium nitrate concentration as nitrogen was 0.025 per cent.

³Strain no.37 was used throughout this study, but strains no.37A and no.37B were obtained midway through this study from Dr. Ross W. Davidson, Bureau of Plant Industry, Beltsville, Maryland.

TABLE 11

Ability of various types of inocula to utilize
lignin before and after exposure to
*Polyporus versicolor*¹

Type of Inocula (1 ml)	Basal Medium ² plus			
	Residual lignin ³ (0.5 per cent)		Fresh lignin (0.5 per cent)	
	Mycelial yield	Mycelial N	Mycelial yield	Mycelial N
Rehomogenized	5.8 mg	0.31 mg	45.6 mg	2.45 mg
Fresh	7.9 "	0.49 "	59.8 "	3.12 "
Mycelial ⁴ Pellets	3.5 "	0.24 "	5.4 "	0.32 "

¹ Mycelia extracted with dioxane after an incubation period of 2 weeks.

² Basal media same as described under Materials and Methods except ammonium nitrate concentration as per cent nitrogen was 0.025.

³ Residual lignin previously exposed to fungal attack and extracted from mycelia with hot dioxane.

⁴ Mycelial pellets produced in lignin shake-culture over a 12-day incubation period.

Rehomogenized pellets and fresh inoculum produced good growth when inoculated into media containing fresh lignin.

B. Sodium lignosulphonate

Screening experiments to determine ability of wood-rotting fungi to utilize sodium lignosulphonate. In the latter phase of this investigation, the experimental approach was designed to determine whether wood-rotting fungi which previously had shown some indication of being able to utilize native lignin, could also utilize sodium lignosulphonate as a sole source of carbon, in a liquid synthetic medium.

Initial experiments were performed to compare the ability of P. versicolor with 5 other white rot fungi studied previously, (Day et al., 1949) to utilize sodium lignosulphonate. Results of such an experiment (table 12) indicated that all organisms employed grew feebly in the sodium lignosulphonate medium. However, P. versicolor did display slightly better ability to utilize the substrate than any of the other organisms tested. The final pH in all instances was slightly alkaline, and although the results were not recorded herein, the pH at no time approached a level lower than 6.0.

Determination of the utilization of methoxyl contained in sodium lignosulphonate when glucose was incorporated as an additional carbon source. Since sodium lignosulphonate did not serve as an adequate carbon source, 2 per cent glucose, which supports luxuriant growth of the fungus P. versicolor, was added to the media along with 0.5 per cent sodium

TABLE 12

Comparison of the ability of 6 cultures of wood-rotting fungi to utilize sodium lignosulphonate as a sole source of carbon in a liquid synthetic medium¹

Organism	Analytical results ²			
	Final pH ³	Mycelial wt (mg)	Lignosulphonate removed ⁴ (mg)	Mycelial N (mg)
<u>Polyporus versicolor</u>	6.50	18.0	5.0	0.78
<u>Ustulina vulgaris</u>	6.60	10.0	2.8	0.42
<u>Fomes geotropus</u>	6.55	4.6	1.4	0.24
<u>Polyporus abietinus</u>	6.95	5.2	2.0	0.27
<u>Poria subacida</u>	6.52	2.2	0.8	0.08
<u>Daedala unicolor</u>	6.45	7.3	2.4	0.36

¹Basal medium same as described under Materials and Methods plus 0.5 per cent sodium lignosulphonate as a sole carbon source.

²Analyses made after 12 days incubation.

³Initial pH of basal medium was 6.0.

⁴Sodium lignosulphonate measured by ultraviolet absorption method.

lignosulphonate. This experimental procedure was similar to that employed by Adams and Ledingham (1942b). Results of this type of experiment recorded in table 13 showed that the pH level dropped rapidly during 4 days incubation and continued to stay at a low level during the remainder of the incubation period. Mycelial dry weight reached a maximum level after 8 days incubation and then started to decline on the tenth day. Methoxyl values indicated a rapid disappearance of methoxyl from the medium, which at first seemed to indicate rapid utilization of lignosulphonate; however, methoxyl analyses made simultaneously with mycelia from the same culture flasks showed that methoxyl disappearing from the test medium re-appeared 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 methoxyl of the control flask, indicating probable "adsorption"* of the sodium lignosulphonate or methoxyl on the mycelia. Figure 4 illustrates graphically this same 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) suggested that the large amount of methoxyl occurring in the sodium lignosulphonate-glucose-grown mycelia did not represent a normal growth picture.

The possibility was considered that the concentration

*The term "adsorption" is employed here to indicate the removal of lignosulphonate from the media by being deposited on or in the mycelia.

TABLE 13

Analytical results from *Polyporus versicolor* grown in sodium lignosulphonate-glucose medium¹

Growth period (days)	pH	Methoxyl (OCH ₃) Content			
		Medium (mg)	Mycelia (mg)	Mycelia + Medium (mg)	Control (mg)
0	7.00	25.50	-----	-----	25.50
2	4.51	16.50	8.95	25.45	25.65
4	2.12	7.13	17.75	24.88	25.40
6	2.65	5.43	18.65	24.08	24.90
8	2.52	3.10	21.64	24.74	25.40
10	2.41	8.14	16.75	24.89	25.10

¹Basal medium same as described under Materials and Methods plus 0.5 per cent sodium lignosulphonate and 2 per cent glucose as carbon sources.

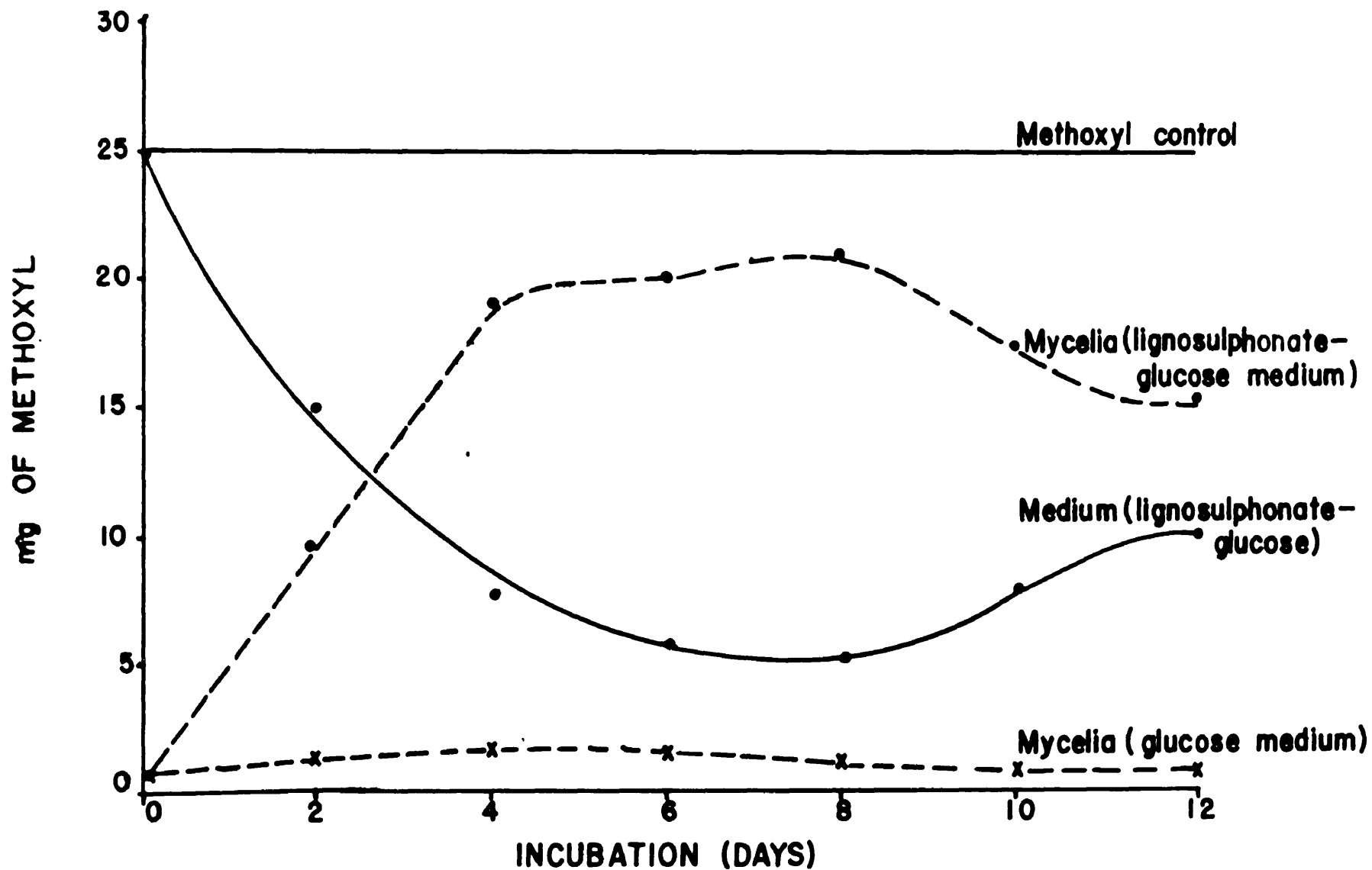


Figure 4. Methoxyl balance for growth of *Polyporus versicolor* in sodium lignosulphonate-glucose and glucose media.

of sodium lignosulphonate 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 certain preparations of lignosulphonate have been employed as tanning agents (Brauns, 1948). Therefore, an experiment such as recorded in table 14 was performed with the addition of 0.5 g of calcium carbonate per culture flask. This cultural condition held the pH level to a much narrower range than previously noted (table 13). From residual glucose determinations recorded in table 14, it can be seen that glucose was completely utilized on the sixth day and that after this time a measureable increase in methoxyl content of the mycelia could not be demonstrated. From these results it would seem that the apparent "adsorption" of sodium lignosulphonate or methoxyl was not necessarily a function of pH per se.

Correlation of mycelial yields, mycelial nitrogen and sodium lignosulphonate disappearance from glucose and sodium lignosulphonate-glucose media. Mycelial nitrogen and dry weight determinations as well as ultraviolet absorption results for determination of sodium lignosulphonate are recorded in table 15.

Comparison of mycelial nitrogen values revealed insignificant differences in amount of nitrogen produced between the 2 media during 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

TABLE 14

Analytical results from Polyporus versicolor culture grown in sodium lignosulphonate-glucose medium plus calcium carbonate

MEDIA ¹							
			Basal + Sodium lignosulphonate (0.5%) and glucose (2%)			Basal + glucose (2%)	
Growth period (days)	pH	Residual glucose (mg)	Methoxyl (OCH ₃) content				
			Media (mg)	Mycelia (mg)	Media + mycelia (mg)	Control (mg)	Mycelia (mg)
0	6.25	800.0	24.40	-----	-----	24.40	-----
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	7.70	0.0	8.50	15.75	24.20	24.00	1.10

¹ Basal medium same as described under Materials and Methods.

mycelia. The dry weight values obtained from the sodium lignosulphonate-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 sodium lignosulphonate-glucose medium only varied from the glucose yield in an amount approximately equal to the sodium lignosulphonate 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 sodium lignosulphonate-glucose medium was due to the presence of sodium lignosulphonate in or on the mycelia.

In previous experiments only one type of nitrogen source was employed in the basal medium (diammonium hydrogen phosphate). According to the results obtained by La Fuze (1937) in his nutritional study of P. versicolor, this fungus was able to produce good growth when various organic nitrogen sources were incorporated into liquid media. Therefore, an experiment was performed in which several amino acids and casein were employed as nitrogen sources and compared at the same time with diammonium hydrogen phosphate and 2 per cent glucose as an additional carbon source. Results of this experiment recorded in table 16 indicated that regardless of the nitrogen source, glucose must be present in the media in order for the fungus to produce good growth. It was possible that the small amount of mycelia produced in

TABLE 15

Results of mycelial nitrogen and dry weight determinations and their relationship to lignosulphonate disappearance from solution

MEDIA¹

Growth period (days)	Basal + glucose (2%)		Basal + lignosulphonate (0.5%) and glucose (2%)		
	Mycelial N (mg)	Mycelial Wt (mg)	Ligno-sulphonate removed ² (mg)	Mycelial Wt (mg)	Mycelial N (mg)
0	0.045	1.0	----	1.0	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

¹Basal medium same as described under Materials and Methods.

²Lignosulphonate determined by ultraviolet absorption method.

TABLE 16

The effect of different nitrogen sources on the decomposition of sodium lignosulphonate by *Polyporus versicolor*¹

Basal medium ² and ligno- sulphonate (0.5%) plus nitrogen source	Analytical results			
	Initial pH	Final pH	Ligno- sulphonate removed ³	Mycelial N
Asparagine	6.20	7.92	3.0 mg	0.75 mg
Glutamine	6.25	7.90	2.0 "	0.75 "
Arginine	6.10	8.10	2.4 "	1.62 "
Glycine	6.10	8.30	2.1 "	1.38 "
Casein	6.20	8.65	2.5 "	1.59 "
(NH ₄) ₂ HPO ₄ + Glucose (2%)	6.20	2.00	110.5 "	10.31 "

¹ Analyses made after 12 days incubation.

² Basal medium same as described under Materials and Methods except for different nitrogen sources (concentration 0.025 per cent as nitrogen).

³ Sodium lignosulphonate measured by ultraviolet absorption method.

amino acid media was due to the presence of available carbon in these compounds.

In order to follow the formation of mycelial nitrogen more closely, mycelia were harvested and examined for nitrogen content at 3-day intervals over a 30-day incubation period. Diammonium hydrogen phosphate was employed as the nitrogen source and sodium lignosulphonate plus glucose and glucose alone were employed in separate media as carbon sources. The nitrogen data obtained is illustrated graphically in figure 5. Nitrogen figures given are for the entire culture flask. The slight increase of nitrogen in the sodium lignosulphonate-glucose medium as compared to the glucose medium was again consistently observed. The rapid decrease after the sixth day of incubation was attributed to autolysis of the mycelia.

Effect of pH and different types of inocula on sodium lignosulphonate "adsorption" phenomena. To further examine the effect of pH on sodium lignosulphonate disappearance from the culture media an experiment was conducted at 3 pH levels and simultaneously the effect of the addition or subtraction of glucose from test medium was examined. In this experiment a large amount of mycelia was used (250 mg) as inoculum. This inoculum was described under Materials and Methods as "normal inoculum". This inoculum was transferred to 2 different test media, one containing sodium lignosulphonate and glucose as carbon sources and the other lignosulphonate. These media were incubated in the usual procedure for 12 days.

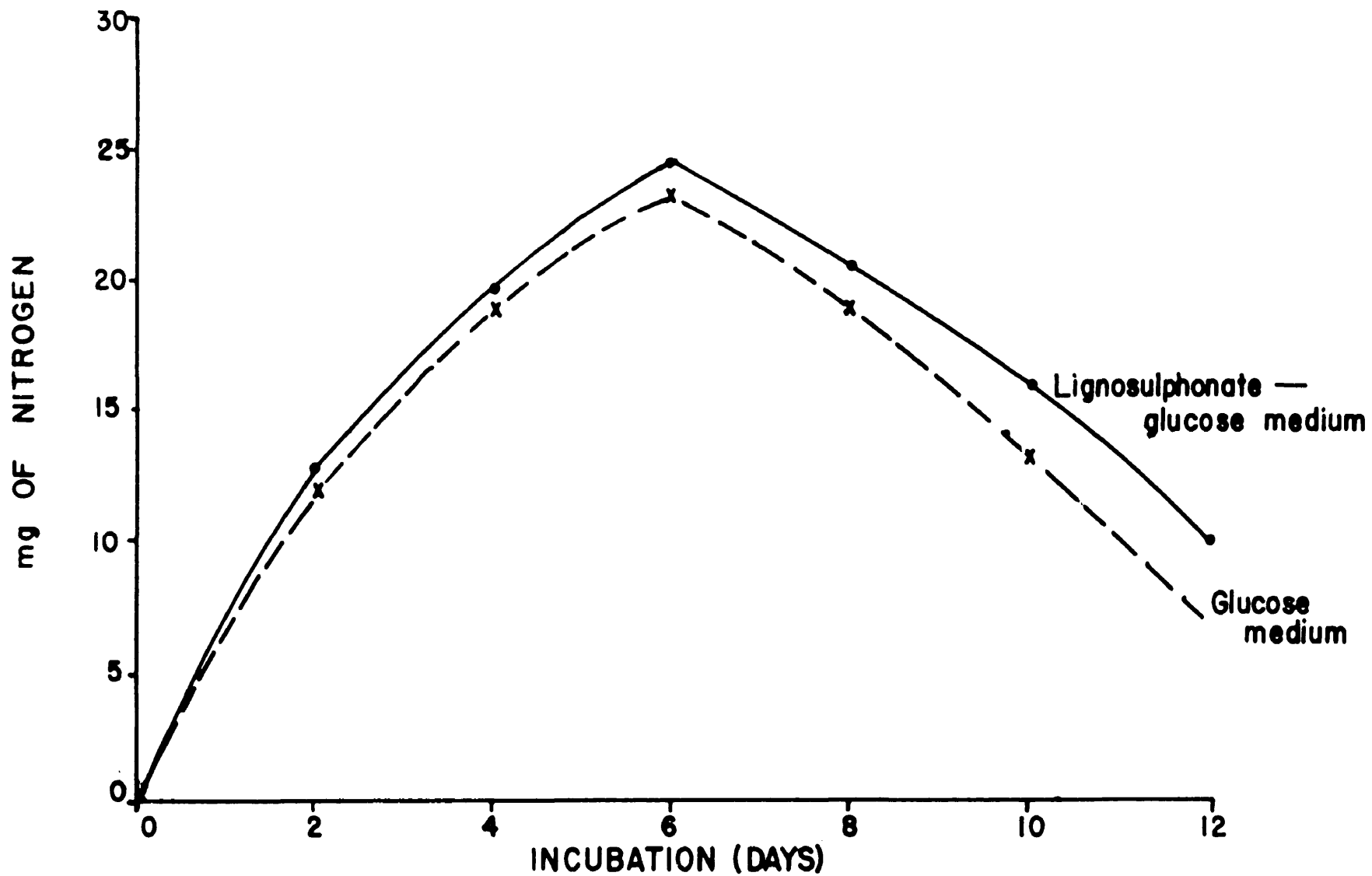


Figure 5. Comparison of mycelial nitrogen produced by *Polyporus versicolor* in sodium lignosulphonate-glucose and glucose media.

Ultraviolet absorption and pH results of this experiment are recorded in table 17. It can be seen that when 2 carbon sources were present in the medium approximately twice as much sodium lignosulphonate was removed or "adsorbed" from the media as when lignosulphonate was present alone. It was also noted that the 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 source of carbon 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 (cultivated and harvested as described in Methods and Materials). The amount of mycelial inoculum per culture flask was the same as used in a previous experiment (table 17). The 3 different types of inocula were inoculated separately into the same media as shown in table 17 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 18. It can be seen from the results that the starved mycelia "adsorbed" approximately as much sodium lignosulphonate as the normal in either medium. The killed cells, for practical purposes, did not remove any sodium lignosulphonate from either media.

TABLE 17

Effect of different pH levels and glucose on lignosulphonate disappearance from solution¹

MEDIA²

Initial pH	Basal + lignosulphonate (0.5%)		Basal + lignosulphonate (0.5%) and glucose (2%)	
	Ligno- sulphonate removed (mg)	Final pH	Ligno- sulphonate removed (mg)	Final pH
7.20	50.0	7.00	110.0	2.20
3.00	48.0	3.00	109.0	2.00
1.50	49.5	1.65	104.0	1.60

¹ Analyses made after 12 days incubation by ultraviolet absorption method.

² Basal medium same as described under Materials and Methods.

TABLE 18

Effect of the condition of mycelium
on lignosulphonate disappearance from solution¹

MEDIA²

Type of inoculum	Basal + lignosulphonate (0.5%)		Basal + lignosulphonate (0.5%) and glucose (2%)	
	Ligno- sulphonate removed (mg)	Final pH	Ligno- sulphonate removed (mg)	Final pH
Normal	49.0	6.50	108.4	2.50
Starved	40.0	6.60	100.2	2.80
Killed	6.0	6.85	7.0	6.75

¹Analyses made after 12 days incubation by ultraviolet absorption method.

²Basal medium same as described under Materials and Methods and initial pH was 7.0.

DISCUSSION

A. Native lignin

From the results of the investigation with native lignin, it could be inferred that the lignin-decomposing ability of the fungi employed was one of a limited and variable nature.

Dry weight determinations of mold mycelium indicated in almost every instance that the organisms employed were utilizing the substrate native lignin to a limited extent. It was obvious from the results in most cases, that the maximal yield of mycelium occurred during a 9-day incubation period which correlated with all other determinations during this period of time. However, after this time the mycelial yield did not increase, but had a tendency to gradually decrease. This condition may have been due to autolysis, since the nature of the mycelial pellets examined at intervals between the twelfth and thirtieth day of incubation was characterized in many instances by a lack of turgor or rigidity and had the appearance of numerous soft, pulpy, slimy masses. The test medium during this same incubation period gradually assumed a color of ordinary tea, and at times became deep reddish-brown as the pH of the medium gradually became more alkaline. This same condition was observed by Bortels (1927) between autolysis and a violet-red coloration of the medium, upon

addition of alkali. The loss in dry weight, therefore, after 9 days could have been due to solubilization and release of cellular constituents into the medium through cell membranes otherwise impermeable to this passage. If it can be assumed that autolysis had taken place, several factors may be considered, either the depletion of nutrients was responsible or the inhibition of further development by creation of an unfavorable growth environment. Depletion of nutrients did not seem likely, since the analytical results revealed that ample carbon substrate was available at all times. A peripheral portion of the native lignin molecule, however, could have been entirely utilized, which was not measurable by the analytical methods employed. The creation of unfavorable growth conditions appeared more feasible, since during the 9-day growth period lignin became firmly adhered to the mycelial pellets. This phenomena may have created unfavorable nutritional conditions for the majority of cells in the interior of the pellet either by blocking the normal metabolic assimilations or creating anaerobic conditions for an organism which is highly aerobic. According to Burkholder and Sinnott (1945) in their study of the morphogenesis of fungi in shake-culture, many mycelial pellets when bisected gave off strong alcoholic odors indicating a lack of oxygen and incomplete oxidation of substrate by the cells in the interior of the mycelial pellet.

The production of inhibitory or toxic products must also be considered, since in a compound like native lignin there are undoubtedly many phenolic-like structures which could be

oxidized to form inhibitory compounds such as quinones. It is believed by Lindeberg (1949) that catecholase-producing hymenomycetes can oxidize o-diphenols in the soil, giving rise to quinones, which in certain cases inhibit the growth of soil fungi. It has also been suggested by other workers (Waksman and Iyer, 1932; Levine et al., 1935) that cessation of lignin decomposition results from the formation of ligno-protein complexes that are inhibitory or very resistant to further microbial action.

Colorimetric determinations of residual lignin in most instances revealed that between 20 to 30 per cent of native lignin was removed from the test media after 9 days incubation. This data did not agree on all occasions with methoxyl data, which usually ranged between 15 and 25 per cent. This difference may have been due either to experimental error, or to an unusual metabolism by the organism.

The slight stimulation of growth and utilization of lignin, apparently brought about by addition of spawn extract to the media, could be explained by the presence of a polyphenol oxidase in this extract, since this type of oxidase was isolated by Gottlieb and Geller (1949) from the mycelium of Agaricus campestris in the form of commercial mushroom spawn. This enzyme was shown by the above investigators to have considerable specificity for the native lignin molecule and may have aided the enzyme system of the fungus Polyporus versicolor in its degradation of the native lignin molecule. Stimulation of growth by addition of wood extract, may have

been caused by unknown factors associated with growth and utilization of lignin by the fungus under natural conditions.

A comparison of results from nitrogen determinations with other data indicated that as the mycelial nitrogen values increased the mycelial weight and lignin utilization also increased. This condition was especially prevalent when ammonium nitrate was employed at a concentration of 0.025 per cent as nitrogen. A similar type of picture was detected by Hilpert *et al.*, (1937) in his nutritional study of Aspergillus niger in which increasing concentrations of ammonium sulfate produced a gradual increase in mycelial weight and mycelial nitrogen, when the glucose concentration was constant. Steinberg (1939) employing the same organism found that regardless of sugar concentration, dry weight and mycelial nitrogen were virtually linear with respect to the initial nitrogen content in media ranging from 0 to 560 mg of nitrogen per liter as ammonium nitrate, ammonium chloride or sodium nitrate.

Results relating to the pH level and different sources of nitrogen indicated that the fungus Polyporus versicolor grew better at an extreme acid pH than at extreme alkaline levels, regardless of the nitrogen source.

Three different strains of Polyporus versicolor used in this study showed approximately equal ability to utilize native lignin as a sole carbon source.

Variations in types of inocula showed that, regardless of the nature of the inoculum, native lignin which had

previously been exposed to the fungus Polyporus versicolor would not support growth upon subsequent re-exposure. This may have been due to alteration or distortion of the residual native lignin by isolation procedures. It was also possible that certain end-products of metabolism were not removed from the recovered lignin which inhibited growth. Another possibility was, that during the initial incubation period, the enzyme system of the fungus became saturated so that secondary enzyme systems might produce some changes in the native lignin molecule. If this was the case it is feasible to postulate that the altered native lignin molecule upon re-exposure to the fungus Polyporus versicolor was not a suitable substrate for normal metabolic assimilation.

The inability of mycelial pellets (previously grown in lignin shake-culture medium) to produce further growth when reincubated in fresh lignin media may have been due to the creation of anaerobic conditions, since these pellets grew well on potato dextrose agar and also produced good growth when rehomogenized.

Although the evidence presented in the results concerning native lignin did not indicate significant utilization of this substrate, it must be remembered that the laboratory conditions employed were far removed from a duplication of the natural environment under which native lignin is thought to be utilized.

B. Sodium lignosulphonate

The initial results obtained from experiments employing

sodium lignosulphonate as a sole carbon source indicated that this substrate did not serve as an adequate carbon source for the fungi employed. Initial experiments employing glucose as an additional carbon source showed that sodium lignosulphonate did not inhibit growth and was apparently removed from the test media, indicating probable utilization. Further examination of the mycelia for methoxyl content indicated an abnormal amount of methoxyl therein. These results compared along with mycelial nitrogen and dry weight values obtained from glucose and sodium lignosulphonate-glucose media revealed that the apparent utilization of sodium lignosulphonate was due to the presence of unutilized substrate "adsorbed" in or on the mycelia.

The slight increase in mycelial nitrogen in sodium lignosulphonate-glucose medium over that in the glucose medium may have been due either to a small amount of lignosulphonate being utilized, or to some synergistic phenomenon.

From this study it may be said that the amount of methoxyl detected on the mycelia compared favorably with the ultraviolet data on sodium lignosulphonate removal. It is believed that these 2 methods should be used to examine both the media and mycelia in order to obtain valid analyses when using soluble sodium lignosulphonate substrates as carbon sources. In a similar work Adams and Ledingham (1942c) using the ultraviolet absorption technique for analysis, concluded that several wood-rotting fungi could utilize approximately 7 per cent of sodium lignosulphonate (3.75 per cent) when incorporated

into synthetic liquid media with 2 per cent glucose as an additional carbon source. From the results of this investigation it is very likely that the data acquired by the above-mentioned investigators was misinterpreted, since no examination was made of the mycelia or media for methoxyl content.

Although the results thus far had indicated an apparent "adsorption" of sodium lignosulphonate on the mycelia, the nature of this "adsorption" phenomena was not understood. Further experiments with a large amount of inoculum employing lignosulphonate and lignosulphonate-glucose media adjusted to various pH levels, indicated that pH had no effect upon adsorption, whereas the presence of glucose did. Experiments employing various types of inocula indicated that "adsorption" did not take place when dead inoculum was used. These results seemed to indicate that the "adsorption" of sodium lignosulphonate from the media was not a mechanical process, but was a reaction associated with actively metabolizing mycelia. It would seem, therefore, that in the experiments previously described, sodium lignosulphonate 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.

VI

SUMMARY AND CONCLUSIONS

It was demonstrated by the application of methoxyl analysis and colorimetric estimation of residual native lignin, that Polyporus versicolor over a 9-day incubation period (shake-culture) could utilize between 15 to 25 per cent methoxyl and 25 to 30 per cent native lignin; however, after this time no further evidence of lignin utilization was observed.

Comparative studies made of mycelial dry weights of 3 white rot fungi Polyporus abietinus, Poria subacida and Polyporus versicolor grown in mineral-salt media, containing native lignin as the sole source of carbon at 4 different concentrations, indicated that Polyporus versicolor grew best in media containing native lignin at concentrations of either 0.25 or 0.5 per cent.

Nitrogen and pH determinations indicated that Polyporus versicolor grew best at pH levels ranging between 6.0 and 7.5 when ammonium nitrate was employed at a concentration of 0.025 per cent as nitrogen. Spawn and wood extract appeared to have some stimulatory effect on utilization of lignin while other factors such as vitamins, extracts and mineral supplements had no significant effect.

Three different strains of Polyporus versicolor studied for their lignin-utilizing ability indicated that all of the

strains possessed the same ability to utilize native lignin.

Studies made with various types of inocula introduced in media containing either fresh or residual lignin as the sole carbon source revealed that, regardless of the type of inoculum, no growth was produced in media containing lignin which had been previously exposed to attack by Polyporus versicolor. Rehomogenized and fresh inoculum grew well in fresh lignin media, but mycelial pellets previously grown in lignin media under shake-culture conditions did not grow in either type of media.

Comparative studies of Polyporus versicolor with 5 other white rot fungi (Daedalea unicolor, Fomes geotropus, Poria subacida, Polyporus abietinus and Ustulina vulgaris) indicated that Polyporus versicolor was the only organism of this group which displayed some tendency of sodium lignosulphonate utilization. Other quantitative examinations made to determine the ability of Polyporus versicolor to utilize sodium lignosulphonate, such as dry weight measurements, mycelial nitrogen, methoxyl and glucose determinations and ultraviolet absorption recordings of residual lignosulphonate, revealed that sodium lignosulphonate was not metabolized but physically removed from the liquid test medium. Proof for this was established by methoxyl and ultraviolet results which showed sodium lignosulphonate to be present in or on the mycelia in an amount equivalent to that removed from the liquid test media. It was also noted that 2 per cent glucose incorporated in the basal medium as an additional carbon source increased the

removal of sodium lignosulphonate from the test medium by Polyporus versicolor twofold. Viable cells were required for this phenomena to occur.

A technique for the colorimetric estimation of native lignin when isolated by dioxane extraction from mycelia and culture media was devised.

VII

REFERENCES

- Adams, G. A., and Ledingham, G. A. 1942a Biological decomposition of chemical lignin. I. Sulphite waste liquor. *Can. J. Res.*, 20C, 1-12.
- Adams, G. A., and Ledingham, G. A. 1942b Biological decomposition of chemical lignin. II. Studies on decomposition of calcium lignosulphonate by wood-destroying and soil fungi. *Can. J. Res.*, 20C, 13-27.
- Adams, G. A., and Ledingham, G. A. 1942c Biological decomposition of chemical lignin. III. Application of a new ultraviolet spectrographic method to the estimation of sodium lignosulphonate in culture media. *Can. J. Res.*, 20C, 101-107.
- Bayliss, J. S. 1908 The biology of *Polystictus versicolor* *J. Econ. Biol.*, 3, 1-24.
- Benson, H. K., and Partansky, A. M. 1934 The rate and extent of anaerobic decomposition of sulphite waste liquor by bacteria of sea water mud. *Proc. Nat. Acad. Sci.*, 20, 542-551.
- Berl, E., and Koerber, W. 1938 Fermentation of cellulose and cellulose humic acid and lignin and lignin humic acid. *J. Am. Chem. Soc.*, 60, 1596-1598.
- Bortels, H. 1927 Uber die Bedeutung von Eisen, Zink und Kupfer fur Mikroorganismen. *Biochem. Z.*, 182, 301-358.
- Boruff, C. S., and Bushwell, A. M. 1934 The anaerobic fermentation of lignin. *J. Am. Chem. Soc.*, 56, 886-888.
- Bose, S. R., and Sarkar, S. N. 1937 Enzymes of some wood-rotting Polypores. *Proc. Roy. Soc. (London)*, B123, 193-213.
- Brauns, F. E. 1939 Native lignin. I. Its isolation and methylation. *J. Am. Chem. Soc.*, 61, 2120-2127.
- Brauns, F. E. 1948 Lignin -- a botanical raw material. *Econ. Bot.*, 2, 419-435.
- Brauns, F. E. 1951 *The Chemistry of Lignin*. The Academic Press, New York, N. Y.

- Burkholder, P. R., and Sinnott, E. W. 1945 Morphogenesis of fungus colonies in submerged cultures. *Am. J. Bot.*, 32, 424-431.
- Campbell, W. G. 1930 The chemistry of the white rots of wood. I. The effect on wood substance of Polystictus versicolor. *Biochem. J.*, 24, 1235-1243.
- Cartwright, K. St. G., and Findlay, W. P. K. 1943 Timber decay. *Biol. Revs.*, 18, 145-158.
- Clark, E. P. 1932 The Viebock-Schwappach method for the determination of methoxyl and ethoxyl groups. *J. Assoc. Off. Agri. Chem.*, 15, 136-140.
- Clark, N. A., and Ogg, C. L. 1942 A wet combustion method for determining total carbon in soils. *Soil Sci.*, 53, 27-35.
- Day, W. C., Pelczar, M. J., Jr., and Gottlieb, S. 1949 The biological degradation of lignin. I. Utilization of lignin by fungi. *Arch. Biochem.*, 23, 360-369.
- Erickson, Dagny 1941 Studies on lake-mud strains of Micromonospora. *J. Bact.*, 41, 277-300.
- Fahreus, G., Nilsson, R., and Nilsson, G. 1949 Studies on the decomposition of wood by means of some white rot fungi. *Svensk Botan. Tid.*, 43, 343-356.
- Garren, K. H. 1938 Studies on Polyporus abietinus. II. The utilization of cellulose and lignin by the fungus. *Phytopathology*, 28, 875-878.
- Gottlieb, S., and Geller, J. H. 1949 Enzymatic decomposition of lignin. *Science*, 110, 189-190.
- Gottlieb, S., Day, W. C., and Pelczar, M. J., Jr. 1950 The biological degradation of lignin. II. The adaptation of white rot fungi to growth on lignin media. *Phytopathology*, 40, 926-935.
- Gottlieb, S., and Pelczar, M. J., Jr. 1951 Microbiological aspects of lignin degradation. *Bact. Revs.*, 15, 55-76.
- Hilpert, R. S., Friesen, G., and Rossee, W. 1937 Der Einfluss des Nahrbodens auf die chemische Zusammensetzung des Aspergillus niger. *Biochem. Z.*, 289, 193-197.
- Kazanskii, A. S., and Mikhailova, M. A. 1936 Biochemical decomposition of lignosulphonic acids in sulphite lye. *Lesokhim. Prom.*, 5, 16-20.

- Konetzka, W. A., Pelczar, M. J., Jr., and Gottlieb, S. 1952 Biological degradation of lignin. III. The bacterial degradation of alpha-conidendrin. J. Bact. In press.
- La Fuze, H. H. 1937 Nutritional characteristics of certain wood-destroying fungi, Polyporus betulinus Fr. Fomes pinicola (Fr.) Cooke, and Polystictus versicolor Fr. Plant Physiology, 12, 625-646.
- Law, Kathleen 1950 Phenol oxidases in some wood-rotting fungi. Ann. Bot., 14, 69-78.
- Levine, M., Nelson, G. H., Anderson, D. Q., and Jacob, P. B. 1935 Utilization of agricultural wastes. I. Lignin and microbial decomposition. Ind. Eng. Chem., 27, 195-200.
- Lindeberg, G. 1946 On the decomposition of lignin and cellulose in litter caused by soil-inhabiting Hymenomyces. Ark. for Botanik., 33A, 1-16.
- Lindeberg, G. 1949 Influence of enzymatically oxidized gallic acid on the growth of some Hymenomyces. Svensk Boton. Tid., 43, 438-447.
- Norman, A. G. 1936 The biological decomposition of lignin. Science Progress, 30, 442-456.
- Pelczar, M. J., Jr., Gottlieb, S., and Day, W. C. 1950 Growth of Polyporus versicolor in a medium with lignin as the sole carbon source. Arch. Biochem., 25, 449-451.
- Perlman, D. 1949 Studies on the growth and metabolism of Polyporus anceps in submerged culture. Am. J. Bot., 36, 180-184.
- Phillips, M. 1934 Chemistry of lignin. Chem. Revs., 14, 103-170.
- Pringsheim, H., and Fuchs, W. 1923 Uber den bakteriellen Abbau von Ligninsaure. Ber deut. chem. Ges., 56, 2095-2097.
- Robbins, W. S., and Kavanagh, F. 1938 Vitamin B, or its intermediates and growth of certain fungi. Am. J. Bot., 25, 229-236.
- Scheffer, T. C. 1936 Progressive effects of Polyporus versicolor on the physical and chemical properties of red gum sapwood. U. S. Dept. Agr. Tech. Bull., 527, 1-45.
- Smith, R. G. 1924 A chemical and pathological study of decay of the xylem of the apple caused by Polystictus versicolor Fr. Phytopathology, 14, 114-118.

- Smith, F. B., and Brown, P. E. 1935 The decomposition of lignin and other organic constituents by soil fungi. J. Am. Soc. Agron., 27, 109-119.
- Sobell, A. E., Yuska, H., and Cohen, J. 1937 A convenient method of determining small amounts of ammonia and other bases by the use of boric acid. J. Biol. Chem., 118, 443-446.
- Somogyi, M. 1945 Determination of blood sugar. J. Biol. Chem., 160, 69-73.
- Steinberg, R. A., and Bowling, J. D. 1939 Optimum solutions as physiological reference standards in estimating nitrogen utilization by Aspergillus niger. J. Agr. Res., 58, 717-732.
- Umbreit, W. W., and Bond, V. S. 1936 Analysis of plant tissue -- Application of a semi-micro Kjeldhal method. Ind. Eng. Chem., 8, 276-278.
- Waksman, S. A. 1944 Decomposition of lignin, in Wise, L. E. Wood Chemistry, p. 853. Reinhold, New York
- Waksman, S. A., and Hutchings, I. J. 1936 Decomposition of lignin by microorganisms. Soil Sci., 42, 119-130.
- Waksman, S. A., and Iyer, K. R. N. 1932 Contribution to our knowledge of the chemical nature and origin of humus. I. On the synthesis of the humus nucleus. Soil Sci., 34, 71-79.
- Wilson, Armine, T., and Bruno, Pauline 1950 The sterilization of bacteriological media and other fluids with ethylene oxide. J. Exptl. Med., 91, 449-458.
- Zobell, C. E., and Stadler, J. 1940 Oxidation of lignin by bacteria from ponds or lakes. Arch. Hydrobiol., 37, 163-171.

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Biochem., 23, 360-369.

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