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Abstract approved: _____

Annual ryegrass (Lolium multiflorum) a major grass seed crop in the Willamette Valley, Oregon, is presently burned in the fields after harvest, causing considerable air pollution and general wastage of a material which could be used in the manufacture of economically important products. The cellulosic component of straw is relatively susceptible to biodegradation but the lignin fraction, approximately 15%, is extremely resistant. Modification of the lignin to relieve recalcitrance would be valuable in the complete biological degradation of the straw and under controlled conditions, the cellulosic and modified lignin components could be used as substrates for the propagation of microbial populations leading to the production of single cell protein.

Klason lignin prepared from Lolium multiflorum was subjected

as a 2.5% w/v suspension, to 1.0, 2.5, 5.0, 10 and 15% H_2O_2 concentrations at pH 1 and 100 C until the peroxide was consumed. Rhodotorula mucilaginosa, which was unable to utilize the non-oxidized lignin as a sole source of carbon, was used to monitor the effect of lignin oxidation on growth.

Lignin oxidation substrates obtained from all concentrations of H_2O_2 exhibited increased growth response to the Rhodotorula. The substrate from the 2.5% H_2O_2 treatment gave the maximum growth response of yeast in terms of cellular dry weight. A soluble polymer-like material which formed during the initial stages of oxidation was not utilized and reduced the overall efficiency of substrate conversion to cellular material. The maximum conversion efficiencies, 21 and 86%, measured as dissolved lignin solids and total carbon respectively occurred in the 10% H_2O_2 substrate. However, maximum growth, 5%, relative to total dissolved lignin was observed in the 1.0% H_2O_2 substrate.

Gas-liquid chromatography-Rapid-Scan mass spectrometry and gas-liquid and thin-layer chromatography were used to determine lignin oxidation products. Succinic, lactic, maleic, malonic and oxalic acids were identified and methoxyhydroquinone, acetaldehyde, methyl alcohol, protocatechualdehyde, p-hydroxybenzaldehyde, vanillin, vanillic acid, and p-hydroxybenzoic acid tentatively identified. All of these compounds except methyl alcohol, oxalic and

maleic acids and methoxyhydroquinone were utilized by the Rhodotorula strain.

The appearance of protocatechualdehyde suggested that the degradation mechanism by H_2O_2 involved hydroxylation of the aromatic fragments. However, lack of muconic acids and the presence of succinic, oxalic, maleic, and malonic acids showed that a para-hydroxylation mechanism was predominant. The occurrence of lactic acid was postulated to occur from the C_3-side chain of the phenylpropanoid building block. This was supported by the oxidation of guaiacylglycerol-β-coniferyl ether and p-hydroxybenzoic, vanillic, and syringic acids. Lactic acid and guaiacol were obtained from the ether whereas only guaiacol was obtained from vanillic acid while the other two acids gave neither guaiacol nor lactic acid.

Formation of quinones and succinic, maleic, malonic and oxalic acids from p-hydroxybenzoic, vanillic, and syringic acids during H_2O_2 oxidation indicated that aromatic carboxylic acids were precursors to hydroquinones which were transformed to non-aromatic quinoid structures prior to cleavage to dicarboxylic acids. The mechanism was postulated to occur by a modified Dakin reaction.

The ability of Rhodotorula mucilaginosa to utilize the oxidation products obtained during treatment of the lignin with H_2O_2 suggests that H_2O_2 oxidation of lignin can greatly improve biodegradation. A similar approach on other lignins utilizing different oxidizing

agents and systems should be explored to obtain the maximum oxidative efficiency.

Hydrogen Peroxide Oxidation of Annual Ryegrass Lignin and Utilization of the Products by *Rhodotorula Mucilaginosa*

by

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INTRODUCTION

Microbial degradation of a potential substrate is related to the organisms enzymatic capabilities and on the structure and environment of the substrate. Most organic structures can be utilized by microorganisms but certain structures are recalcitrant to biological degradation. Lignin represents such a material and is extremely important in the natural biological dissimilation cycle since it is second only to cellulose in abundance.

The randomly polymerized phenylpropanoid subunits comprising the lignin structure are readily utilized by a number of organisms including bacteria, yeast, and filamentous fungi as are the phenylpropanoid oxidative cleavage products. However, the complex interunitary cross-linking of the subunits restricts macromolecular degradation to only a few fungi possessing polyphenoloxidase activity. The largest group within this category is found in the class Basidiomycetes and comprises the white-rot forms.

This study was undertaken to develop a chemical degradation system for ryegrass (Lolium multiflorum) lignin by acid-catalyzed hydrogen peroxide oxidation which would achieve the utilization of the oxidation products by Rhodotorula mucilaginosa. We wished to

determine conditions for maximum degradation of the lignin, to understand the mechanism(s) of oxidation and the ability of the Rhodotorula mucilaginosa to utilize the products and provide information which can be extended to the treatment of other biologically recalcitrant structures.

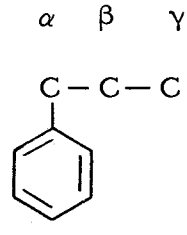
LITERATURE REVIEW

Lignin

Lignin, an encrusting material imparting structural integrity and microbial resistance to plant cell walls, exists in its natural state covalently bonded with celluloses (7, 70). It represents 10-38% of the dry weight of mature plant material, with cellulose, hemicelluloses, and extractants such as tannins, phlobophenes, lignans and coloring materials making up the remainder (6, 13, 15).

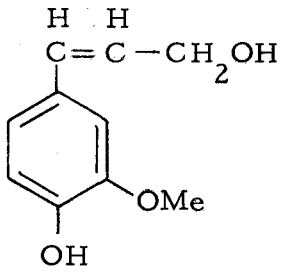
At present no single specific lignin molecule is known and no method has been developed for the isolation of the random structured macromolecule (10, 70). The isolation is particularly complicated by its tendency to repolymerize, particularly in acidic media, by self-condensation reactions of undefined mechanisms (7, 13, 14).

Although no definite structure has been demonstrated, the lignin macromolecule appears to be developed from a basic phenylpropanoid structure of the C_6-C_3 type (I) (14, 63).



(I)

Three main precursors are postulated from this basic phenylpropanoid structure; trans-coniferyl (I), trans-sinapyl (II) and trans-p-coumaryl alcohols (III) (35, 68, 70).



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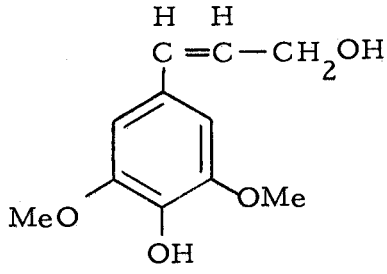

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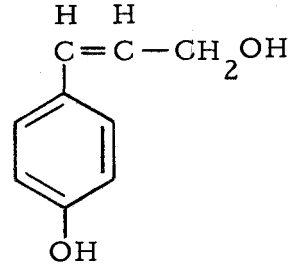
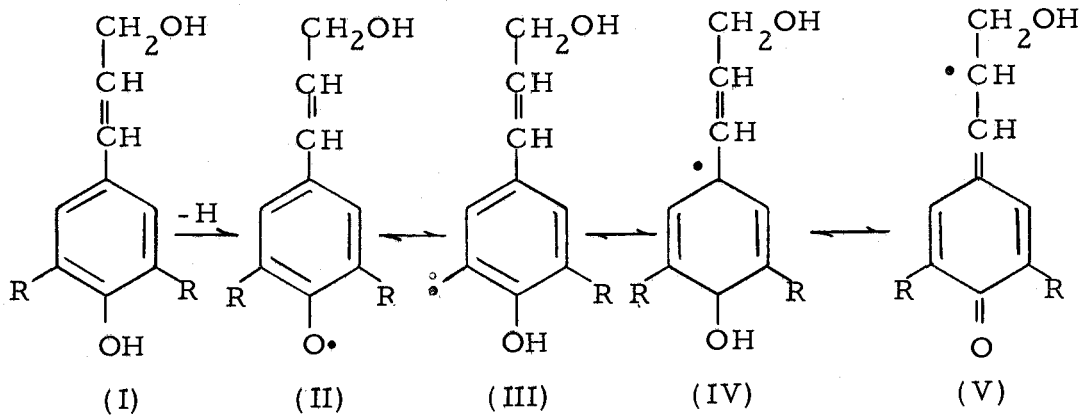


(III)

Early investigations by Klason (55, 56), Erdtman (31) and Freudenberg (34, 36, 37, 44, 64) suggested that lignin was the condensation product resulting from polymerization of these basic phenylpropanoid alcoholic precursors. They postulated that oxidative dehydrogenation of the phenolic hydroxyl group of the alcohol (I) resulted in mesomeric radical forms; namely, phenoxy (II), ortho-quinone (III), alicylic dienone (IV) and para-quinone methide (V) radicals.



(I) (II) (III) (IV) (V)

These various radicals were then considered to be neutralized by random pairing to give the lignin macromolecular structure.

Microbial Utilization of Lignin

In general: 1) only a limited number of organisms are capable of lignin decomposition; 2) the degradation is extremely slow and incomplete; 3) the age, source, chemical nature and isolation method influence its susceptibility to microbial attack; and 4) filamentous fungi such as the Basidiomycetes which produce polyphenoloxidase enzymes are more capable of lignin degradation than other microflora.

The literature (4, 43, 61, 65, 75) indicates that yeast-like fungi and bacteria do not possess polyphenoloxidase activity and are, therefore, incapable of degrading lignin. However, they are capable of degrading numerous lignin-like aromatic compounds representative of the phenolic building blocks (16) and their role in lignin degradation can only be assessed in terms of lignin-like model compound metabolism (47, 65).

The first attempt at elucidating microbial lignin degradation was made in 1955 by Higuchi et al. (50). Beech wood meal previously decayed by several white-rot fungi was extracted and in the extract coniferylaldehyde, vanillin and syringaldehyde were detected. Analysis of vanillin and syringaldehyde yields after nitrobenzene oxidation

of the decayed and fresh beech wood showed that the two aldehydes were reduced in the decayed wood. The ratio of syringaldehyde to vanillin was higher in the decayed wood, indicating that guaiacyl units were preferentially attacked. Further studies showed lignin added to the soil to be resistant to microbial decay (78, 79).

Ishakawa (52) using three Basidiomycetes white-rot fungi to degrade soft-wood lignin, isolated fifteen compounds from the culture supernatant fluid. The principal products were coniferyl alcohol, guaiacyglycerol, and guaiacylglycerol-β-coniferyl ether. These were further metabolized by side chain reduction and converted to protocatechuic acid or catechol, the reactions which form the focal points in the metabolism of aromatic compounds (48).

Formation of the β-O-4 dilignol, guaiacylglycerol-β-coniferyl ether, was common to all culture filtrates, and this phenomenon coupled with the observation that guaiacyl units decreased during decomposition suggested the dilignol was the first lignin cleavage product (50, 51, 53). It is presently considered the more important cross-linkage group relative to susceptibility to microbal degradation (1).

Oxidation of Lignin

Oxidations of the lignin macromoledule have classically been categorized according to the degree of lignin degradation; namely,

1) degradation to aromatic carbonyl and carboxylic acids, 2) cleavage of the aromatic structure, and 3) group-specific oxidations (71).

The first category includes oxidation in an alkaline medium with nitrobenzene, molecular oxygen, or metal oxides. This category is important in the elucidation of lignin structure because at least a semblance of the original form is represented by the aromatic oxidation products (11, 21). It is also industrially important for the production of useful chemicals such as vanillin and its homologs (11).

The second category comprises oxidation under acidic or neutral conditions, using peracetic acid, nitric acid, chlorine, chlorine dioxide, or hydrogen peroxide (71). These oxidants cause fragmentation of the aromatic nucleus and, therefore, are not satisfactory for lignin structural studies (11) but are industrially useful in the bleaching of pulp by selectively attacking holocellulose structures and destroying chromophoric groups (71, 74).

The last category includes reactions involving specific functional group analysis such as phenolic hydroxyl, benzyl and methoxyl groups (71).

The major distinction between oxidations under acid and alkaline conditions can be summarized as follows:

1. Alkaline oxidations require a para-hydroxyl group to the propyl side chain for effective lignin degradation to aromatic units, predominately aldehydic in nature.

2. Any condition which prevents formation of a quinone methide will greatly reduce the effectiveness of alkaline oxidation.
3. Acidic oxidation is generally initiated by ortho or para hydroxylation followed by quinone formation and finally cleavage of the non-aromatic ring to dicarboxylic acids.

Acid-Catalyzed Oxidations

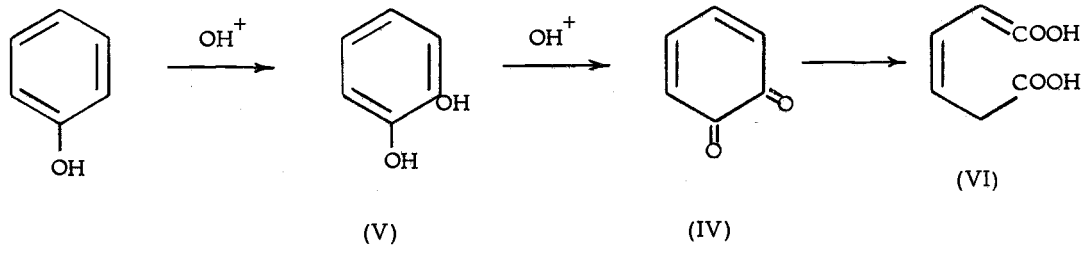
While lignin oxidation under alkaline conditions results primarily in lignin-like aromatic structures, oxidation in an acidic medium results in cleavage of the aromatic nuclei to dibasic acids (71).

Peracetic Acid Oxidation

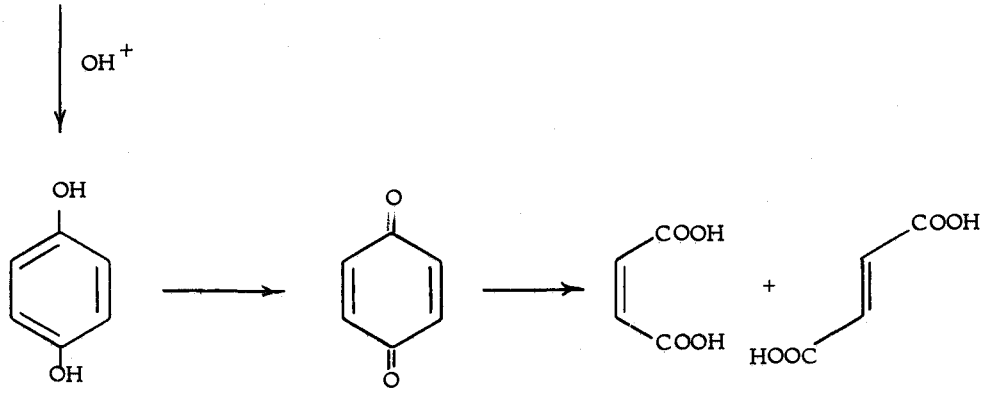
Perhaps the most thoroughly investigated acid-catalyzed oxidation system involves the effect of peroxy acids, mainly peracetic and perbenzoic acids, on lignin-like model compounds (72). However, their role in lignin-macromolecular degradation is still unknown (72).

Peracetic oxidation of phenols leads to ortho and para-hydroxylation reactions with the peroxy acid ^{+}OH ion attacking at an electron-rich site on the aromatic nucleus (26). Further substitution produces the corresponding quinone. Para-quinones are unusually stable in the presence of peroxide but ortho-quinones immediately undergo ring-fission to dibasic acids. Henderson (46), found that the oxidation

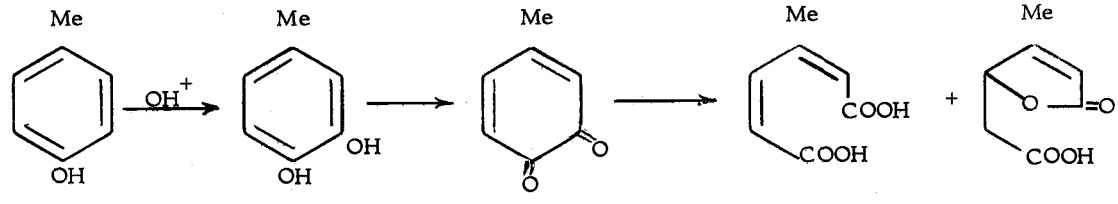
products detected depended on the degree and nature of phenolic substitution. Phenols which have only hydrogen in a position para to the hydroxyl group give predominately para-quinones (I) or para-dihydric phenols (II) depending on the reaction conditions. When the para position to the phenolic hydroxyl group is occupied (III) the formation of ortho-quinones (IV) or ortho-dihydric phenols (V) prevails. Continued oxidation of the ortho-phenols and ortho quinones results in the formation of muconic acids (VI) and lactones (VIII). Para-phenols and para-quinones are degraded predominately to maleic and fumaric acids (VII) (71).



(V) (IV) (VI)

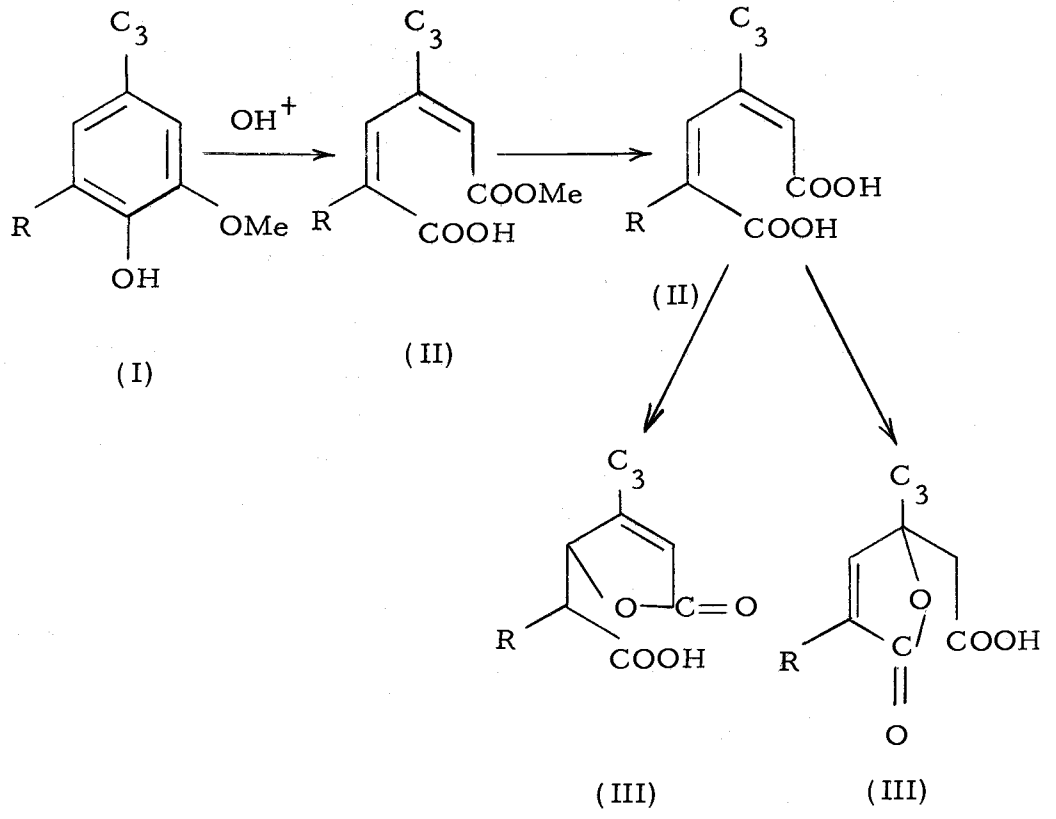


(II) (I) (VII)

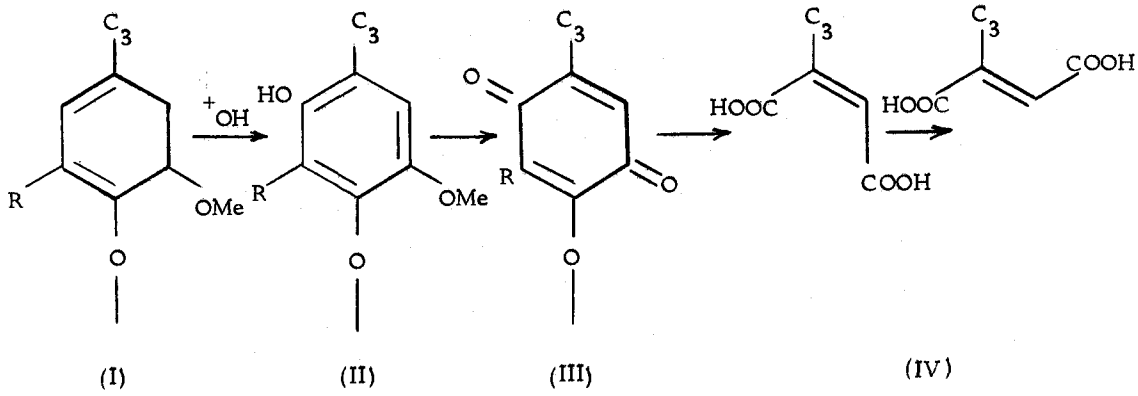


(III) (V) (IV) (VI) (VIII)

Paracetic oxidation studies of Douglas-fir meal (a softwood lignin) and red alder (a hardwood) showed that the majority of aromatic nuclei were cleaved to muconic acid structures via an ortho-quinone intermediate (58, 72). Muconic acid esters which contained some of the original methoxyl groups present in the aromatic precursors were also formed. The formation of lactones (III) was found to occur from muconic acid intermediates (II) which in turn resulted from direct cleavage of aromatics within the lignin molecules containing free phenolic hydroxyl groups (I).



However, aromatic units with the phenolic unit etherified (I) were the first oxidized to 3, 6 quinone intermediates (III) via a para-hydroxylation mechanism (II) and further to maleic acid (IV).



(I) (II) (III) (IV)

Hydrogen Peroxide Oxidation

Hydrogen peroxide is considered a major oxidant during alkaline pulp bleaching by forming benzoquinones from the unetherified units with subsequent fragmentation to colorless dicarboxylic acids (3, 71).

The oxidation of lignin by hydrogen peroxide under neutral or acidic conditions, however, has not been adequately investigated either with lignin or lignin-like model compounds (71). Two studies relating the effect of hydrogen peroxide on spruce lignin at pH 7 resulted in the identification of oxalic, malonic, succinic, glycolic, lactic, tartronic, malic, hydroxyglutaric, acetic, and formic acids in addition to several aromatic structures (2, 67). However, no mechanisms were proposed.

Hydrogen peroxide oxidation under acidic conditions is postulated to be analogous to peracetic acid oxidation because of the similarity of the ion species (71). The pH dependent ion species are shown below with the HO^+ species predominating at neutrality (76).

$$H_3O_2^+ \rightleftharpoons HO^+ \rightleftharpoons H_2O_2 \rightleftharpoons HO_2^-$$

A general survey of the literature relating hydrogen peroxide oxidation of lignin or lignin-like model structures in an acidic medium produced by the addition of such acids as sulfuric, hydrochloric, or nitric was uneventful. The conclusion was that the few studies of

hydrogen peroxide treatment of lignin have been carried out in a neutral to alkaline environment.

Specific information to date related to microbial growth response to oxidized lignin products is also lacking. Earlier studies have shown that sodium ligninsulfonate (29, 57, 69) and annual ryegrass (Lolium multiflorum) straw (62) can be oxidized photolytically to structures enhancing microbial growth. This information led to the present study of acid-catalyzed hydrogen peroxide oxidation of annual ryegrass (Lolium multiflorum) lignin and the effect on the growth response of a yeast (Rhodotorula mucilaginosa) when grown on the various oxidation products.

Lolium multiflorum lignin and Rhodotorula mucilaginosa were selected because this study is one phase of a two phase investigation concerned with alternate means of disposal of annual ryegrass straw (which is currently burned in the fields after seed harvest) to produce yeast single cell protein. The first phase involves the sulfuric acid hydrolysis of the cellulose and hemicelluloses leaving, predominantly, the lignin fraction. The second phase, the subject of this study, is concerned with 1) the mechanism of hydrogen peroxide oxidative modification of the lignin in a sulfuric acid environment and 2) the growth of a non-lignin utilizing yeast (Rhodotorula mucilaginosa) on the oxidation products.

MATERIALS AND METHODS

Straw Lignin Preparation

Klason lignin was prepared from annual ryegrass (Lolium multiflorum) using a modification of the technique recommended by Brauns (8).

Approximately 4000 g of annual ryegrass were homogenized with distilled water in a Waring blendor. The water was evaporated off in vacuo at 45 C and the straw residue dried at 100 C for 24 hours. The dried residue was shaken with glass beads in a large glass jar to break up the matted straw. Three and one-half kilograms were added to a large 40 liter carboy containing 35 liter of 72% reagent grade sulfuric acid. Cold tap water was allowed to flow down the outside of the jar to maintain a temperature of less than 15 C. The mixture was kept for 2 hours at 15 C while continuously mixing with a large magnet-magnetic stirrer combination. The contents were removed in five liter portions and each volume diluted with 10 liters of distilled water at a rate such that the temperature did not exceed 20 C. The lignin from each dilute volume was collected on a Buchner fritted disc coarse-pore funnel. The combined lignin samples were washed with 17.5 liters of 3% sulfuric acid, filtered and suspended in 35 liters of 3% sulfuric acid. Five liter portions were siphoned

off, refluxed 4 hours, filtered, and washed with distilled water until neutral to pHydrion paper. The lignin fractions were combined, dried at 100 C for 48 hours and placed in a desiccator over anhydrous calcium chloride. The lignin dry weight totaled 490 g which was 14% of the original straw. A lignin determination on five separate straw samples using TAPPI Standard T13m gave an average value of 13.9%. The lignin was a finely dispersed light brown powder and was shown to be carbohydrate-free by applying the Molisch test to a solution prepared by refluxing 1 g of the lignin preparation in 50 ml of 3% sulfuric acid for 24 hours.

The carbon content (see page 19) of the lignin was 62% and agreed well with Brauns who gave a range of 61-64% for graminae lignins (9). The methoxyl content (see page 31) was 5.74%.

Acid-Catalyzed Hydrogen Peroxide Oxidation

Lignin Oxidation

Ryegrass straw lignin was oxidized as a 2.5% w/v suspension in 1.0, 2.5, 5.0, 10 and 15% H_2O_2 concentrations prepared from 30% reagent grade H_2O_2 (Mallinckrodt Chemical Works, St. Louis, Mo.). The mixtures were prepared by adding 10.0 g of pre-dried constant weight lignin to 400 ml of the desired H_2O_2 solution and adjusting the pH to 1 with concentrated sulfuric acid. The mixture was refluxed

under a gentle boil. The concentration of H_2O_2 before and during refluxing was followed by iodometry (38). When the peroxide level reached zero, refluxing was stopped and the oxidation mixture centrifuged in 250 ml polyethylene bottles at 35,000 x g. The supernatant fluid was decanted in 100 ml portions into glass bottles and frozen until needed. Any remaining residue was washed twice with 250 ml of distilled water and centrifuged to remove residual water soluble oxidation products. The residue was added to pre-dried constant weight aluminum pans, dried at 100 C to constant weight and stored in a vacuum desiccator over anhydrous magnesium perchlorate.

Lignin-Like Model Compound Oxidation

Solutions were prepared by dissolving 2.5 g of p-hydroxybenzoic acid, vanillic acid (4-hydroxy-3-methoxy benzoic acid), syringic acid (4-hydroxy-3, 5-dimethoxy benzoic acid) and guaiacylglycerol-β-coniferyl ether in 100 ml of 2.5% H_2O_2. The pH was adjusted to 1 with sulfuric acid and the solutions refluxed under a mild boil for 96 hours. The samples were frozen until needed for analysis.

Polymer-Like Material Oxidation

A water soluble polymeric material was formed during the initial stages of the lignin oxidation. In order to study the chemical

character of this material, 1 liter of 1% H_2O_2 and 25 g of straw lignin mixture at pH 1 was refluxed until the peroxide was consumed. The dark brown supernatant fluid was filtered and lyophilized. The residue was dissolved in 100 ml of distilled water and 75 ml were then mixed with 740 ml of 95% ethyl alcohol. The resulting 85% ethyl alcohol solution caused a precipitate to form. Earlier work has shown that lignin-like polymers can be fractionated according to molecular weight ranges by adjusting the ethyl alcohol concentration (32). Compounds having molecular weights greater than 700-1500 are precipitated from an 85% ethyl alcohol solution. This range was selected to eliminate low molecular weight compounds which might be monomeric aromatic units because a relatively high molecular weight range was desired for oxidation studies without interference from these smaller compounds. The light brown precipitate which formed was collected on a porous glass medium pore filtering funnel and washed with 500 ml of 85% ethyl alcohol. The residue was dried at 100 C for 24 hours, 1.0 gram mixed with 100 ml of 2.5% H_2O_2 and the pH adjusted to 1 with concentrated sulfuric acid. Reflux was commenced at a gentle boil and continued for 96 hours. The supernatant fluid was then used for structural studies.

Carbonyl Determination

Carbonyl group content was determined using the 2, 4-dinitrophenylhydrazine colorimetric method of Critchfield (22).

Dissolved Lignin Solids

The concentration of dissolved lignin solids derived from solubilized lignin in the liquid phase of the H_2O_2 oxidation supernatant fluids was determined by adding 10 ml of the oxidation samples to tared aluminum pans, and drying for 24 hours at 100 C. The weight was expressed as mg/ml.

Carbon Dioxide-Carbon Determination

Carbon loss as CO_2 was measured gravimetrically as follows: The reflux unit containing the lignin-H_2O_2 mixture was fitted with a gas tight tube connection at the condenser outlet. The other end of the tube was attached to a flask containing 500 ml of concentrated sulfuric acid which was in turn connected to two drying tubes containing 20-30 mesh anhydrous magnesium perchlorate. A tube, attached to the outlet side of the second magnesium perchlorate tube, was joined to a cartridge containing 20-30 mesh caroxite (Fisher Scientific Co., St. Louis, Mo.). The evolved carbon dioxide was absorbed by the caroxite after passing through the drying train.

After each oxidation, the preweighted caroxite cartridge was disconnected and weighted. The drying train was recharged after each oxidation with fresh acid and anhydrous magnesium perchlorate. The complete oxidation of the lignin to carbon dioxide to determine the total carbon content was achieved by refluxing in 30% H_2O_2.

Carboxyl Determination

The neutralization equivalent (17) of the H_2O_2 oxidation supernatant fluids was determined by titration with 0.10 N sodium hydroxide standardized against potassium acid phthalate. A Corning Model 5 pH meter (Scientific Instruments, Medfield, Mass) with an 8 mm combination electrode #9007 (Broadley-James Corp., Fullerton, Calif.) was used to determine the end point.

Total Carbon Analysis

The total carbon (TC) analyzer system used in this study was a modification of the original unit described by Rockhill (69). The unit has a sensitivity of ±2.5 μg/ml and an operation time of 1 min/sample. The major changes were 1) the incorporation of silicon dioxide resistance heating elements which allowed the combustion temperature to be increased from 900 C to 1100 C, and 2) the design of a zirconium oxide combustion tube fused to a pyrex injection port.

A parts list of the components replacing the original items is

given below. The remainder of the unit is identical with the system described earlier.

1. Silicon dioxide rod resistance heating furnace. (Lindberg Engineering Co., Chicago, Ill).
2. Zirconium oxide combustion tube, 24 x 7/8 inch I. D. #7-586A, (Fisher Scientific Co., St. Louis, Mo.) fused to a pyrex injection port.

Twenty micro-liter samples were injected through the injection port. Three determinations per sample were made and their values averaged.

Standards were prepared from reagent grade acetic and benzoic acids within a range of 10-1000 μg/ml. A straight line was obtained by plotting carbon concentration (μg/ml) versus recorder reading. Necessary sample dilutions were made using distilled water previously boiled to eliminate dissolved carbon dioxide.

Gel Filtration

The brown, water soluble, polymeric-like material obtained during the initial stages of lignin oxidation was fractionated using Sephadex G-10 and G-25 gels (Pharmacia Fine Chemicals, Inc. Piscataway, N. J.). These gels have molecular weight exclusion values for dextrans of 700 and 5000 respectively. Only the material eluting in the last 5 ml of the void volume was collected for TC

analysis without regard for smaller structures eluting later. Adsorption of certain low weight aromatic structures in addition to wide range molecular weight polymers such as ligninsulfonates has been reported to occur on Sephadex (33, 41, 54, 60). This was obviated by the use of a .05 M salt solution in an alkaline environment (54).

One ml of the sample to be fractionated was added directly to the column after determining the void volume with blue dextran 2000 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The column and fractionation conditions were as follows:

1. Column
   A. K15/30 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.).
      Sephadex G-10 swollen in pH 11, 0.05 M sodium chloride, sodium hydroxide solution; volume-38 ml; void volume-10 ml.
   B. K 25/100 column (Pharmacia Fine Chemicals, Inc. Piscataway, N. J.)
      Sephadex G-25 medium swollen in pH 11, 0.05 M sodium chloride, sodium hydroxide solution; bed volume-61 ml; void volume - 30 ml.
2. Conditions:
   A. Sample size - 1.0 ml
   B. Flow rate - 0.5 ml/min through an automatic fraction

collector (LKB Ultrovac Fraction Collector, Type 7000, LKB-Producter AB, Stockholm-Brommal, Sweden)

C. Eluent - 0.05 M sodium chloride in pH 11 sodium hydroxide solution

D. Fraction size - 5 ml

E. Room temperature (23-25 C)

F. Column equilibrated by flowing 200 ml of eluent before applying sample.

Thin-Layer Chromatography (TLC)

Thin-layer chromatography determinations were performed on ether extracts and 2, 4-dinitrophenylhydrazone derivatives of the oxidation supernatant fluids before and after microbial growth. Continuous ether extraction for 48 hours was carried out on 50 ml samples after adjusting the pH to 1 with concentrated hydrochloric acid. The ether phase was then evaporated to dryness with a stream of nitrogen gas. The residue was dissolved in 5.0 ml of 95% ethyl alcohol. Twenty µl samples were applied directly to TLC plates. Lignin-like reference compounds were prepared by dissolving 10 mg in 2 ml of 95% ethyl alcohol. Twenty µl were applied as above.

The 2, 4-dinitrophenylhydrazones were prepared by mixing 5 ml of supernatant fluids with 5 ml of 0.01% 2, 4-dinitrophenylhydrazine (2, 4-DNPH) reagent in 4 N hydrochloric acid (77). The tubes were

allowed to set for at least 1 hour before the precipitate was removed by centrifugation, the precipitate washed twice with water and dried in vacuo. The precipitate was then dissolved in 2 ml of methyl alcohol and 20 µl applied to the TLC plate. Lignin-like reference compounds containing carbonyl functional groups were derivatized by adding 10 mg to 4.0 ml of 2,4-DNPH reagent, allowing to set one hour, centrifuging the precipitate, washing twice with distilled water and drying in vacuo. The precipitate was dissolved in 2 ml of methyl alcohol and 20 µl applied to the TLC plate.

Thin layer plates were prepared by spreading a 0.25 mm thick layer of 50% w/v silica gel G #08075 (American Optical Corp., Buffalo, N.Y.) in water on 8 x 8 inch glass plates. The plates were air dried and activated at 105 C for 30 minutes (66). Trimming 5 mm of silica gel off each edge increased the Rf reproducibility and increased the uniformity of the solvent front.

Benzene/dioxane/acetic acid (90:25:4), benzene/methanol/acetic acid (45:8:4) and toluene/2-butanone (230:20) were used as solvent systems (3, 66). Two hundred ml were added to 4.5 x 9 x 9 inch glass tanks (American Optical Corp, Model 02041, Richmond, CA) closed with glass lids. Tank saturation was achieved by immersing two 7 x 7 inch and two 4 x 7 inch pieces of Whatman #1 filter paper in the solvent. These sheets were placed against the sides of the tank with the lower edges immersed in the solvent.

Spray reagents for phenolic, carbonyl and carboxyl functional groups were prepared as follows:

1. Tetrazotized benzidine (66)

   Solution I - 5 g benzidine dissolved in 25 g of 25% hydrochloric acid and diluted to 1 liter with distilled water.

   Solution II - 10% aqueous sodium nitrite.

   Equal volumes of the two solutions were mixed before use.

   Phenolic - various colors depending on structure.

2. 2, 4-dinitrophenylhydrazine (77)

   0. 01% 2, 4-dinitrophenylhydrazine dissolved in 42 ml concentrated hydrochloric acid and the volume made up to 250 ml with distilled water.

   Carbonyls - yellow or orange spots

3. Bromocresol purple (66)

   0. 04 g bromocresol purple in 100 ml of 50% ethanol, pH 10,

   Carboxylics - yellow spots on blue background.

Solvent was removed from the developed TLC plates by placing in a 75 C forced air oven for at least twelve hours before spraying with the reagents. Rf values were reported as the mean of five separate developments. Quinone structures gave their own yellow color.

Gas-Liquid Chromatography (GLC)

GLC analysis was performed on a F & M instrument, model #402, equipped with a hydrogen flame ionization detector (Hewlett Packard, Avondale, Penn.) The standard chromatograph conditions were as follows:

Silyl Ethers and Esters

Injector temperature - 190 C

Detector temperature - 180 C

Isothermal column temperature - 150 C

H_2 - 55 ml/min

He - 50 ml/min

Air - 190 ml/min

Sample Size - 5 µl

A thirteen foot, 1/8 inch, stainless steel column was prepared according to Clark (18) with the exception that chromosorb W-AW, 100/120 mesh, #82-000044-00, (Varian Aerograph, Walnut Creek, Calif.) was used in place of Diatoport (F & M Scientific, Avondale, Penn.). The column packing was prepared by coating chromosorb W-AW 100-120 mesh with 25% Apiezon L, #LP-6, (Hewlett Packard, Avondale, Penn).

Silyl derivatives were prepared according to Dalgliesh et al. (24).

Pyridine was prepared by refluxing reagent grade pyridine (J. T. Baker, Morristown, N. J.) over potassium hydroxide pellets for four hours, and then collecting by distillation. To a 10 ml glass tube were added 2.5 ml of the ethyl alcohol solution from the ether extract of the H_2O_2 oxidation supernatant fluids or 1 ml of the lignin reference compound in ethyl alcohol. Aliphatic acids were weighed directly into the tubes. The alcohol and any residual water were evaporated off in a stream of dry nitrogen gas. Pyridine, 1.0 ml containing 20 mg/ml of veratrole (Aldrich Chem. Co., Milwaukee, Wis.) as an internal standard was added to each tube followed by 0.90 ml hexamethyldisilazane (Sigma, St. Louis, Mo.) and 0.60 ml trimethylchlorosilane (Sigma, St. Louis, Mo.). The tubes were sealed with teflon-lined screw caps and the contents mixed and allowed to set for at least 24 hours. Quantitative conversion of phenolic and carboxyl groups to silyl ethers and esters was then considered complete. Complete conversion was determined by silylating approximately 10 mg of the reference compounds for 5, 10, 20, 30, and 40 hours as above and injecting into the GLC with veratrole as the internal standard. Quantitative conversion was complete by 20 hours. The tubes were centrifuged to remove the ammonium chloride which formed and 5 µl of the supernatant fluid were injected into the GLC.

Identification of GLC peaks was made by injecting standard reference silylated compounds mixed with the silylated oxidation

solution and determining peak addition. Also, a standard plot of homologous series carboxylic acids was made to give additional supportive evidence for structural identification. Mass spectrometric identification of methylated structures present in the oxidation solution also made it possible to use known reference compounds for peak identification. Peak area was determined by multiplying the height by one-half the width at half peak height. The concentration was then directly proportional to the veratrole standard concentration. Methyl alcohol was quantitated by injecting 5 μl of H_2O_2 supernatant fluid directly into the GLC and comparing to the veratrole standard.

Methyl Ethers and Esters

Methyl ethers and esters were prepared from dimethyl sulfate and sodium hydroxide (3), methyl alcohol and sulfuric acid (77) and diazomethane prepared from Diazald (Aldrich Chemical Co., Milwaukee, Wis.). Quantitative methylation could not be achieved by any of these methods and the phenolic materials appeared to polymerize into a black viscous material which interefered with the overall reaction. However, qualitative conversion was adequate to allowed GLC-mass spectrometric identification of dicarboxylic aliphatic structures. The following operating conditions were maintained.

Injector temperature 165 C

Detector temperature 170 C

Isothermal column temperature 135 C

H_2 = 50 ml/min

He = 45 ml/min

Air = 190 ml/min

Sample size - 1.0 μl

A ten foot, 1/8 inch, stainless steel column was prepared according to Bicho (5). Twenty percent Carbowax 20 M, # LP36, (F and M Scientific, Avondale, Penn) was adsorbed onto Chromosorb W-AW 60-80 mesh. The column was cured for 24 hours at 200 C before use.

GLC-Rapid-Scan Mass Spectrometry

Mass spectra (MS) of the methylated derivatives already described, were obtained using a flame ionization GLC, Model 810, (F & M Model, Avondale, Penn) integrated with a rapid-scan mass spectrometer (Atlas MAT CH-4 Nier type). The column conditions were identical with those described for GLC-methyl ethers. One-fifth of the column effluent was led into the GLC ionization detector with the remainder passing to the heated electron-capture (EC-1) inlet. Constant MS monitoring of the structures entering the instrument was maintained with a 20 eV source while a 70 eV source

gave ionization spectra.

Spectrophotometric Determination

Phenolic Hydroxyl Groups

Difference spectra at 300 nm according to Goldschmid (42) to determine the phenolic content of the H_2O_2 oxidation supernatant fluids were obtained on a Zeiss absorption spectrophotometer, Model PMQII (Oberkochen, West Germany), integrated with a quartz prism monochromator, Model M4QIII (Carl Zeiss, Oberkochen, West Germany). The units were powered by a Zeiss transformer, Model #506964/50HZ. Matched 1 cm quartz cells, #46005 (Beckman Instruments Co., Fullerton, California) were used to contain the sample. Appropriate serial dilutions with buffer were prepared when necessary to keep the optical density between 0.3 and 0.6.

Aromatic Nuclei

Absorption at 280 nm was used to determine the presence of aromatic nuclei in the oxidation supernatant fluids. The percent of aromatic nuclei was calculated using an absorptivity value of 3810 for Bjorkman lignin at 280 nm (72). Appropriate serial dilutions were prepared as above.

Methoxyl Determination

The residues remaining after refluxing with 1.0, 2.5, 5.0, 10 and 15% H_2O_2 concentrations were sent to Mikroanalytisches Laboratorium, 53 Bonn, Buschstrate for analysis.

Reference Model Compounds

Analytical grade phenolic lignin-like model compounds and aliphatic acids were purchased when available. However, quinone structures had to be synthesized in this laboratory. The preparative procedures are given below:

1. Methoxyhydroquinone (3)

   This compound was produced in a 25% yield, from vanillin and hydrogen peroxide. After recrystallization from ether it had a melting point of 88 C; the literature reports 88.5-89 C (30).

2. Methoxy-p-benzoquinone (30)

   This compound was prepared from methoxyhydroquinone and lead peroxide with a 29% yield. Recrystallization from hot benzene gave yellow crystals with a melting point of 143 C; the literature reports 143-144 C (30).

3. 2,6-dimethoxy, 1,4-benzoquinone (40)

   Reacting 1,2,3-trimethoxybenzene with perbenzoic acid produced a 3% yield. The melting point was 255 C; the literature

reports 226 C (40).

Growth Conditions for Rhodotorula muscilaginosa on Oxidized Straw-Lignin Supernatant Fluids and Model Compounds

Rhodotorula mucilaginosa was obtained from Dr. Ronny Ferm, Department of Microbiology, Uppsala Sweden. The Rhodotorula strain was originally isolated from sulfite liquor waste and was routinely transferred onto 2% agar slants containing 10 ml of a ten-fold mineral salts solution and 90 ml of 0.1% glucose solution. The concentrated mineral salts solution with a final pH of 5.5, contained the following in g/ml: NH_4Cl-10, KH_2PO_4-50, Na_2HPO_4-10, $MgSO_4 \cdot 7 H_2O$-5 and thiamine hydrochloride-5×10^{-4}.

Fifteen ml of mineral salts solution were added to 135 ml of H_2O_2 oxidation supernatant fluid previously adjusted to pH 5.5 with 5 N sodium hydroxide or hydrochloric acid. The solution was passed under pressure through a sterilizing Millipore filter, # RAW PO47 (Millipore Corporation, Bedford, Mass). The sterile solution was split aseptically into three 50 ml portions and each portion added to a sterile baffled 300 ml sidearm flask, 14 x 130 mm (Bellco Glass, Inc., Vineland, N.J.), inoculated and secured on a gyrotory shaker, Model S-3 (New Brunswick Scientific Co., New Brunswick, N.J.). The speed was set at 250 rpm and the inoculated samples maintained at room temperature (23-25 C).

The cells to be used for inoculation were taken from a mid-exponential growth in a medium containing mineral salts and glucose, and washed 3 times with sterile mineral salts solution. The cells were then diluted with mineral salts solution to a Klett-Summerson (Klett Manufacturing Co., New York) colorimeter, containing a number 42 filter, reading of 100 and 0.1 ml of the suspension added to each flask.

All growth responses were monitored with the same instrument. Klett readings were converted to Rhodotorula dry weight units since it was found that a direct proportionality existed between Klett units and cellular dry weight within the range used. Earlier experimental data relating Rhodotorula growth on glucose, succinic acid, p-hydroxybenzoic acid, vanillin, and the lignin oxidation supernatant fluids showed that there was a direct correlation between the Klett reading and cellular dry weight throughout the incubation period.

Dry weight determinations were performed as follows: after incubating for the desired time, the contents were added to 100 ml plastic centrifuge tubes. The tubes were centrifuged at 4000 x g for 10 minutes and the supernatants decanted into clean, dry, teflon lined screwcap glass tubes. The cellular residue remaining in each flask was washed into its corresponding centrifuge tube with distilled water and the contents gently swirled on a Vortex mixer, model #S8223 (Scientific Products, Evanston, Ill). Enough water was added to

make a total volume of approximately 80 ml and the tubes centrifuged as before. The supernatant was decanted and discarded. Two more washings were performed. The cellular material was washed into tared aluminum pans with distilled water, dried for 24 hours at 105 C, cooled in a desiccator over anhydrous magnesium perchlorate and weighed. The values from the 3 flasks were averaged.

RESULTS

Hydrogen Peroxide - Lignin Effects

Hydrogen Peroxide Uptake

Hydrogen peroxide uptake by the lignin at 100 C was greatly influenced by the initial H_2O_2 concentration. The time necessary for H_2O_2 elimination, however, was essentially the same for all concentrations used, Figure 1. Straight line kinetics for 1.0, 2.5 and 5.0% H_2O_2 concentrations were displayed over the entire 70 hour interval, showing that the decrease in H_2O_2 was proportional to reflux time. The 10 and 15% samples showed a different type of reaction kinetics with straight line portions assuming steeper slopes between 10 and 30 hours. The lines then leveled off at approximately 30 hours and became essentially congruent with the 1.0 and 2.5% H_2O_2 samples.

Hydrogen Peroxide Dissolution of Straw-Lignin

Lignin dissolution was dependent on but not proportional to the H_2O_2 concentration, Figure 2. One percent H_2O_2 dissolved approximately 64% of the solid lignin and increasing the concentration 2.5, 5.0 and 10 and 15 fold did not produce a proportional increase in lignin dissolution. The data showed that increasing the H_2O_2 concentration to 15% relative to 1.0% H_2O_2 increased lignin dissolution only by

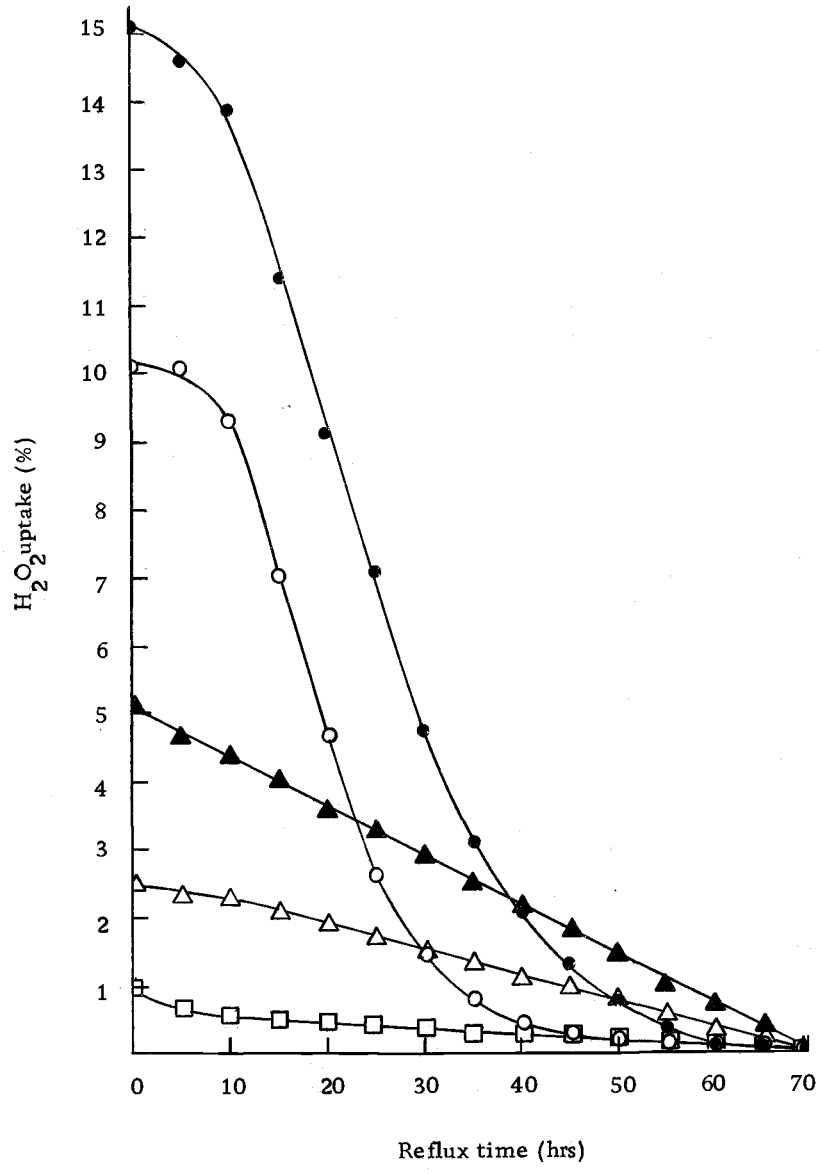


Figure 1. Uptake of 1.0, 2.5, 5.0, 10 and 15% H_2O_2 by 2.5% straw-lignin suspensions at pH 1 and 100 C.

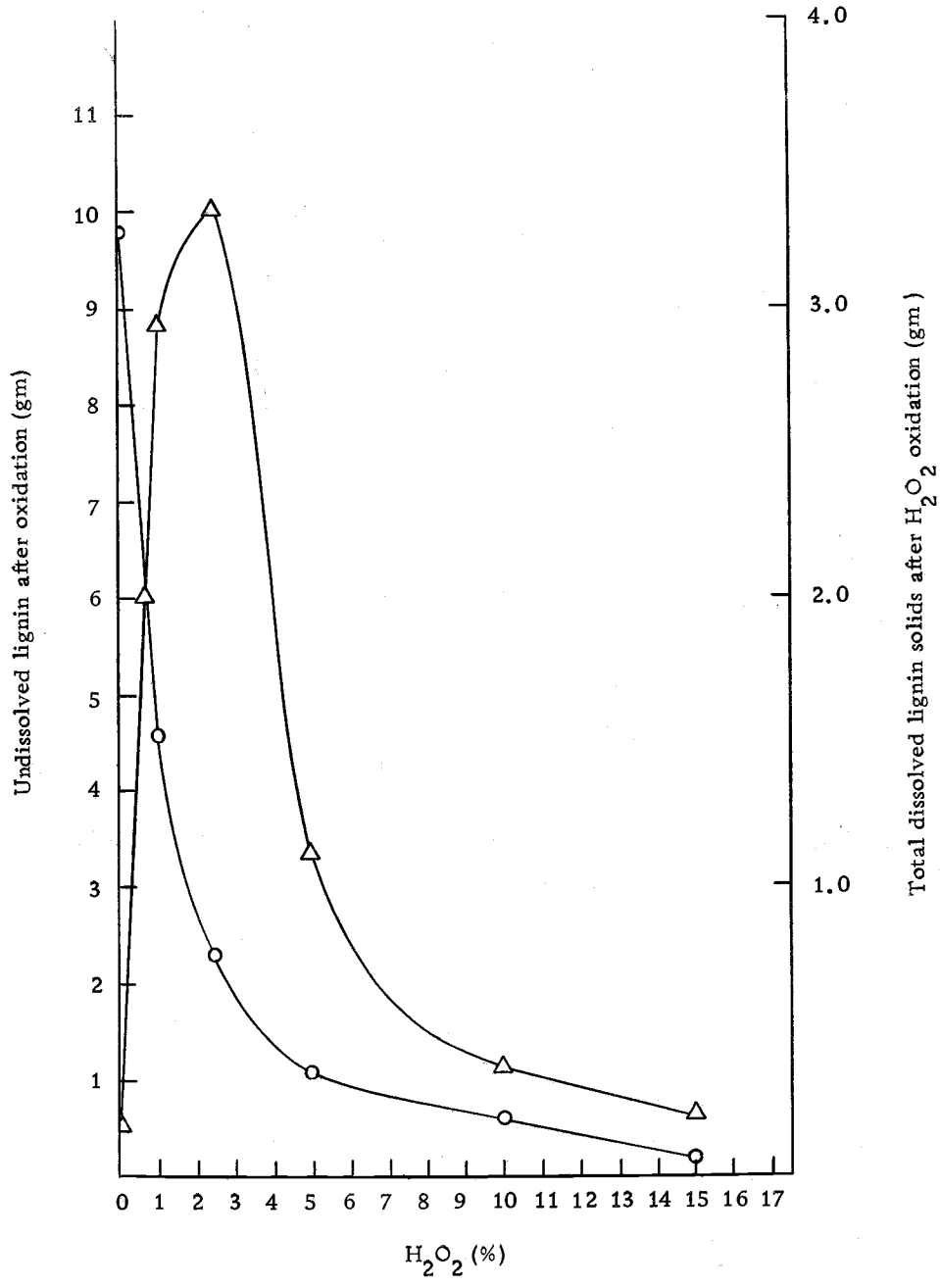


Figure 2. Effect of 1.0, 2.5, 5.0, 10.0 and 15.0 percent H_2O_2 on the dissolution (o-o) of straw-lignin and dissolved solids (Δ-Δ) at pH 1 and 100 C.

35% over that obtained at 1% concentration. Extrapolation of the solid lignin curve from 10.0 on the ordinate to the abscissa suggests that total dissolution of the lignin should have been complete by using 2.0% H_2O_2 had there been a proportionality established.

Dissolved lignin solids followed a similar curve to undissolved lignin. Maximum dissolved solid content was attained in the 2.5% H_2O_2 sample. A shoulder in the curve between 1.0-2.5% H_2O_2 also showed that lignin dissolution was not proportional to H_2O_2 concentration in this range. Hydrogen peroxide concentrations greater than 2.5% decreased the amount of dissolved lignin solids.

CO_2-Carbon and Soluble Total Carbon Effects

Figure 3 shows the effect of H_2O_2 concentration on carbon loss as carbon dioxide and total carbon (TC) remaining in solution. Maximum TC occurred with 2.5% H_2O_2. At this point, 3500 µg/ml TC remained while 42% of the lignin carbon was lost as carbon dioxide. No carbon loss was detected in the 1.0% H_2O_2 oxidation sample.

Carbonyl and Carboxyl Group Formation

The oxidation of straw-lignin with varying concentrations of peroxide had an effect on the formation of soluble carbonyl and carboxyl functions found in the supernatant fluid, Figure 4. Maximum carbonyl group formation was found in the 1.0% H_2O_2 oxidation

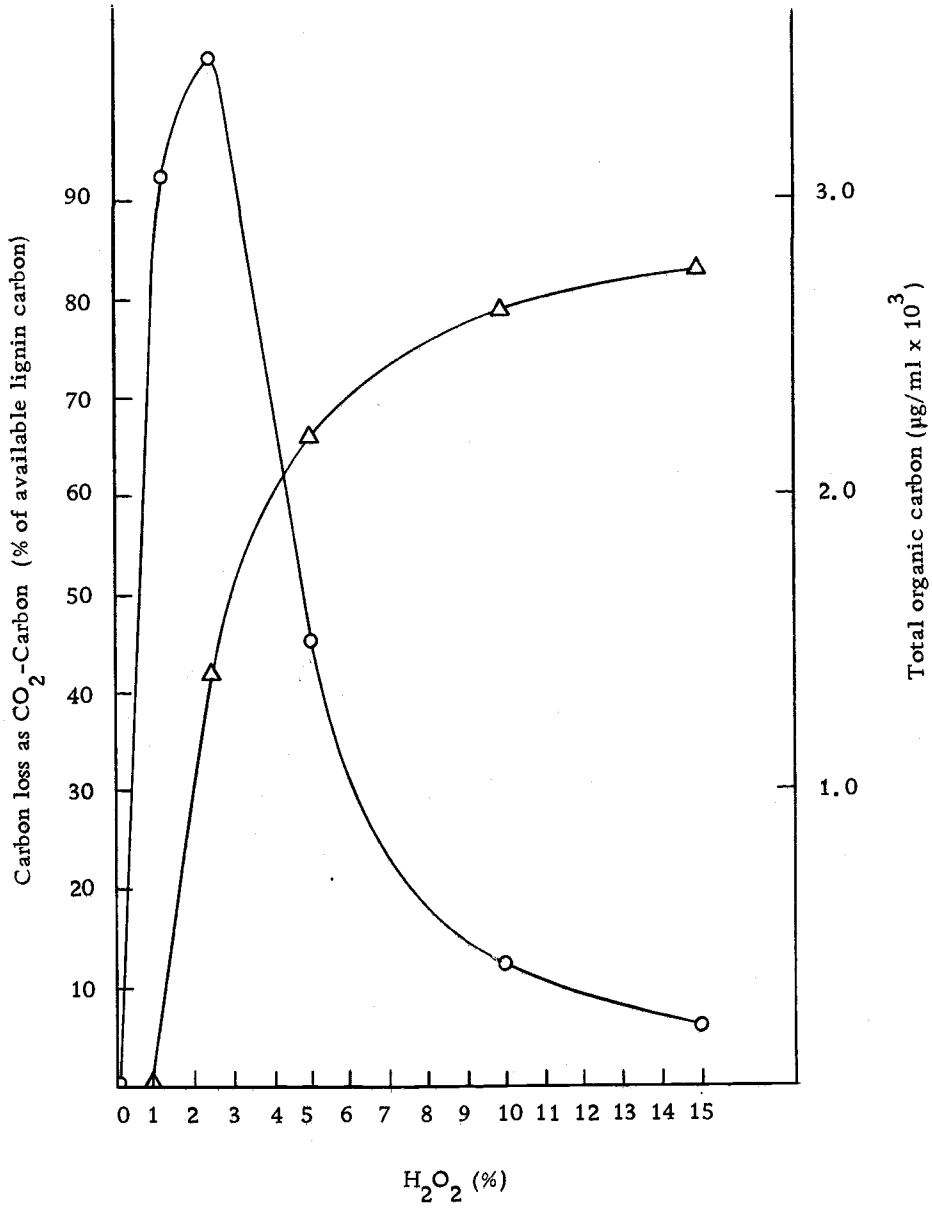


Figure 3. Effect of H_2O_2 concentration on total carbon content (o-o) remaining in solution and loss as CO_2-Carbon (Δ-Δ) during oxidation of straw lignin at pH 1 and 100 C.

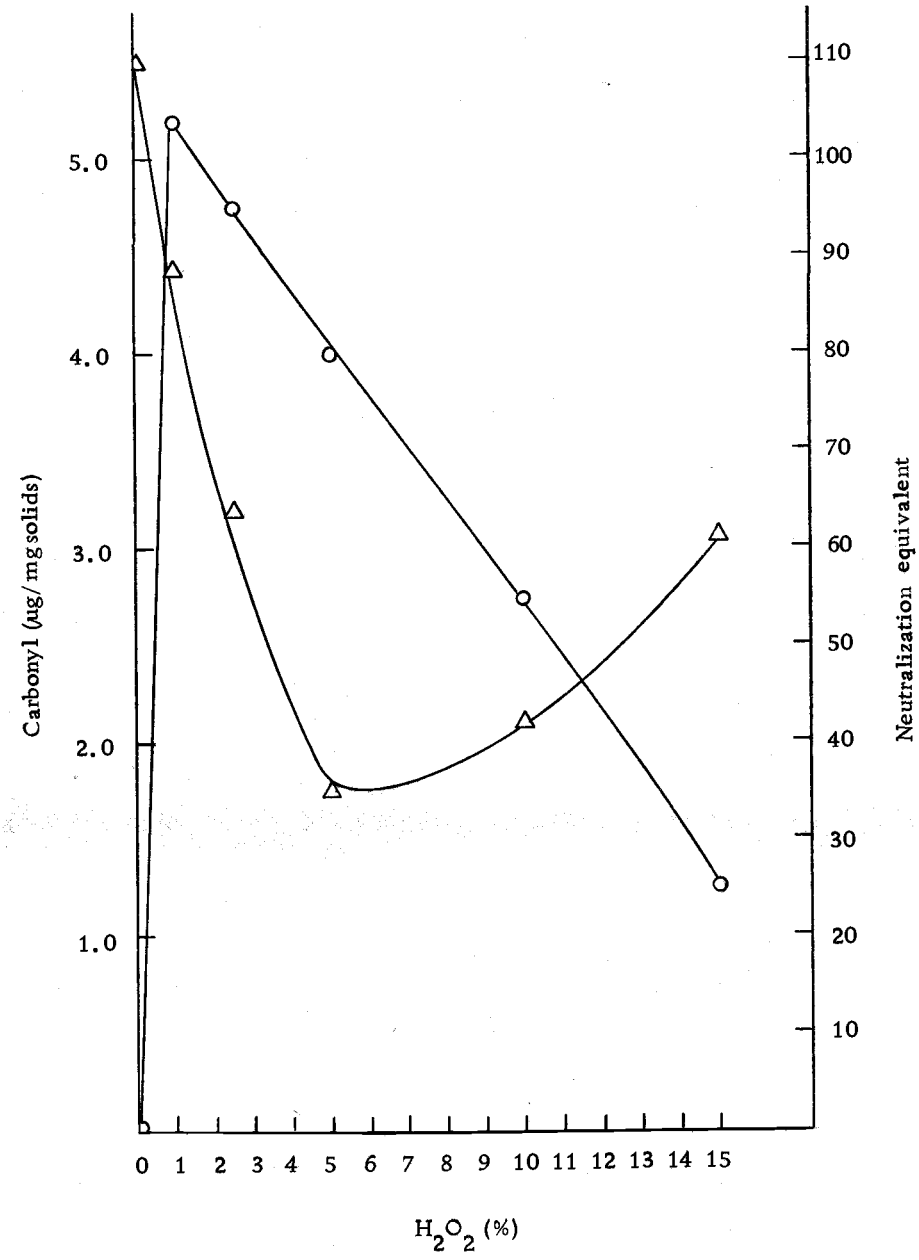


Figure 4. Effect of 1.0, 2.5, 5.0, 10 and 15% H_2O_2 oxidation of lignin on the formation of carbonyl (o-o) and carboxyl (Δ-Δ) groups at pH 1 and 100 C.

mixture, reaching a value of 5.2 μg/mg of supernatant fluid solids. A linear decline in carbonyl content was then established reaching a minimum value of 1.2 μg/mg of the dissolved solids at 15% H_2O_2. The neutralization equivalent reached the minimum value, 35, at 5.0% H_2O_2 concentration. At this peroxide concentration, maximum carboxyl functional group formation had occurred and was then followed by a decrease in acidic groups as evidenced by the increasing neutralization equivalent values.

Aromatic Nuclei and Phenolic Hydroxyl Formation

Figure 5 shows that the highest content of aromatic nuclei occurred in the 1.0% H_2O_2 oxidation mixture. Rapid destruction then occurred with 2.5-15% H_2O_2. At 1.0 and 2.5% H_2O_2 concentrations, 4.0 and 1.0% respectively of the aromatic nuclei were destroyed. Oxidation with 5.0% H_2O_2 however increased the proportion of aromatic nuclei destroyed to 75%. The increase in destruction beyond that level was then more gradual, reaching values of 89 and 91% for 10 and 15% H_2O_2 concentrations, respectively.

A maximum value of 1.4% phenolic hydroxyl occurred in the 1.0% H_2O_2 oxidation mixture. This peak value agreed nicely with the aromatic nuclei peak. A rapid decline then occurred reaching a value of zero in the 5.0% H_2O_2 oxidation mixture.

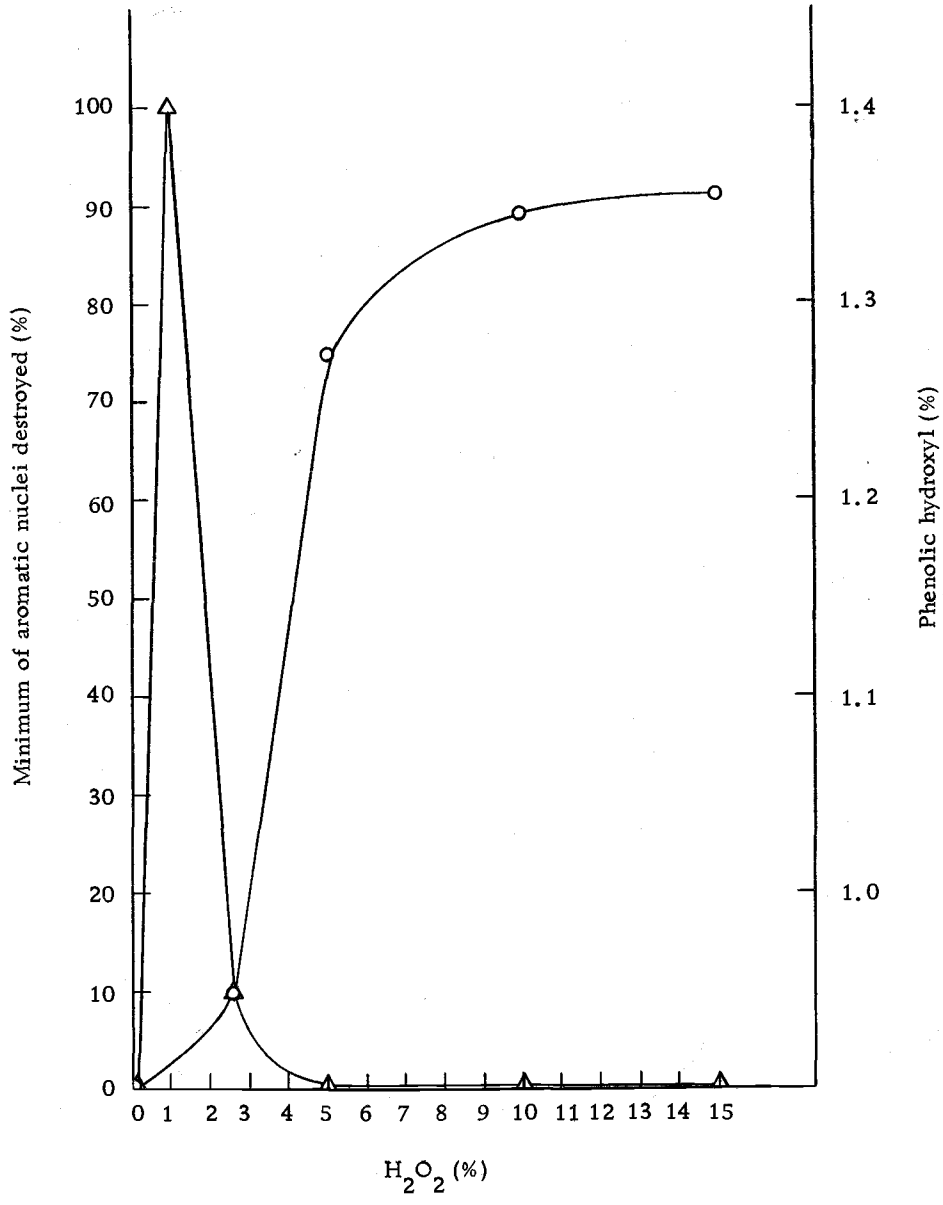


Figure 5. Effect of H_2O_2 concentration, at pH 1 and 100 C, on the destruction of lignin aromatic nuclei (o-o) and formation of phenolic hydroxyl groups (△-△).

Demethoxylation and Methyl Alcohol Formation

Lignin demethoxylation and methyl alcohol formation are shown in Figure 6. At least 22, 47 and 87% methoxyl group loss occurred in the 1.0, 2.5 and 5.0% H_2O_2 oxidation mixtures, respectively. Oxidizing with 15% H_2O_2 only increased the demethoxylation to 96%. Again the greater differential loss of methoxyl groups occurred with 1.0% H_2O_2 followed closely by the 2.5 and 5.0% samples. The greatest increase in methyl alcohol, approximately 19% of the theoretical amount, also occurred in the 1.0% H_2O_2 oxidation solution. This value represented 66% of the total methyl alcohol level achieved with the 15% sample. The theoretical amount of methyl alcohol was based on the stoichiometric converesion of methoxyl to alcohol by the formula, $CH_3O \xrightarrow{H} CH_3OH$.

Oxidation Structures

The concentration and distribution of the aliphatic acids determined by GLC at the various H_2O_2 concentrations is shown in Table 1. Lactic acid reached a maximal level in the 2.5% H_2O_2 oxidation solution, decreased in the 5.0% of H_2O_2 solution and became constant at 152 μg/ml in the 10 and 15% H_2O_2 samples. Succinic and oxalic acids reached maximum values in the 5.0% H_2O_2 oxidation solution and both decreased rapidly at higher H_2O_2 concentrations. Maleic and malonic

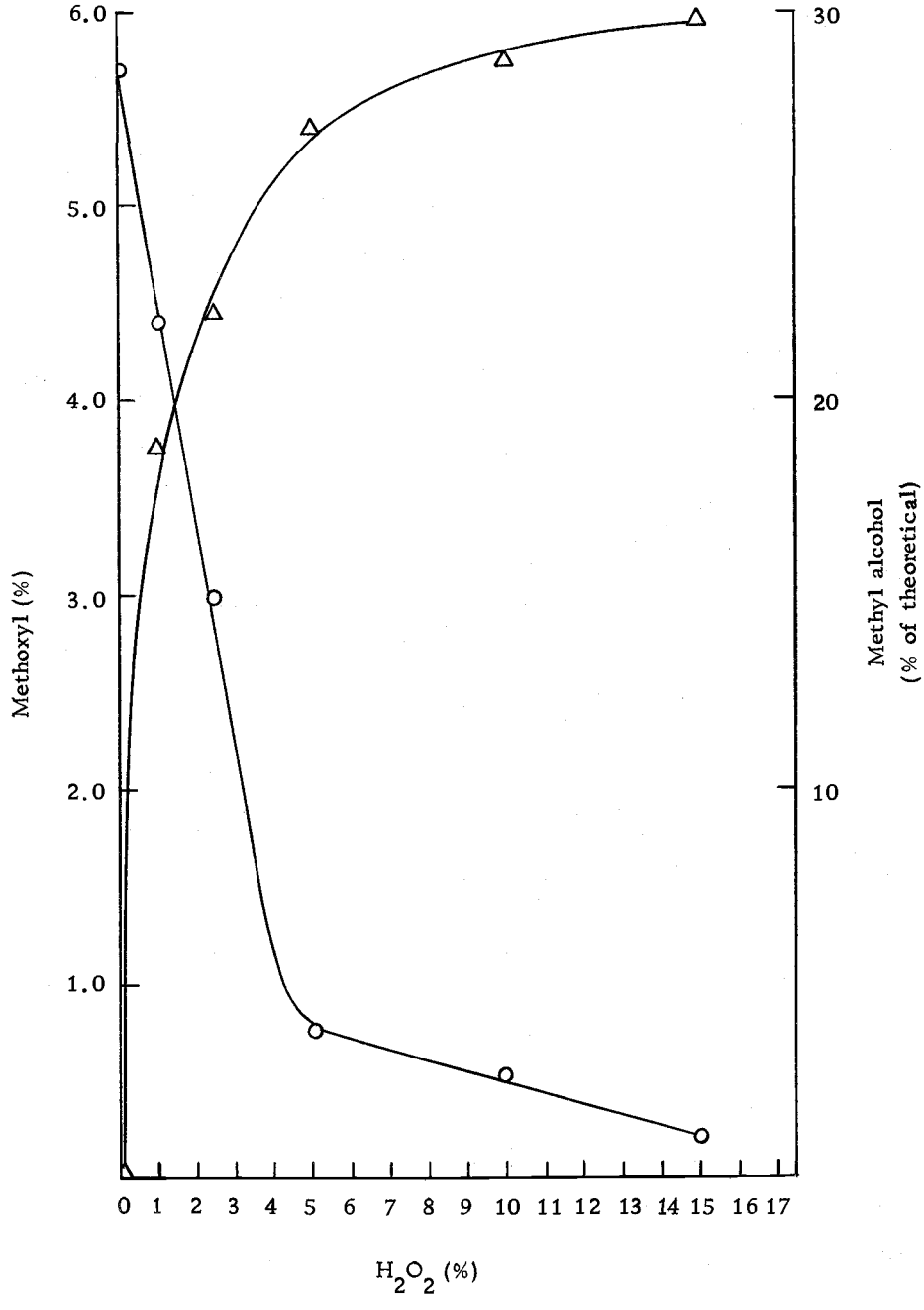


Figure 6. Loss of methoxyl (o-o) and formation of methyl alcohol (Δ-Δ) during H_2O_2 oxidation of straw lignin at pH 1 and 100 C.

Table 1. Gas-liquid chromatographic retention times (RT) and concentrations (μg/ml) of aliphatic carboxylic acids extracted from hydrogen peroxide-lignin oxidation solutions, also presence (+) of acids in polymer-like oxidation solution.

Carboxylic Acids	RT (min)	hydrogen peroxide (%)						polymer
		0.0	1.0	2.5	5.0	10.0	15.0	
unidentified	9.2	--	--	8	11	76	63	+
lactic	10.0	--	336	796	203	152	152	-
oxalic	11.7	--	128	150	162	13	6	+
unidentified	14.1	--	339	337	283	232	126	+
malonic	19.1	--	71	50	25	16	--	+
maleic	26.0	--	185	82	40	15	--	+
succinic	35.0	--	1220	1560	2490	380	285	+

acids attained maximum levels in the 1.0% of H_2O_2 oxidation mixture and were undetectable in the 15% H_2O_2 samples.

The unidentified acid with a retention time of 9.2 minutes increased to a maximum value in the 10% H_2O_2 mixture and was not detectable in the 1.0% oxidation mixture. The other unidentified acid, retention time of 14.1 minutes, attained a maximum level in the 1.0% H_2O_2 mixture and decreased at a slow rate in higher H_2O_2 concentrations.

The acid structures detected in the oxidation solution of the polymer-like material were oxalic, maleic, malonic, succinic, and the two unidentified structures. No lactic acid was detected.

Carboxylic structures identified by GLC as silyl derivatives in the ether extract of the supernatant fluids after H_2O_2 oxidation of p-hydroxybenzoic, vanillic, and syringic acids and guaiacylglycerol-β-coniferyl ether are shown in Table 2. Succinic acid was common to all four lignin-like model compounds. Malonic, maleic and oxalic acids were common to p-hydroxybenzoic and vanillic acids, and guaiacylglycerol-β-coniferyl ether. Guaiacol was formed from vanillic acid and guaiacylglycerol-β-coniferyl ether. Para-hydroxybenzoic acid also gave the unidentified product (refer to Table 1) with a retention time of 14.1 minutes while lactic acid was produced only from guaiacylglycerol-β-coniferyl ether. All four model structures formed a known water insoluble polymer-like material.

Table 2. Carboxylic acid structures identified by GLC and benzoquinones by TLC after hydrogen peroxide oxidation, pH 1 and 100 C, of lignin-like reference aromatic structures.

reference structures			
p-hydroxybenzoic acid	Vanillic acid	syringic acid	guaiacylglycerol-β-coniferyl ether
malonic acid	malonic acid	-	malonic acid
maleic acid	maleic acid	-	maleic acid
succinic acid	succinic acid	succinic acid	succinic acid
oxalic acid	oxalic acid	-	oxalic acid
benzoquinone	-	-	-
unidentified (RT 14.1)	-	-	-
-	methoxy-p-benzoquinone	-	methoxy-p-benzoquinone
-	-	3,6-dimethoxy-p-benzoquinone	-
-	-	-	lactic acid
-	guaiacol	-	guaiacol

Para-benzoquinone structures were also formed from their respective acids and methoxy-p-benzoquinone was formed from guaiacylglycerol-β-coniferyl ether. The oxidation of glycerol, not shown, did not result in lactic acid formation.

Growth Response

The growth response of Rhodotorula mucilaginosa to the straw lignin oxidation supernatant fluids was varied as evidenced by Figure 7. Rhodotorula growth in supernatant fluids obtained from 1.0, 2.5 and 5.0% H_2O_2 oxidations resulted in an initial lag time of approximately 10 hours. This was followed by rapid growth which reached cellular dry weight values of 0.36, 0.44 and 0.33 mg/ml for the 1.0, 2.5 and 5.0% H_2O_2 solutions, respectively, 20 hours after inoculation. The substrates from the 1.0 and 2.5% H_2O_2 oxidation supernatant fluids then exhibited a slower growth rate between 20 and 86 hours where cellular dry weights obtained were 0.68 and 0.78 mg/ml for the two peroxide concentrations, respectively. The 5% oxidation supernatant fluid supported a true diauxic growth response with the first stationary phase occurring at 28-68 hours and the second growth phase reaching a maximum dry weight yield of 0.60 mg/ml 144 hours after inoculation. The 10% H_2O_2 oxidation supernatant fluid exhibited an initial 10 hour lag followed by normal growth response which reached a maximum cellular dry weight value after 30 hours

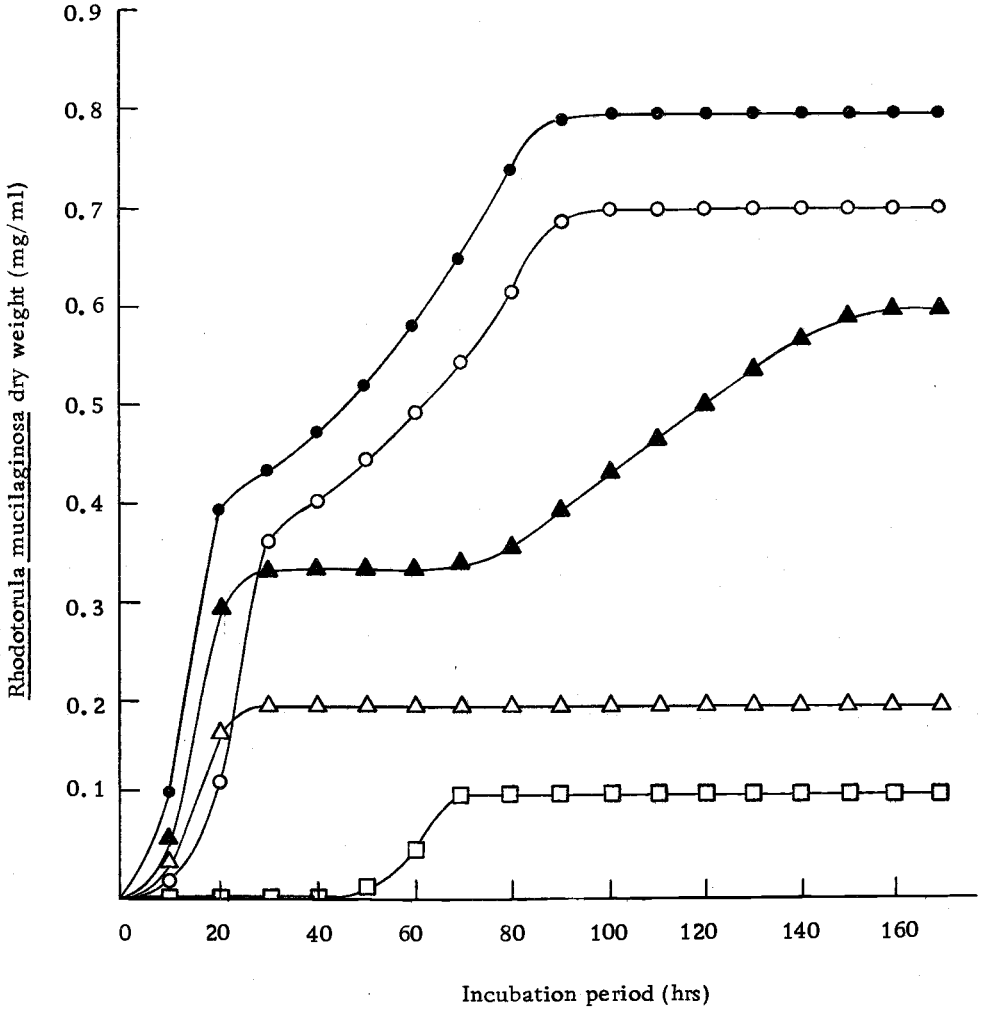


Figure 7. Growth response of Rhodotorula mucilaginosa to supernatants from 1.0 (o-o), 2.5 (●-●), 5.0 (▲-▲) 10 (△-△) and 15% (□-□), H_2O_2, pH 1 and 100 C, straw-lignin oxidation supernatant fluids.

incubation. A similar pattern was exhibited by the 15% H_2O_2 sample except the initial lag lasted for approximately 44 hours.

Table 3 shows the relationship between Rhodotorula dry weight, hydrogen peroxide concentration used to oxidize the straw lignin, and TC content at 24 and 144 hours. The percent utilization of available TC by Rhodotorula increased as the concentration of H_2O_2 was increased for both 24 and 144 hour growth conditions. However, 1.0, 2.5 and 5% H_2O_2 oxidation supernatant fluids gave the greatest increase at 144 hours over their counterparts at 24 hours. Total carbon utilization in the 10 and 15% H_2O_2 oxidation supernatant fluids was identical at 24 and 144 hours.

Conversion of the dissolved lignin and soluble solids in the oxidation solutions to cellular dry weight is also shown in Table 3. A relatively low conversion rate was noted for 1.0 and 2.5 H_2O_2 samples both at 24 and 144 hours incubation and for 5.0% H_2O_2 at 24 hours. However, there was an increase to approximately 21% after 144 hours with 5.0% H_2O_2. The percentage of dissolved lignin converted directly to cellular dry weight was quite low with 5.2% being the highest obtained at 144 hours in the 1.0% H_2O_2 sample. These values represent the efficiency of conversion of total dissolved lignin into cellular material. It is evident that as the concentration of H_2O_2 increases the efficiency decreases.

Table 3. Conversion of dissolved lignin solids (DLS), total dissolved lignin (TDL) and total carbon (TC) to Rhodotorula mucilaginosa cellular dry weight.

H_2O_2 (%)	Cellular dry weight (mg/ml)		DLS %		TDL %		TC before growth (µg/ml)	TC after growth (µg/ml)		TC utilization %	
	24 hr	144 hr	24 hr	144 hr	24 hr	144 hr		24 hr	144 hr	24 hr	144 hr
0.0	0.0	0.0	0.0	0.0	0.0	0.0	5	4	4	20	20
1.0	0.4	0.7	5.5	9.2	2.9	5.2	2400	1900	1200	21	50
2.5	0.4	0.8	4.8	9.6	2.1	4.2	3000	2350	1850	22	38
5.0	0.3	0.6	10.8	21.5	1.3	2.6	1240	793	280	64	77
10.0	0.2	0.2	21.5	21.5	0.8	0.8	345	49	48	86	86
15.0	0.0	0.1	0.0	17.5	0.0	0.4	227	220	29	3	87

Pre- and Post-Growth Total Carbon-Sephadex Profile

Sephadex fractionation of the polymeric-like material before and after growth response to the H_2O_2 oxidation supernatant fluid solutions is shown in Table 4. Organic carbon eluted in the void volumes of Sephadex G-10 and G-25 columns were not utilized as a carbon source by Rhodotorula because near quantitative recovery was achieved after growth. The polymeric-like material which represented a major portion of the TC content in the 1.0 and 2.5% H_2O_2 supernatant fluids was greatly reduced in the 5.0, 10 and 15% solutions. The material in the void volume representing 5000 and greater molecular weight material was not detectable in 5.0, 10 and 15% H_2O_2 samples whereas it represented approximately 10 and 5.0% of the 1 and 2.5% H_2O_2 oxidation supernatant fluids, respectively.

Pre- and Post-Growth Absorbance at 280 nm and 300 nm Relative to Rhodotorula Growth

Absorbance at 280 nm, measure of the aromaticity, was found to be highly dependent on the degree of lignin oxidation, Table 5. High absorbance was noted with 1.0 and 2.5% H_2O_2 oxidation supernatant fluids. Growth of Rhodotorula on these substrates did not reduce the absorbance significantly at the end of 24 hours incubation. However, a reduction of 6.0 and 16% respectively was detected at the end of 144 hours incubation. The percent decrease after growth

Table 4. Total carbon (μg/ml) profile of Sephadex G-10 and G-25 excluded fractions before and after Rhodotorula mucilaginosa growth response to hydrogen peroxide, pH 1 and 100 C, oxidized straw-lignin.

H_2O_2 (%)	TC before growth	Fractionated TC before growth		Fractionated TC after growth		Percent recovery of fractionated after growth	
		G-10	G-25	G-10	G-25	G-10	G-25
0.0	5	--	--	--	--	--	--
1.0	2400	1160	240	1158	240	99	100
2.5	3000	1250	150	1250	149	100	99
5.0	1240	180	--	179	--	99	--
10.0	345	24	--	23	--	96	--
15.0	227	5	--	5	--	100	--

Table 5. Absorbance (A) at 280 and 300 nm before and after growth response of Rhodotorula muciloginosa to hydrogen peroxide, pH 1 and 100 C, oxidized straw-lignin.

H_2O_2 (%)	before growth		24 hr after growth		144 hr after growth		% decrease 24 hr after growth		% decrease 144 hr after growth	
	280 nm	300 nm	280 nm	300 nm	280 nm	300 nm	280	300	280	300
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1.0	66.2	23.8	65.0	22.3	62.1	20.2	1.8	6.3	6.2	70
2.5	30.3	10.8	29.0	9.2	25.3	4.8	4.3	15.0	16.0	84
5.0	3.1	1.7	2.8	1.2	1.2	0.4	9.7	29.0	29.0	87
10.0	0.5	0.6	0.4	0.3	0.3	0.1	20.0	50.0	40.0	80
15.0	0.2	0.2	0.1	0.1	0.1	0.1	50.0	50.0	50.0	50

became greater as the concentration of H_2O_2 was increased and reached a maximum value of 50% in the 15% H_2O_2 oxidation supernatant fluid both at 24 and 144 hours of incubation. The greatest overall decrease in absorbance at 280 nm occurred in all substrates incubated for at least 144 hours.

Absorbance at 300 nm, measuring phenolic hydroxyl groups, showed a much greater decrease after growth. Generally, the absorbance decreased as the H_2O_2 concentration increased. The largest percentage decrease occurred in the substrates incubated 144 hours. These substrates also showed the greatest differential between 24 hours and 144 hours for 1.0 and 2.5% H_2O_2 oxidation supernatant fluids.

TLC of Lignin-Like Model Compounds and Lignin Oxidation Products

Rf values of lignin-like monomeric structures having corresponding Rf values to spots obtained from lignin oxidation solutions are shown in Table 6. The structures shown were selected from 115 chromatographed lignin-like model compounds. The Rf value or color produced before or after spraying eliminated all but methoxyhydroquinone, protocatechualdehyde, p-hydroxybenzaldehyde, p-hydroxybenzoic acid, vanillic acid and vanillin as likely structures corresponding to the lignin oxidation products. All chromatographic spots, developed in the benzene/dioxane/acetic acid solvent system,

Table 6. Corresponding R_f values between monomeric lignin-like structures and hydrogen peroxide, pH 1 and 100 C, oxidized straw-lignin products.

	benzene/dioxane/acetic acid (90:25:4)												benzene/methanol/acetic acid (45:8:4)												toluene/2-butanone (230:20)									
	0.00	0.06	0.20	0.31	0.34	0.38	0.39	0.44	0.48	0.51	0.54	0.70	0.81	0.00	0.04	0.15	0.21	0.26	0.34	0.37	0.47	0.52	0.53	0.71	0.79	0.00	0.07	0.09	0.11	0.16	0.18	0.26	0.28	0.78	0.83
A		+														+															+				
B				+															+									+								
C						+														+												+			
D									+												+								+						
E										+												+					+								
F											+												+									+			
G*		R_f = 0.37													R_f = 0.35												R_f = 0.03 (Streak)								
H*		R_f = 0.15													R_f = 0.19												R_f = 0.00								
I													+												+									+
J	C	-	A	C	A	C	A	A	A	A	C	C	C	C	A	-	A	A	C	C	A	A	C	C	C	C	C	A	A	A	-	C	C	C	C

A. Methoxyhydroquinone
B. Protocatechualdehyde
C. p-hydroxybenzaldehyde
D. p-hydroxybenzoic acid
E. Vanillic acid
F. Vanillin
G.* methoxy-p-benzoquinone
H.* 3, 6-dimethoxy-p-benzoquinone
I. Acetaldehyde
J. A = acidic; C = carbonyl

detected in the lignin H_2O_2 oxidation samples except Rf values 0.81, disappeared in the 10 and 15% H_2O_2 oxidation supernatant fluids. These spots also were quite predominant in the 1.0% H_2O_2 sample then decreased as the H_2O_2 concentration increased. Carbonyl structures, except the fraction showing an Rf value of 0.81, were predominant in the 1.0% H_2O_2 sample with the spot corresponding to protocatechualdehyde exhibiting the greatest visual intensity. Carboxyl structures were predominant in the 2, 5 and 5.0% H_2O_2 samples. The spots corresponding to authentic vanillic and p-hydroxybenzoic acids were quite intense.

The spot corresponding to methoxyhydroquinone was detected only in the 1.0% of H_2O_2 sample. The apparent intensity of the spot with Rf value of 0.81 increased as the H_2O_2 concentration was increased.

When TLC was performed on the lignin oxidation solutions before and after growth, only spots corresponding to authentic vanillic and p-hydroxybenzoic acid and the spot with an Rf value of 0.20 in the benzene/dioxane/acetic acid solvent system were absent after 24 hours growth. Spots with Rf values of 0.34 and 0.44 in the same solvent became lighter after growth. After 144 hours incubation, all spots containing carbonyl and carboxyl groups, except the spots with Rf values 0.70 and 0.81 and the unknown spot corresponding to methoxyhydroquinone were absent.

With the exception of spots with Rf values of 0.70 and 0.81, other chromatographic spots developed in the benzene/dioxane/acetic acid system indicated the occurrence of unsaturation when exposed to iodine vapor. These spots (except Rf-0.70 and 0.81) were also phenolic in nature as evidenced by spraying with tetrazotized benzidine. The characteristic fluorescence of the phenolic structures, mainly phenolic acidic components, agreed also with the reference structures. The chromatographic migration in the benzene/methyl alcohol/acetic acid and toluene/2-butanone solvent systems were similar to that observed in the benzene/dioxane/acetic acid system. The Rf values in all these solvent systems were quite close (Table 6).

Corresponding Rf values obtained in the benzene/dioxane/acetic acid solvent system for authentic 2,4-dinitrophenylhydrazones, lignin oxidation supernatant fluids, and preparative amounts of the spot with Rf-0.70 are shown in Table 7. These reference carbonyl structures were the only ones from a total of 11 lignin-like reference compounds which had Rf values corresponding to spots from the lignin oxidation solutions. The spots corresponding to vanillin, p-hydroxybenzaldehyde and protocatechualdehyde were most intense in the 1.0% H_2O_2 sample but decreased through the 5.0% H_2O_2 oxidation supernatant fluid and disappeared in the 10 and 15% H_2O_2 oxidation supernatant fluids. The spot with Rf 0.70 appeared to have equal intensity in the 1.0, 2.5 and 5% H_2O samples but disappeared in the 10% H_2O_2

sample. All spots except those with Rf values of 0.69 and 0.72 disappeared after 144 hours of growth but were unaffected after 24 hours of growth.

Characterization of Carbonyl Structure With an Rf Value of 0.70 From Benzene/Dioxane/Acetic Acid (45:8:4)

A white, steam volatile, water insoluble, waxy material accumulated in the condenser from the straw lignin-H_2O_2 mixture during the beginning of the refluxing period. The material was soluble in 95% ethyl alcohol, chloroform and concentrated sulfuric acid but insoluble in ether, benzene, 5.0 percent sodium hydroxide, 50% hydrochloric acid and concentrated phosphoric acid. The melting point was 51 C. It also exhibited unsaturation in iodine vapor. The presence of a carbonyl function was shown during reaction with 2,4-dinitrophenylhydrazine. No typical phenolic reaction occurred with tetrazotized benzidine and bromocresol green spray showed that a carboxyl structure was absent. According to these criteria, the compound would belong to the N_2 solubility class (73).

Mass Spectra Identification of Lignin Oxidation Products

The elution sequence for the methyl ethers and esters of the carboxylic acids identified by mass spectrometry was found to be identical to the corresponding silyl ethers and esters. This

information was used to establish the retention times and identity of the silyl derivatives.

Mass spectrometry allowed the identification of succinate, malonate, maleate, lactate and oxalate. However, two structures with retention times of 9.2 and 14.1 minutes as silyl derivatives and retention times of 6.2 and 8.0 minutes, respectively, as methyl derivatives, were not identified. The structure with a 6.2 minute retention time was assigned m/e values of 88, 31, and 15. The m/e value of 88 was considered to be the probable parent ion, P^+. A literature search of mass spectrographic data for compounds with a $P^+ = 88$ suggested that a five carbon secondary alcohol or ether was the most likely structure. No exact structure could be deduced from available literature. The structure with an 8.0 minute retention time had ion-fragments with m/e values of 74 and 31 but no other assignments could be made. Investigation of the literature, using $P^+ = 74$ suggested an acetal or ether structure with four carbon atoms.

DISCUSSION

The reaction kinetics associated with acid-catalyzed H_2O_2 oxidation appear to be complex as suggested by the equation, rate of oxidation = $k_2 H_2O_2$ nucleophile$^-$ + $k_3 H_2O_2$ nucleophile$^-$ H^+ (27). This equation describes a mechanism analogous to a consecutive first-order reaction where the original substrate goes through a series of reactions to obtain the final product (25). A single definition of the reaction kinetics of H_2O_2 lignin oxidation is difficult because the response is variable (Figure 1) with increasing peroxide concentration. The decomposition rate of H_2O_2 is greatly influenced by the concentration and temperature, becoming an exponential function as the concentration increases (28). The variability in response may be related to oxygen evolution. Approximately 8.0 g of H_2O_2 would be required to decompose 2.5 grams of lignin to carbon dioxide assuming a 62% carbon content for the lignin macromolecule. This would indicate that total destruction of the lignin to carbon dioxide should have been theoretically accomplished at least in the 10% H_2O_2 mixture. However, in practice this was not the case because 20% of the lignin carbon still remained in this system.

Hydrogen peroxide uptake efficiency by the lignin when 1.0% of the oxidant was used approached 100%. The uptake efficiency decreased, however, as the H_2O_2 concentration increased. At 10

and 15% H_2O_2 concentrations the uptake efficiency was 20 and 13%, respectively, indicating extensive loss of oxygen. This might account for the apparent rapid peroxide uptake curves for these latter concentrations, as seen in Figure 1.

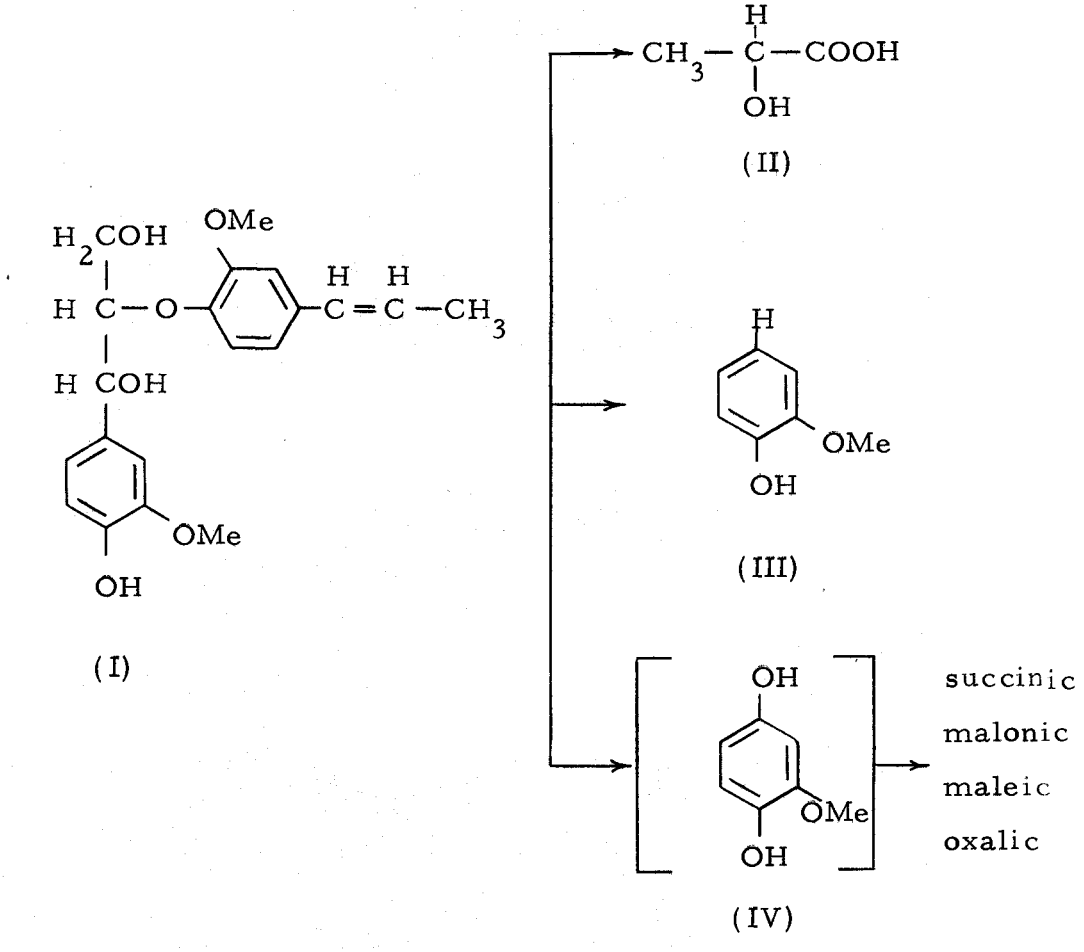
The initial rapid lignin dissolution (Figure 2) concomitant with carbonyl and carboxyl group formation (Figure 4) and phenolic hydroxyl formation (Figure 5) suggested a considerable degree of macromolecular alteration.

The types and distribution of interunitary linkages would have an effect on the dissolution rate. An investigation of softwood lignin structures indicates that approximately 60% of the bonds are ethereal and 40% carbon-carbon linkages, predominantly between C_3 - C_3 side chains (12). Peroxide oxidation of the RC-O-CR linkage would provide the necessary combination for hydroxylation (page 12, literature review), whereas the oxidation of the RC-CR linkages would be more conducive to the formation of carboxyl groups (12). The initial lignin dissolution could be predominately attributed to the cleavage of ethereal linkages. The fact that 64% of the lignin was dissolved in the 1.0% H_2O solution while the phenolic hydroxyl content increased from zero to 1.4% (Figure 5) would indicate RC-O-CR interunitary bond cleavage. If this were the case, the rapid dissolution rate could be explained. However, the interunitary linkages are considered using a softwood lignin as a model and not a graminal lignin because

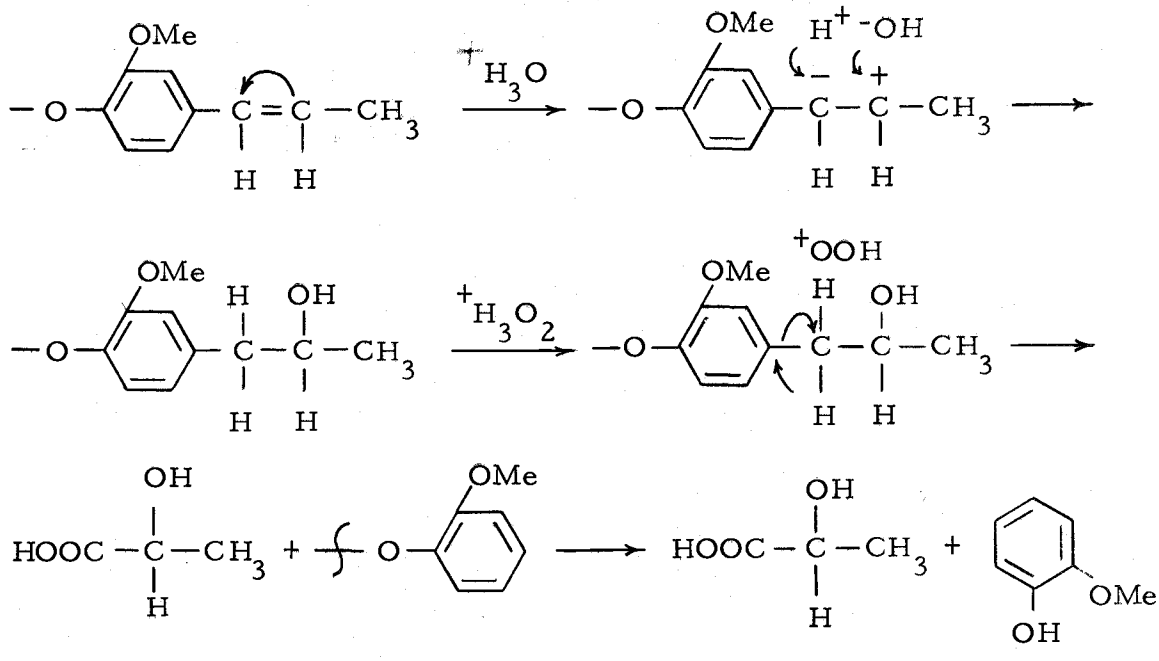
a common graminal lignin structure could not be found in the literature. However, it appears safe to assume that the basic linkage types are similar (45) since p-hydroxyphenyl and guaiacyl units predominant and would have the same number of radical resonance forms available during random polymerization to form the lignin structure. Demethoxylation was also important during the initial stages of oxidation (Figure 6). The rapid decrease in methoxyl content coupled with phenolic hydroxyl and protocatechualdehyde formation would suggest that syringyl and guaiacyl moeities were being altered to ortho-dihydroxy structures which were then further oxidized to muconic acids (72). The 1.3% loss in methoxyl closely approximates the 1.4% increase in phenolic hydroxyl during 1.0% H_2O_2 oxidation. This suggests that a stoichiometric conversion has taken place. However, only 19% of the methoxyl groups were recovered as methyl alcohol and no formaldehyde or formic acid was detected in this reaction mixture. Evidently phenolic hydroxyl formation must have been dependent on other parts of the aromatic molecule in addition to the methoxyl center. This assumption was borne out by the presence of dicarboxylic structures derived from p-hydroxyphenolic moeities. Succinic, malonic, and maleic acids were formed and probably resulted from an aromatic cleavage via a p-hydroxy precursor (72). Muconic acids were not detected in the reaction products in this study. The continued oxidation of

protocatechualdehyde would lead through an ortho-quinone to a muconic acid. Therefore, some muconic acid structure probably was present but not isolated.

The tentative identification of methoxy hydroquinone gave support that a major portion of the hydroxylation was in a para position. If this were the case, then extensive side-chain oxidation would occur and the formation of lactic acid could be explained. The acid-catalyzed H_2O_2 oxidation of guaiacyglycerol-β-coniferyl ether (I) yielded lactic acid (II) as well as guaiacol (III) and structures consistent with a hydroquinone intermediate (IV).



Similar oxidation of vanillic acid gave guaiacol, maleic, malonic and succinic acids and methoxy-p-benzoquinone but no lactic acid. The formation of lactic acid from guaiacylglycerol-β-coniferyl ether must have resulted from one of the C_3 side chain moeities by direct cleavage between C_1 and the aromatic nucleus because the guaiacol formed could come from either aromatic nucleus. The possibility of direct C_1-aromatic cleavage of the glycerol group and hydrolysis of the β-4' ether bond appears unlikely. The hydroxyl group either at C_1 or C_3 would have to leave forming a carbonium ion. Formation of a methyl group would then require a hydride ion transfer which would be difficult under the imposed reaction conditions. However, hydration, cleavage and oxidation of the propene side chain on the coniferyl moeity could lead to lactic acid as follows:



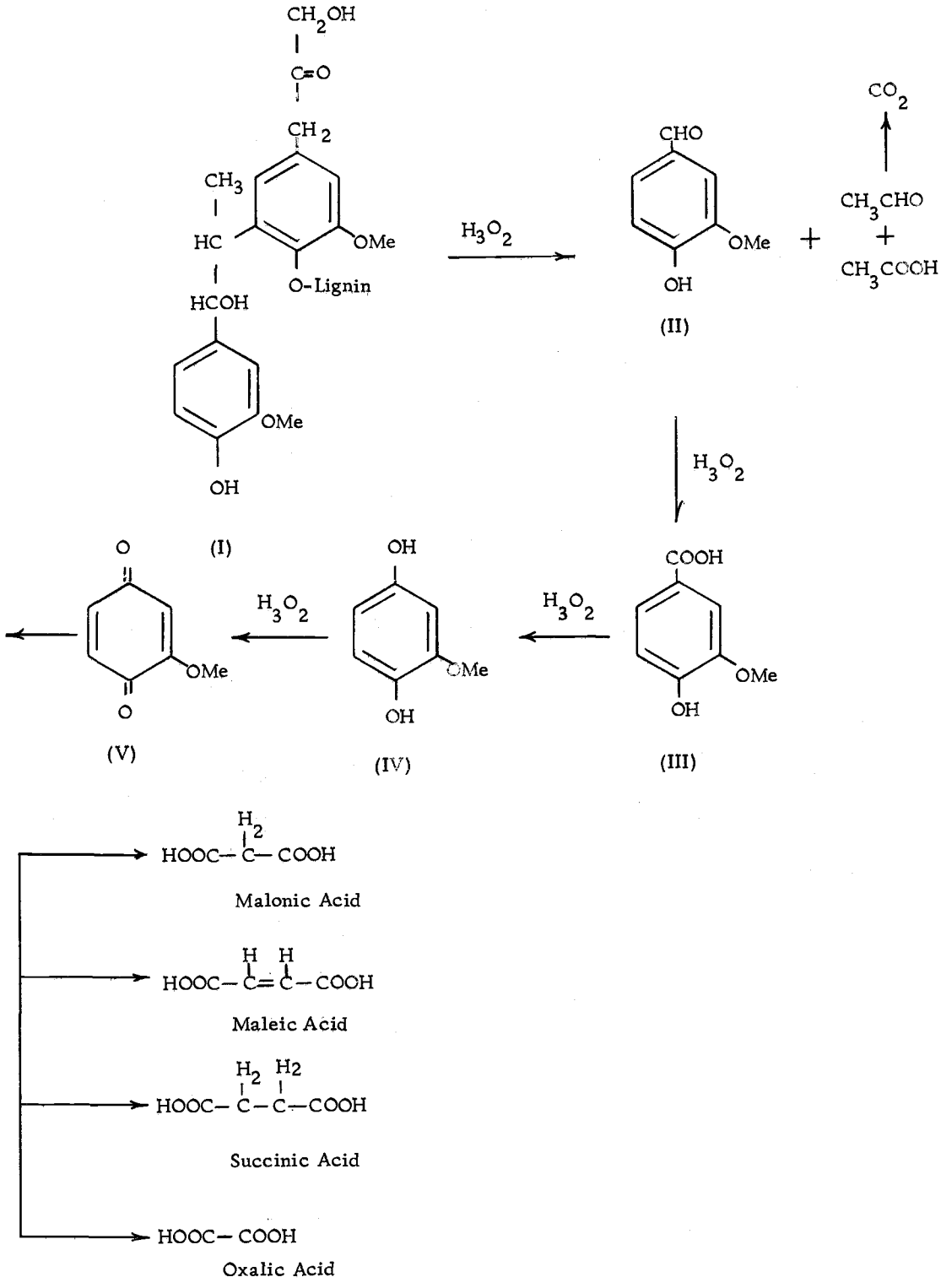
The occurrence of the free propene side chain in lignin is rare (12). However, its presence has been postulated in gamma-5', C-C linkage to an adjoining aromatic constituent (39). As outlined earlier, cleavage of the C-C linkage could be followed by a similar reaction sequence resulting in lactic acid.

The formation of polymer must have involved extensive side-chain oxidation and elimination. Oxidation of the polymeric material produced the common dicarboxylic acids associated with para-quinone type cleavages, however, no lactic acid was detected. This suggested that the C_3 side-chain was cleaved prior to quinone formation and polymerization of the quinonoid units then followed. This assumption was further supported by the oxidation of p-hydroxybenzoic, vanillic, and syringic acids and guaiacylglycerol-β-coniferyl ether. In each case, a polymer-like material was obtained during the acid-catalyzed H_2O_2 oxidation. The polymerization was not apparent until the solution became colored, presumably from quinone formation. The corresponding benzoquinones were tentatively shown to be present and could have polymerized. Previous studies have shown that quinonoid structures polymerize under acidic oxidative conditions to form linear polymers (19, 45, 59).

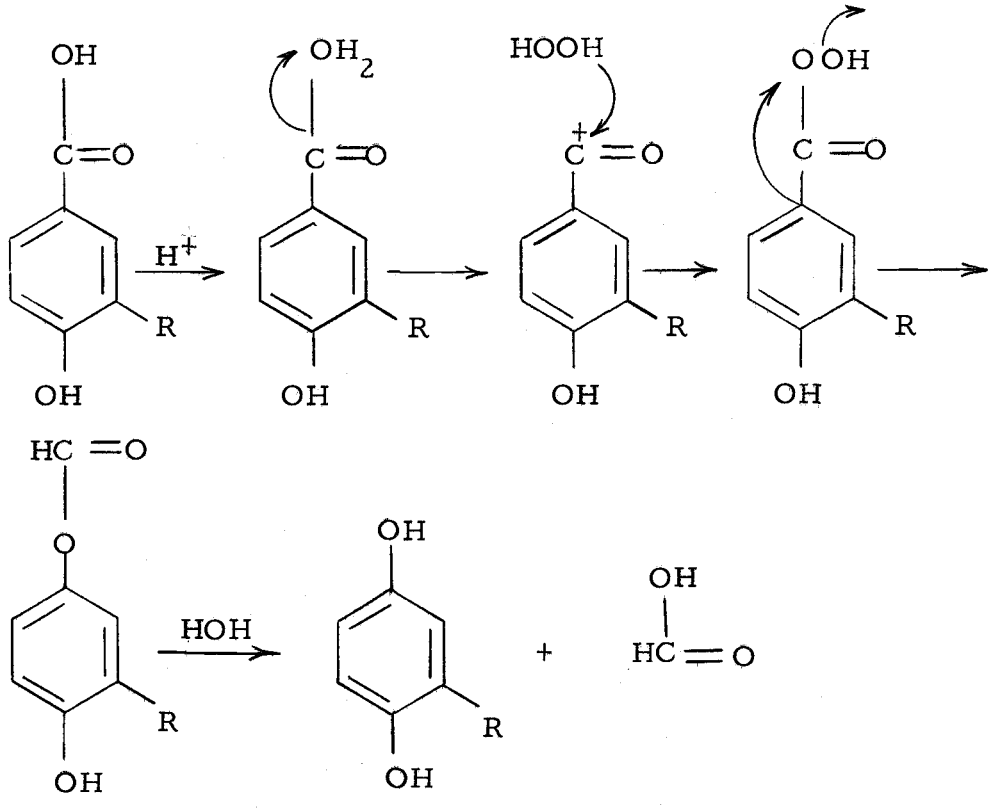
The extensive aromatic degradation occurring during lignin oxidation with 5.0, 10 and 15% H_2O_2 (Figure 5) was paralleled by an increase in succinic, maleic, malonic and oxalic acids. This

would be expected if side chain elimination was followed by hydroxylation of the aromatic constituents to p-quinone structures.

Using a basic lignin structure (I) the following reaction sequence is suggested: Oxidation of the C_3 side-chain between C_1 and C_2 would yield an aromatic aldehyde (II) plus acetaldehyde and acetic acid from the C_2 and C_3 carbons after hydrolysis of the β-5' linkage. Continued oxidation would lead to a carboxylic group (III) then through the hydroquinone (IV) and quinone (V) state. The para-benzoquinone nucleus (V) would then be cleaved forming the dicarboxylic acids shown. A minor competing reaction through the ortho-quinone would also occur with formation of muconic acids. Insufficient information regarding the two unidentified structures passing through the mass spectrograph and the one structure accumulating in the reflux condenser prevented postulating their formation.


CH2OH
C=O
CH2
CH3
HC
HCOH
OMe
O-Lignin
OMe
OH
(I)
H3O2
CHO
OMe
OH
(II)
+
CO2
CH3CHO
+
CH3COOH
H3O2
COOH
OMe
OH
(III)
H3O2
OH
OMe
OH
(IV)
H3O2
O
OMe
O
(V)
H2
HOOC– C– COOH
Malonic Acid
H H
HOOC– C= C– COOH
Maleic Acid
H2 H2
HOOC– C– C–COOH
Succinic Acid
HOOC– COOH
Oxalic Acid

Formation of para-benzoquinone and methoxy-para-benzoquinone from p-hydroxybenzoic and vanillic acids, respectively, would add supportive evidence that the C_3 side chain was cleaved between C_1 and C_2 with further oxidation of the C_1 atom to a carboxyl group. Oxidation to this stage followed by oxidative decarboxylation and hydroxylation by the peroxide would then lead to the para-benzoquinone structures. No prior evidence for acid-catalyzed H_2O_2 oxidation of aromatic acids to quinones was found in the literature. A modified mechanism similar to the Dakin reaction might explain the results (23). The Dakin reaction is an oxidative type of rearrangement involving aldehydes and takes place in acidic or alkaline media. The main feature of this mechanism is the migration of the aromatic constituent from its carbonyl carbon to the adjacent oxygen linking the peroxide to the carbonyl carbon. The OH group in the carboxyl group on p-hydroxybenzoic and vanillic acids can become a good leaving group in a reaction involving an electrophilic attack by peroxide. This configuration would be similar to the Dakin reaction configuration and could explain the results eventually leading to quinone formation.



The concept originally employed (29, 57, 62, 69) that a natural polymer such as ligninsulfonate or straw could be modified to structures dissimilable by microorganisms was further supported in this study. However, the degree of lignin oxidation greatly influenced the growth response of Rhodotorula to the substrate (Figure 7). The presence of phenolic structures, which the Rhodotorula can utilize as a carbon source, resulted in a diauxic

growth response. The diauxic lag would allow the induction of the enzymatic system necessary for aromatic utilization (47). The initial growth response involved predominately low molecular weight non-aromatic structures as evidenced by absorbance at 300 nm (Table 5). After enzymatic induction for aromatic dissimilation the predominant lignin aromatic oxidation fragments were utilized. These fragments were probably monomeric phenolic structures since Rhodotorula cannot utilize dimeric and larger structures (16). All of the aliphatic acid structures detected in the oxidation solution (Table 1) were utilized except oxalic and maleic acids. The highest total concentration of these structures occurred in the 2.5% H_2O_2 oxidation mixture where the greatest overall cellular dry weight was obtained (Table 3). However, it was not until the majority of the aromatic constituents were destroyed in the 5.0% H_2O_2 system that the utilization efficiency of the available total carbon (Table 2) and conversion of soluble solids to cellular dry weight (Table 3) were relatively high compared to the 1.0 and 2.5% peroxide systems. The efficiency values were shown to be dependent on the formation of the water soluble polymer (Table 4). Approximately 40% of the total carbon in the lignin oxidation solution was bound up in the polymer. This fraction was not

utilized by the Rhodotorula, shown by near quantitative recovery after growth of the yeast (Table 4). Hydrogen peroxide concentrations of 5.0, 10 and 15% resulted in a lower polymer content, presumably by oxidative degradation, and as a result the growth efficiency increased (Table 3). Even though there was less total carbon and soluble solids in the oxidation solution, the major constituents were the aliphatic dissimilable structures. The loss of carbon as carbon dioxide (Figure 3) influenced the conversion efficiency of total dissolved lignin to yeast cellular dry weight. The highest efficiency values were obtained with 1.0 and 2.5% H_2O_2. Carbon dioxide loss was minimum at these concentrations. As the peroxide concentration increased carbon dioxide formation increased. This reduced the concentration of biologically dissimilable structures and greatly reduced the efficiency of converting dissolved lignin to yeast.

Acid-catalyzed H_2O_2 oxidation of the straw lignin appears to be similar to paracetic acid oxidation. A great deal of lignin macromolecular alteration involving concomitant hydroxylation and cleavage to similar aliphatic constituents is common to both these reagents. The presence of muconic acids with peracetic acid but their absence with H_2O_2 seems to suggest a different reaction mechanism. Also, the presence of lactic acid during H_2O_2 oxidation would also suggest

that the two peroxides act differently on the lignin structure. However, this study was carried out on a graminae-derived lignin while the majority of lignin oxidations reported have been with gymnosperm and angiosperm lignins. The variation of guaiacyl, syringyl and p-hydroxbenzyl moeities in these different types of lignins has been well documented (20, 49). Graminae in general have a ratio of 0.60 for syringyl/guaiacyl and p-hydroxybenzyl/guaiacyl units while gymnosperms and angiosperms give ratios of 1.0 and 2.6 respectively for syringyl/guaiacyl and zero for p-hydroxybenzyl/guaiacyl. Thus, while H_2O_2 and peracetic acetic oxidation of lignin appear to have similarities, differences cannot be accurately described because different lignins were used.

A more complete understanding of acid-catalyzed H_2O_2 oxidation of straw lignin will allow development of more efficient conditions to maximize biologically assimilable carbon. To achieve such an understanding, factors influencing lignin degradation to aromatics which are then repolymerized to biologically recalcitrant structures must be established. Additional factors such as other catalytic agents and peroxy acids should be investigated as a means of improving lignin conversion to biologically available structures. New techniques such as high pressure oxidation, ultrafiltration of low molecular weight biologically dissimilable structurs as they are formed, and

yeast production related to mixed culture and continuous fermentation should also be investigated.

SUMMARY

Sulfuric acid lignin, 2.5% w/v, isolated from Lolium multiflorum was subjected to 1.0, 2.5, 5.0 and 10 and 15% H_2O_2 concentrations at pH 1 and 100 C until the peroxide was consumed. The lignin oxidation substrates were then used to elucidate the oxidative mechanism and to determine the feasibility of using modified lignin to support growth of a Rhodotorula mucilaginosa strain. From the data obtained it appeared that:

1. The degree of lignin modification was greatly influenced by the types of interunitary linkages between the phenylpropanoid building blocks. Approximately 64% of the lignin was dissolved during oxidation by 1.0% H_2O_2. Because approximately 60% of interunitary lignin linkages are postulated to be of the RC-O-CR' type, it can be suggested that these are oxidatively cleaved in preference to the remaining RC-CR' types.

2. Hydrogen peroxide stability at pH 1 and 100 C during lignin oxidation decreased as the concentration increased, resulting in a loss of gaseous oxygen. If lignin dissolution was proportional to H_2O_2 concentration, complete oxidation of the lignin to carbon dioxide should have been accomplished using 8.0% H_2O_2. However, increasing the peroxide concentration 15 fold relative to 1% H_2O_2 only increased lignin dissolution by 35% with 20% of the lignin

remaining after oxidation. This unstable characteristic would have to be considered carefully in analysis of economic efficiency.

3. The concentration of H_2O_2 greatly influenced the formation of carbon dioxide and total carbon content in the oxidation mixture. Minimum carbon dioxide formation occurred in the 1.0% H_2O_2 substrate while maximum total carbon occurred in the 2.5% peroxide substrate. Increasing H_2O_2 concentration decreased soluble total carbon and increased carbon dioxide formation, but not proportionately.

4. Hydrogen peroxide oxidation of the lignin resulted in formation of smaller subunits by cleavage of interunitary linkages and the C_3-side chain of the aromatic constituents to form phenolic carbonyl and carboxyl functional groups. This was supported by the detection of vanillin, p-hydroxybenzaldehyde, protocatechualdehyde, vanillic acid, and p-hydroxybenzoic acid during the initial stages of oxidation.

5. The oxidative cleavage of interunitary linkages was followed by considerable hydroxylation since the phenolic content was increased to 1.4% in the 1.0% H_2O_2 substrate. The detection of methoxyhydroquinone and protocatechualdehyde confirmed this since they would be cleavage products resulting from para- and ortho-hydroxylation and would probably be the major source of protocatechualdehyde because this structure is an ortho-dihydric phenol.

6. The initial increase in carbonyl content was followed by

carboxyl formation. This observation coupled with an increase in phenolic hydroxyl groups suggested that the formation of methoxyhydroquinone resulted from an attack of H_2O_2 on the carboxyl site of the aromatic acid followed by hydroxylation. The mechanism seemed to fit a modified Dakin reaction which involves coupling of H_2O_2 to the carboxyl carbon while the protonated hydroxy group is eliminated. The aromatic moeity then migrates to the peroxide oxygen atom attached to the carboxyl carbon. At this stage, the carboxyl carbon and the attached oxygens were eliminated during hydrolysis forming a phenolic hydroxyl group on the aromatic constituent.

7. Loss of aromaticity and formation of succinic, maleic, malonic, and oxalic acids indicated that the predominant oxidative route occurred through a para-hydroxylation mechanism as opposed to ortho-hydroxylation. These acids are indicative of an oxidative cleavage of para-hydroxylated structures. Ortho-hydroxylated phenols on the other hand are cleaved predominately to muconic acids which were not detected.

8. Lactic acid was postulated to occur from the C_3-side chain of the C_6-C_3 lignin subunit. Supporting evidence came when guaiacylglycerol-β-coniferyl ether and p-hydroxybenzoic, vanillic and syringic acids were oxidized with H_2O_2. The ether gave lactic acid and guaiacol whereas only guaiacol was formed from vanillic acid.

Para-hydroxybenzoic and syringic acids gave neither guaiacol nor lactic acid. Delineation of the nature of the side chain came when glycerol was oxidized and lactic acid was not detected. This suggested that the α, β unsaturated C_3-side chain on the coniferyl moeity was the unit being oxidized to lactic acid and not the glycerol structure on the guaiacyl unit. Hydrolysis of the α, β unsaturated site added a hydroxyl to the β-carbon and proton to the α-carbon. Oxidative cleavage between the α-carbon and the aromatic nucleus resulted in the formation of the carboxyl group necessary for the completion of the lactic acid structure liberating guaiacol by protonation at the aromatic C_1 position.

9. Formation of the brown, water-soluble, polymeric-like material was postulated to occur by condensation of quinonoid units after elimination of the C_3-aliphatic side chain. Oxidation of the polymer gave succinic, maleic, malonic and oxalic acids but no lactic acid. Also, oxidation of p-hydroxybenzoic, vanillic, and syringic acids gave a similar appearing polymer along with their respective para-benzoquinones. Oxidative polymerization of quinones has been reported previously in the literature. The molecular weight was generally greater than 700 with 80-88% representing weights of 700-5000 in the 1.0 and 2.5% H_2O_2 substrates. Fractions with weights of at least 700 were present in all peroxide oxidation solutions but substances with molecular weights greater than 5000 were

not detected in 5.0, 10 and 15% H_2O_2 oxidation substrates. The content of polymer dropped to 2% in the 15% peroxide oxidation substrate. The polymeric material was not utilized as a carbon source by Rhodotorula mucilaginosa as evidenced by near quantitative recovery from the inoculated growth media.

10. Growth of Rhodotorula mucilaginosa on the lignin oxidation substrates was varied. The greatest growth in terms of cellular dry weight occurred in the 2.5% H_2O_2 substrate. This was followed closely by growth on the 1.0 and 5.0% H_2O_2 oxidation supernatant fluids. The incubation period for all growth studies was 144 hours. The initial growth response on all three substrates reached a peak at approximately 24 hours, followed by a diauxic lag period then further growth terminating near 89 hours. The initial growth was attributed to the aliphatic acid constituents present in the medium followed by the diauxic lag necessary for aromatic dissimilatory enzyme induction. At the end of this interval, utilization of the aromatic constituents proceeded. This concept was further supported by the fact that no diauxic effect occurred with 10 and 15% H_2O_2 substrates where the free non-polymerized phenolic and aromatic content was very low. Absorbance values in the 1.0, 2.5 and 5.0% H_2O_2 oxidation supernatant fluids at 280 and 300 nm were also greatly reduced after the second growth phase of the diauxy but not during the second lag interval. In addition, TLC of the substrates

before and after 24 and 144 hours of growth showed that aromatic constituents were still present at 24 hours but essentially gone after 144 hours of incubation.

11. Maximum conversion efficiency by Rhodotorula mucilaginosa of dissolved lignin solids, total dissolved lignin and substrate total carbon to vegetative cells varied relative to the peroxide concentration. Twenty-one percent of the dissolved lignin solids and 86% of the total carbon were utilized in the 10% H_2O_2 substrate whereas 5% total dissolved lignin was utilized in the 1.0% peroxide supernatant fluid. The increased utilization of dissolved lignin solids and TC in the 10% H_2O_2 supernatant fluid could be attributed to the reduced concentration of polymer and recalcitrant aromatics. The expression of a relatively low value for total dissolved lignin during oxidation with low peroxide concentration occurred because the total dissolved lignin number was the sum of carbon dioxide and other volatiles lost during drying in addition to dissolved lignin solids. Since loss of carbon dioxide and volatiles was minimum during lignin oxidation by 1.0% peroxide the total dissolved lignin conversion efficiency value should become larger relative to higher peroxide concentrations where carbon dioxide and other volatiles such as methyl alcohol were formed and eliminated; thus not entering into the total dissolved lignin expression.

12. Lignin oxidation by peracetic acid and sulfuric acid

catalyzed-H_2O_2 appear to follow similar mechanisms. Mainly, a large amount of lignin macromolecular alteration involved with concomitant ortho- and para-hydroxylation of the benzenoid nucleus and finally cleavage to similar aliphatic constituents. The main differences appear to be reflected in the lack of muconic acids and the presence of lactic acid both associated with H_2O_2 oxidation. However, differences may be more dependent on the types of lignin than the oxidizing agents since a graminal lignin was used in this study while the available literature reflects work predominantly on gymosperm and angiosperm lignins.

13. A sidelight to this study was the observation that acid catalyzed-H_2O_2 oxidation, pH 1 and 100 C, of para-hydroxybenzoic, vanillic and syringic acids gave para-benzoquinone, methoxy-para-benzoquinone and 2, 6-dimethoxy-para-benzoquinone respectively. A survey of the literature indicated that general peroxide quinone syntheses center around aromatic carbonyl and phenolic oxidation and not aromatic carboxyl structures. This finding suggested a new route of syntheses for quinones and also supported the postulation that aromatic carboxylic structures are also converted to hydroxylated hydroquinones before being further oxidized to the non-aromatic quinonoid state prior to cleavage to aliphatic constituents.

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