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Title: PHOTOOXIDATIVE DEGRADATION OF ANNUAL RYEGRASS STRAW TO SUBSTRATES ENHANCING RHODOTORULA MUCILAGINOSA GROWTH

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Annual ryegrass (Lolium multiflorum) is a major seed crop in the Willamette Valley of Oregon. Persistent crop diseases have necessitated annual field burning to protect future crops. This practice, while very effective for disease eradication, produces large quantities of air polluting smoke in the valley and a general wastage of a material which could be used in the manufacture of economically important products. The cellulosic portion of straw is susceptible to microbial dissimilation but the lignin fraction (10-14%) is extremely resistant. Modification of the straw to relieve this resistance would be valuable in the biological utilization of straw and, under controlled conditions, the modified components could be used as substrates for microbial growth in the production of single cell protein.

Ryegrass straw hydrolysed with 3% sulfuric acid at 121 C,

homogenized to a fine consistency and then Soxhlet extracted with hot water prior to irradiation was subjected as a 1.0% w/v suspension to photooxidation with ultraviolet light and gaseous oxygen for 5 hour intervals to a maximum of 30 hours. After each photooxidation period the photolyzate was removed and subjected to both chemical analyses and growth by Rhodotorula mucilaginosa, a non-lignin utilizing yeast.

Gas-liquid chromatography-Rapid-Scan mass spectrometry and gas-liquid chromatography were used to determine straw photooxidation products. Glyceraldehyde, erythrose, threose, arabinose, xylose, mannose, rhamnose and glucose were identified. All compounds isolated were utilized by the Rhodotorula strain.

The appearance of glyceraldehyde, erythrose and threose which are not normally found in straw suggested a 1-carbon photodecomposition of arabinose and xylose leading to erythrose and threose. Glyceraldehyde would result from a 1-carbon photodecomposition of erythrose and threose. Photodecomposition of the C_3 side chain in the C_3-C_6 lignin phenylpropanoid structure may be an additional source of glyceraldehyde.

Ryegrass straw lignin concentration decreased from 33% to 12% after 12 hours photooxidation suggesting a demethoxylation of the phenylpropanoid structure and a concomitant increase in sulfuric acid solubility. Extensive photodecomposition of the C_3-C_6 lignin

structure was ruled out because the concentrations of aromatic nuclei and phenolic hydroxyl groups were only 0.09% and 0.43% respectively and considered to be insignificant levels.

The ability of Rhodotorula mucilaginosa to utilize the photo-oxidation products obtained indicates that this type of treatment of recalcitrant materials can greatly improve biological utilization. A similar approach on other recalcitrant materials using different oxidizing agents and systems should be explored.

Photooxidative Degradation of Annual Ryegrass Straw to Substrates Enhancing *Rhodotorula mucilaginosa* Growth

by

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INTRODUCTION

The ability of microorganisms to degrade the wide variety of substrates in nature is related to the structure and environment of the substrate and the microorganisms enzymatic capabilities. While most organic structures can be degraded by organisms, certain structures are very resistant to biological dissimilation. The lignin in straw represents such a material.

Most grass straws have lignin content around 10-14%. A major portion of the remaining straw is comprised of cellulose and hemicelluloses which are readily degraded in nature. Even so, straw is very slowly degraded because of the lignins present.

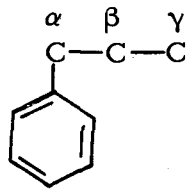
This study was undertaken to develop a photooxidative system for straw treatment which would enhance the release of readily metabolizable substrates for dissimilation by a *Rhodotorula mucilaginosa* strain. It was the purpose of this study to (1) better understand the role of ultraviolet light in photodecomposition of straw, (2) identify the organic structures released during photooxidation and (3) determine to what extent these structures were utilized by *Rhodotorula mucilaginosa*. This would provide information which could be extended to the modification of other biologically recalcitrant materials for production of single cell protein.

LITERATURE REVIEW

Lignin

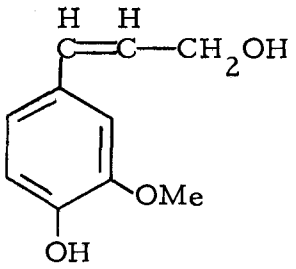
Lignin serves as an encrusting material imparting structural integrity to plant cell walls and exists in its natural state covalently bonded with celluloses (8, 55). Plants contain varying amounts of lignin ranging from a few percent for herbaceous plants to about 30 percent for conifers with cellulose, hemicelluloses, and extractants such as tannins, phlobophenes, lignans, and coloring materials comprising the remainder (8, 9, 19, 51).

The lignin macromolecule appears to be made up of basic C_6-C_3 phenylpropanoid structures (a) (10, 46).

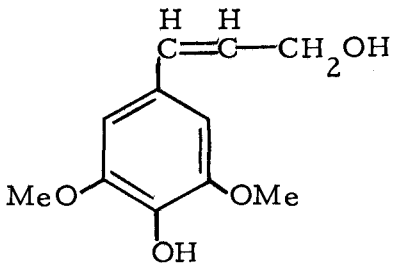


(a)

Many early investigators (16, 18, 19, 20, 23, 33, 34, 42) have suggested that the lignin macromolecule is a condensation product resulting from the polymerization of three phenylpropanoid alcoholic precursors, namely: trans-coniferyl (a), trans-sinapyl (b), and trans-p-coumaryl alcohols (c).



(a)



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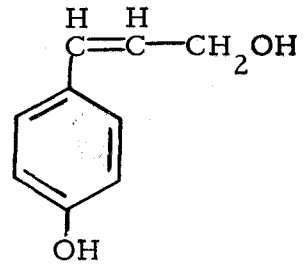


(c)

Lignin Degradation by Microorganisms

An extensive review of lignin degradation by Gottlieb (22) suggests that very few microorganisms are capable of degrading lignin and then only at a very slow rate. A few filamentous fungi, mainly basidiomycetes, which exhibit polyphenyloxidase activity are the only organisms thus far isolated from the vast microflora in nature with this capability.

Yeast-like fungi and bacteria which do not possess this enzyme activity, and therefore, cannot degrade lignin (7, 22, 44, 50, 57) are nevertheless important in lignin degradation. These organisms are capable of degrading numerous lignin-like aromatic compounds which have structures similar to the basic phenolic building blocks found in lignin (12). However, their role in lignin degradation can only be assessed in terms of lignin-like model compound metabolism (24, 50).

Several attempts have been made to elucidate the mode of lignin microbial degradation by isolating metabolic intermediates from

decomposed lignified materials. Higuchi et al. (26) extracted beechwood meal previously decomposed by several white-rot fungi and detected coniferylaldehyde, vanillin, and syringaldehyde in the extract. Nitrobenzine oxidation of decomposed and fresh beechwood showed that vanillin and syringaldehyde were not only reduced in the decomposed wood but the ratio of syringaldehyde to vanillin was higher, suggesting a preferential attack of the guaiacyl units in the lignin structure.

Ishakawa (28) using a white-rot fungus, Fomes fomentarius, found that the principal units derived from the decomposition of a soft wood lignin were coniferyl alcohol, guaiacylglycerol and guaiacylglycerol-β-coniferyl ether. These were degraded further by a shortening of the side chain leading to protacatechuic acid or catechol which are metabolised through the characteristic aromatic pathways (25).

The presence of guaiacylglycerol-β-coniferyl ether in all cultural filtrates used by Ishakawa coupled with the observation of Higuchi that guaiacyl units were decreased during decomposition suggested that guaiacylglycerol-β-coniferyl ether was the first lignin cleavage product (26, 29, 30). This cross-linkage group is now considered to be the more important group relative to microbial degradation susceptibility (1).

Photooxidation

Physico-chemical Fundamentals

Most polyatomic molecules have a number of metastable excited electronic states. Promotion of molecules to these states is accomplished by adsorption of visible or ultraviolet light. The excited molecules can lose their excess energy by either primary or secondary decomposition modes. Three primary decomposition modes are possible for an excited molecule; 1) form ground state products through a chemical reaction, 2) lose energy through radiation emission, and 3) lose excess energy by transfer to another molecule. Secondary photochemical reactions can be divided into six categories: 1) elimination and decomposition, 2) additions and dimerization, 3) atomic abstraction, 4) rearrangements, 5) substitution, and 6) chain reactions. The energy required for most organic chemical bond rupture is 1 to 5 ev with differences in a given molecule of only a few tenths of an electron volt. However, the molecular decomposition mode is greatly influenced by these small differences in energy. This selectivity makes reactions possible that are thermodynamically impractical (43).

Plant Polysaccharides

Several investigators (5, 6, 11) have studied the effects of light

on organic molecules and have found that light with wavelengths shorter than 350 nm will rupture some covalent bonds leading to the formation of new compounds. Kujirai (36) studied the effects of ultraviolet light on cellulose films and found that light containing wavelengths of 185 nm and 253. 7 nm produced aldehydic and carboxylic compounds respectively.

Other investigators (40, 41) have noted significant increases in the number of reducing groups on polysaccharides isolated from groundwood after exposure to ultraviolet light in the presence of oxygen. This observation was attributed to depolymerization resulting from photooxidation of the polysaccharides. They also noted a reduction in pentosan and cellulose content.

The presence of oxygen during photolysis of polysaccharides such as cellulose greatly influences the photolytic rate and the types of end-products formed. At 185 nm a primary dissociation occurs in cellulose due to its strong absorption characteristics at this wavelength. This reaction is oxygen independent. Early in the photolysis period a direct scission of glucosidic linkages predominates, but on prolonged exposure a 2, 3-dialdehyde is formed by splitting off hydrogen atoms from secondary carbon atoms. At 253. 7 nm the presence of oxygen becomes important in the photolytic process because cellulose is transparent to visible and ultraviolet light having wavelengths longer than 200 nm. The oxygen

molecule absorbs readily at 253. 7 nm and is elevated to an excited state where it is capable of abstracting a hydrogen atom from the 1-carbon of the pyranose ring to form a cellulose radical. The radicals formed react with activated oxygen and other cellulose molecules to form cellulose peroxides which further decompose photochemically to form α-lactones. These structures are hydrolyzed photochemically to form hydroxy-carboxylic acid end groups (37).

Lignin and Related Materials

Lignified materials like wood and pulp are also modified when exposed to ultraviolet light. Leary (38) observed a yellowing process when these materials were exposed to 355-500 nm light and suggested that a demethoxylation reaction, resulting from lignin photolysis, was involved.

The presence of oxygen during lignin photolysis also influences the rate of photooxidation and the end-products formed just as it did with cellulose. Free radicals are formed which rapidly combine with oxygen to oxidize the lignin macromolecule (32). These findings support the suggestions that photooxidation is an important intermediate process in the natural degradation of lignified materials (32). Vanillin, syringaldehyde, some higher molecular weight phenolic compounds (17, 53), carbon dioxide, carbon monoxide, water and

methanol are among the products thus far isolated from photooxidized woody materials (32).

Rockhill (52) has shown that sodium ligninsulfonate, a waste product from pulp and paper production, can also be altered photochemically with ultraviolet light in the presence of oxygen to produce similar compounds. Photooxidation of the ligninsulfonate occurred most efficiently when (a) the solution being treated was acidic, and (b) the light contained wavelengths less than 210 nm. The process was also temperature independent and oxygen dependent exhibiting a true primary photodecomposition mode.

Microbial Response to Photooxidized Lignified Materials

Photooxidation of sodium ligninsulfonate in water with ultraviolet light and oxygen followed by growth studies on the oxidation products has shown that some lignified materials can be photochemically modified to allow increased microbial growth (14, 15, 35, 52).

Studies with an Aspergillus isolate (52) and a *Pseudomonas* sp. (14) on ligninsulfonate has shown that 55 percent of the organic carbon remaining after photooxidation is rapidly utilized by these microorganisms. This represented a ten-fold increase in the biological availability of ligninsulfonate.

Annual ryegrass straw, *Lolium multiflorum*, containing 14 percent lignin, also has been photooxidized to structures enhancing

utilization by an Aspergillus sp. (45). Except for these studies, however, specific information relating to microbial growth responses to photooxidized lignin or lignin containing materials and the photolytic by-products is somewhat lacking.

The information obtained from these earlier studies provided the groundwork which led to the present study on annual ryegrass (Lolium multiflorum) straw photooxidation and the resulting growth response of a yeast, Rhodotorula muscilaginosa, to the oxidation products.

The combination of annual ryegrass straw and Rhodotorula muscilaginosa were selected for this study as the last phase of a three phase investigation concerned with alternate methods of annual ryegrass straw disposal (currently burned in the fields after grass seed harvest) to produce single cell protein. The first phase involved a mild sulfuric acid hydrolysis to remove a major portion of the cellulose and hemicelluloses for single cell protein production with a food yeast, Candida utilis, leaving a straw residue containing approximately 33 percent lignin. The second phase was concerned with 1) the mechanism of acid catalyzed hydrogen peroxide oxidative modification of a sulfuric acid lignin derived from annual ryegrass straw and 2) the growth of a non-lignin utilizing yeast, Rhodotorula mucilaginosa, on the oxidation products. The third phase and subject of this study is concerned with 1) the photooxidative modification of the hydrolyzed

straw residues remaining after phase one and 2) the growth of a non-lignin utilizing yeast, Rhodotorula mucilaginosa, on the photo-oxidation products.

MATERIALS AND METHODS

Straw

Source

Annual ryegrass straw (Lolium multiflorum), supplied by the Department of Farm Crops at Oregon State University from a 1971 crop, was milled to pass through a 40 mesh screen.

Hydrolysis

Hydrolyzed straw used in this study was prepared in the following manner (39). Approximately 1200 grams of milled straw were suspended in 12 liters of 3% w/v technical grade sulfuric acid and hydrolyzed by heating in an autoclave for 30 to 45 minutes at 15 psi (121°C). The straw residue was separated from the hydrolyzate by first straining through cheese cloth then compressing in a wine press. The residue was washed with 4 liters of distilled water, pressed a second time and air dryed for 48 hours. The moisture content of the straw was approximately 10%.

Preparation for Photooxidation

Air dryed hydrolyzed straw was oven dryed at 50°C for 24 hours and then prepared for photooxidation in 5 gram quantities by

first homogenizing with 250 ml distilled water for 10 minutes at high speed (Waring Blender, Model #700B) producing particles which would pass through a 100 mesh screen. The slurry was then transferred to a buchner funnel containing a #1 Whatman filter and the water removed under reduced pressure. The residue was placed in a Soxhlet thimble and extracted 24 hours with hot water at pH 2.0 to remove extractable hemicelluloses. This procedure provided an assurance that any carbohydrates detected resulted from photooxidation and not from the photooxidative system (4).

The extracted residue was collected by filtration with a #1 Whatman filter in a buchner funnel and then transferred to 500 mls of distilled water in a one liter beaker. The pH was adjusted to 2.0 with concentrated sulfuric acid prior to photooxidation.

Photolysis System

Ultraviolet Lamp

A type "A" medium pressure 500 watt mercury-vapor lamp (model #673A-36, Engelhard-Hanovia Co., Newark, New Jersey), powered by a step-up transformer (model #20651-1, Engelhard-Hanovia Co., Newark, New Jersey), was used as the photolysis source. The lamp emitted approximately 29 percent of its radiation between 184.9 and 404.5 nm, 16 percent between 435.8 and

623.4 nm, and 55 percent in the infrared region (49).

Immersion Well and Hardware

The basic photolysis system used in this study is shown in Figure 1. A double wall quartz immersion well with a 60/50 male ground glass joint (model #19434, Engelhard-Hanovia Co., Newark, New Jersey) was used to contain the lamp. The mixture to be irradiated was circulated around the outside of the well while coolant water was circulated through the double wall to maintain the temperature at 30-37° C.

The sample reaction vessel, with inlet tube near the base and outlet tube at the top, was designed to fit externally over the immersion well and seal with the well by a 60/50 ground glass female joint. The volume of the vessel was approximately 350 ml. The vessel was connected to a 1 liter external reservoir via a polyethylene-housing centrifugal pump (Chemical Rubber Co., Cleveland, Ohio) to allow continuous circulation of the sample. The pumping rate was eight liters per minute.

Oxygenation System

Compressed oxygen was passed through a needle valve and flowmeter (#19, Gelmon Instruments, Inc., Great Neck, New York) into a fritted disc located in the bottom of the reaction vessel. The

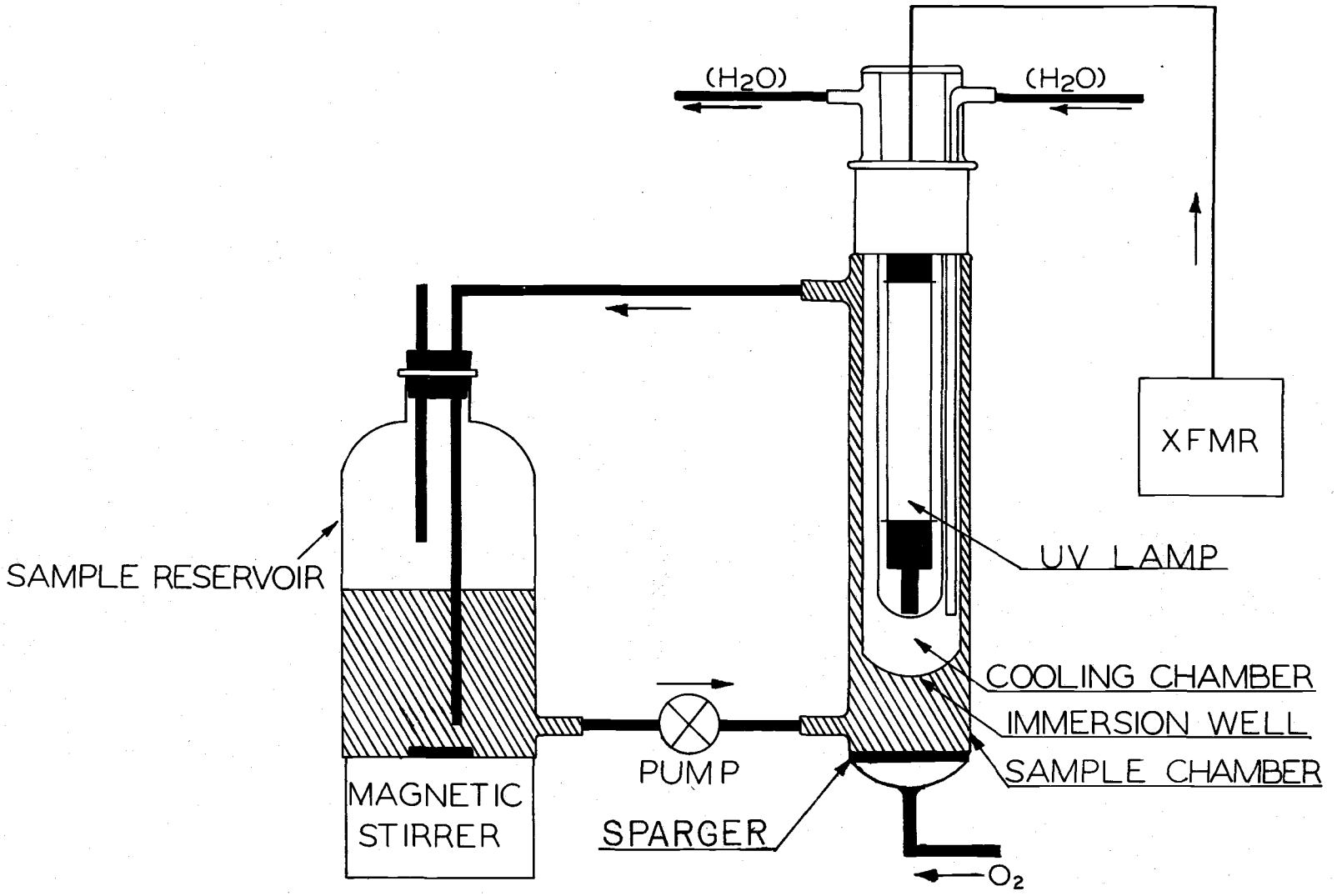


Figure 1. Schematic diagram of straw photooxidation system.

external vessel was sealed off from the atmosphere with a rubber stopper. An outlet tube was covered with a clamped rubber tube containing a slit to allow escape of excess oxygen and gases resulting from photooxidation.

Straw Photooxidation

Basic Procedure

A 1 percent w/v straw-water mixture was prepared with 5 grams of hot water extracted, hydrolyzed straw and 500 ml of distilled water. The sample was adjusted to pH 2.0 with concentrated sulfuric acid and added to the 1 liter external vessel of the photolysis system. The oxygen flow rate was set at 10 ml/min and the pump started and maintained for 15 minutes to allow dissolved oxygen stabilization before activating the lamp.

Differential Photolysis

Photooxidation of the straw sample was discontinued after 5 hours and the sample removed from the external vessel. The photolyzate was collected by filtration through a 32 cm #1 Whatman filter paper in a conical funnel and stored at 4° C until needed for analysis. The residue was removed from the filter paper and Soxhlet extracted 24 hours with hot water. After extraction the photolysis

preparation procedure was repeated with the residue and photooxidation continued another 5 hours. This procedure was repeated until 70 hours of photolysis had accrued at which point the residue could no longer be recovered for further treatment.

Sustained Photolysis

Photooxidation of each straw sample was discontinued at 20, 40 and 70 hours and the samples removed from the photolysis system. The photolyzate was collected as described in differential photolysis. The residue was Soxhlet extracted with hot water, lyophilized and saved for lignin determination. A new sample of hydrolyzed straw was prepared for each sustained photooxidation.

Dissolved Oxygen Determination

The oxygen probe employed was a steam-sterilizable membrane probe described by Johnson et al. (31). The probe was connected to a probe amplifier (31) and the electrical signals transcribed on a Rustrak Recorder (Rustrak Instrument Co., Inc., Manchester, New Hampshire).

Probe calibration was accomplished by passing the following gaseous combinations across the membrane through an extra course pyrex fritted disk in a closed tube (20 mm O. D.) at a flow rate of 100 ml/min: 100 percent nitrogen gas; 50 percent nitrogen-50 percent air;

100 percent air; 50 percent air-50 percent oxygen. Zero chart setting was established by adjusting the probe amplifier with 100 percent nitrogen gas flowing across the membrane.

After calibration the probe was transferred to the straw-water mixture in the external vessel while the lamp was on and the percent oxygen tension recorded for various oxygen flow rates through the reaction vessel fritted disk. A standard curve relating the chart readout to corresponding percent oxygen tension in the solution is shown in Figure 2. Essentially complete saturation occurs above 6 ml/min oxygen flow. A value of 10 ml/min was chosen for these studies in order that oxygen saturation would be assured even if the flow rate fluctuated 2-3 ml/min.

Dissolved Organic Solids

The concentrations of dissolved organic solids in the straw photolyzates were determined by adding 10 ml of the photolyzate to tared aluminum pans and drying to constant weight at 105° C. The weight was expressed as mg/ml.

Percent Lignin Determination

Approximately 1.0 gram quantities of lyophilized photooxidized straw residue were added to tared 25 ml beakers and dryed to constant weight at 105° C. To each beaker was added 15 ml of 72 percent

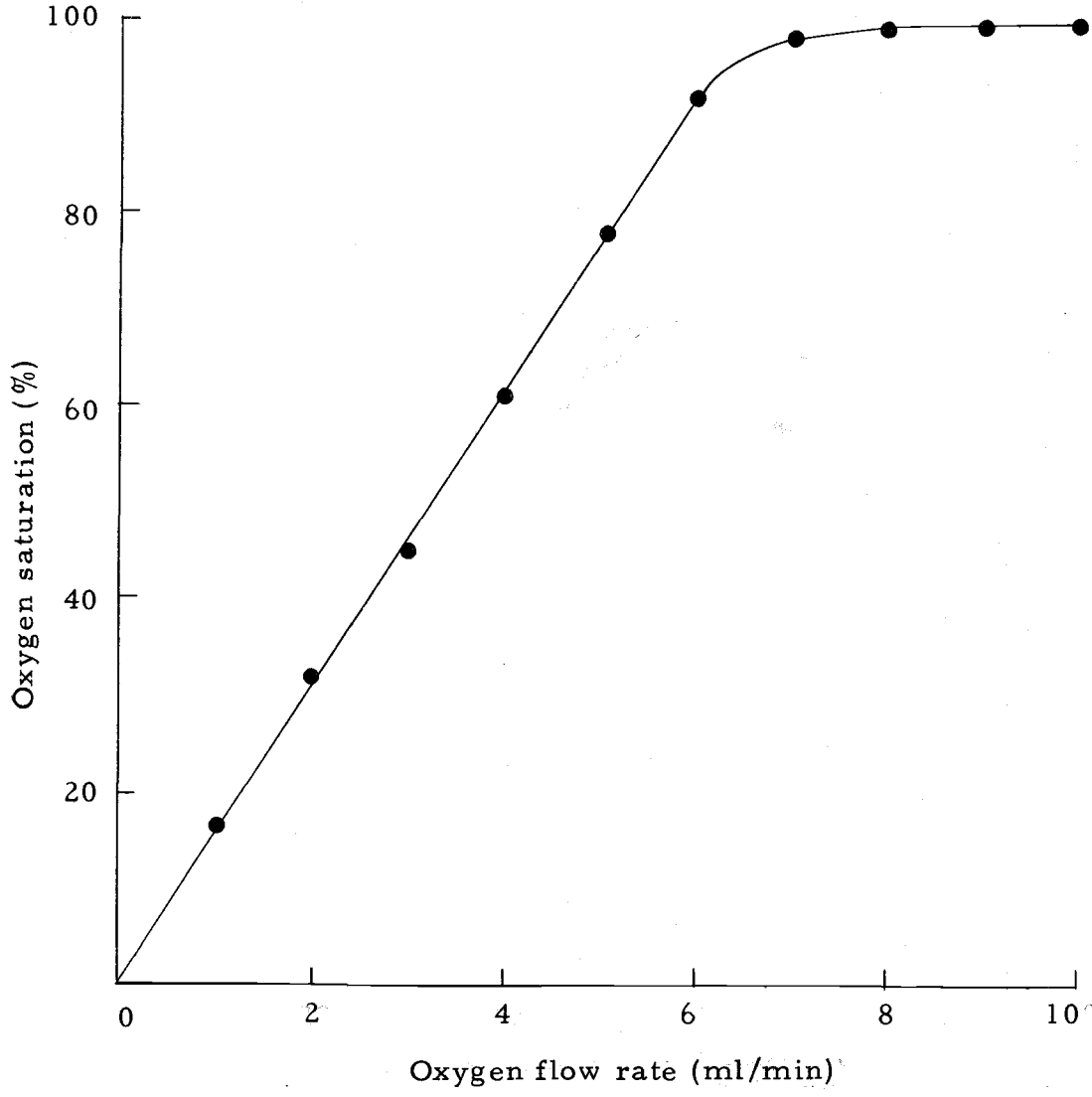


Figure 2. Determination of oxygen saturation point in a ryegrass straw-water photooxidation mixture using a steam-sterilizable membrane probe.

sulfuric acid, which had been pre-cooled to 15° C, and the beaker placed in an 18-20° C water bath for 1 hour with occasional stirring. After 1 hour the mixture was diluted to 3 percent sulfuric acid with distilled water and refluxed 4 hours. The lignin residue was collected under reduced pressure onto predried and tared spun glass filters (S & S No. 24) held in a borosilicate filter holder (VWR Scientific #28153-020) and the residue, representing the lignin fraction, washed free of acid with 500 ml of hot water. The glass filters were then dryed to constant weight at 105° C. The percent lignin in the original sample was determined from the change in residual dry weight.

Total Carbon Analysis

The system used to determine total carbon was a modification of the original unit described by Rockhill (52). The unit had 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 in the above reference.

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.

Ten 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 benzoic acid with 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.

Gas-Liquid Chromatography (GLC)

Instrumentation

GLC analysis was performed on an F & M instrument, model 402, equipped with a hydrogen flame ionization detector (Hewlett Packard, Avondale, Penn.). Chromatographic peak area measurements were made with an electronic integrator (model 3370, Hewlett Packard, Avondale, Penn.). The standard chromatograph conditions were as follows:

Injector temperature - 215° C

Detector temperature - 210° C

Programmed column temperatures - 120-180° C

Isothermal column temperature - 180° C

H_2 - 40 ml/min

He - 70 ml/min

Air - 230 ml/min

Sample size - 5 μl

Preparation of Column

One hundred milligrams of ethylene glycol succinate (F & M Scientific Corporation, Avondale, Penn.) were dissolved in 25 ml of chloroform and 100 mg ethylene glycol adipate (Applied Science, State College, Penn.) were dissolved in acetone. The above mixtures were quickly poured together, mixed with 10 grams of Gas Chrom P, 100-120 mesh (Applied Science, State College, Penn.) and occasionally gently stirred. After the main portion of solvent evaporated the residue was dried in a flow of air at room temperature in a 150 mm diameter petri dish until the odor of chloroform and acetone disappeared. About 6 ml of this powder was packed by vibration into a 4 ft x 1/8 inch o. d. stainless steel column and both ends plugged with glass wool. The newly prepared column was conditioned overnight at 180° C while purging with helium at a flow rate

of 70 ml/min.

Preparation of Alditol-Acetate Derivatives

Alditol-acetate derivatives were prepared on the photolyzates by a modification of the procedure reported by Albersheim et al. (2).

Twenty-five milliliters of photolyzate were placed in a 100 ml beaker and saturated barium hydroxide added to bring the solution to pH 5.5. The barium hydroxide precipitate obtained by this procedure was removed by centrifugation at 2000 x g for 10 minutes and the centrifugate transferred to a 100 ml beaker. A 0.75-0.80 g quantity of sodium borohydride was added to the centrifugate and allowed to stand 2 hours to reduce all aldehydic groups to alcohols. Excess sodium borohydride was removed by adding glacial acetic acid dropwise from a Pasteur pipet until hydrogen gas evolution ceased. The solution was transferred to a 250 ml round bottom flask and 0.1 ml of 10 mg/ml myo-inositol solution added as an internal standard. The solution was evaporated to dryness in vacuo on a rotary evaporator. Residual water and borate were removed by adding 10 ml of methanol and evaporating to dryness. Methanol addition and evaporation were repeated and then the flask placed in an oven at 105° C for 15 minutes to remove any residual moisture. After cooling 10 ml of acetic anhydride and 0.5 ml of concentrated sulfuric acid were added and the solution placed in a 70° C water bath for 1 hour. The solution was cooled 5 minutes and then slowly poured into approximately 70 ml of

ice water to precipitate the acetate derivatives. The water mixture was transferred to a separatory funnel and extracted with 25, 15, and 10 ml of dichloromethane respectively. The solvent phase was removed after each extraction and added together then evaporated on the rotary evaporator to a syrup consistency. One milliliter of distilled water was added to remove excess acetic anhydride and the solution evaporated to dryness.

The alditol-acetate derivatives were taken up in 1 ml of dichloromethane and centrifuged to remove any sediment. Five microliter injections were used for chromatographic analysis.

GLC - Rapid-Scan Mass Spectrometry

Mass spectra of the acetylated derivatives already described, were obtained using a flame ionization GLC, Model 810 (F & M Scientific, Avondale, Penn.) integrated with a rapid-scan mass spectrometer (Atlas MAT CH-4 Nier type). The column conditions were identical with those described in the alditol-acetate derivative GLC analysis. 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 mass spectra 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 (21) for determining the phenolic content of the photolyzates were obtained on a Zeiss absorption spectrophotometer, Model PMZII (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 #506964/60HZ. Matched 1.0 cm quartz cells, #46005 (Beckman Instruments Co., Fullerton, California) were used to contain the sample.

Aromatic Nuclei

Absorption at 280 nm was used to determine the presence of aromatic nuclei in the photolyzates. The percent of aromatic nuclei was calculated using an absorptivity value of 3810 for Bjorkman lignin at 280 nm (54).

Growth Parameters for *Rhodotorula mucilaginosa* on Photooxidized Straw Photolyzates

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 percent agar slants containing 10 ml of a ten-fold mineral salts solution and 90 ml of 0.1 percent 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, $Na_2HPO_4 \cdot 7H_2O$-5 and thiamine hydrochloride-5×10^{-4}.

Fifteen ml of mineral salts solution were added to 75 ml of photooxidized straw photolyzate previously adjusted to pH 5.5 with 5 N sodium hydroxide. The solution was passed in vacuo through a fritted disc bacterial filter, porosity UF-#33990 (Corning Glass Works, Corning, New York). The sterile solution was split aseptically into three 30 ml portions and each portion added to a sterile baffled 300 ml sidearm flask, 14 x 130 (Bellco Glass, Inc., Vineland, N. J.), inoculated with Rhodotorula mucilaginosa 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 incubation temperature held at 25° C.

The cells used for inoculation were taken from mid-exponential growth on a medium containing mineral salts and glucose, and washed three times with sterile mineral salts solution. These were then diluted with mineral salts solution to a reading of 200 on a Klett-Summerson colorimeter, filter #42 (Klett Manufacturing Co., New York) and 0.1 ml of the suspension added to each flask. All growth responses were monitored with the above colorimeter.

RESULTS

Photooxidation Effects on Annual Ryegrass Straw

Total Dissolved Solids

The total dissolved solids present in the photolyzates with respect to time irradiated for both sustained and differential photooxidation are shown in Figure 3. Each point on the sustained photooxidation curve represents an average of dissolved solids in the photolyzates from three photooxidations of new straw samples irradiated for the time indicated. The variance from one run to another never exceeded 0.2 mg/ml. The points on the differential photooxidation curve represent the dissolved solids in the photolyzates obtained since the completion of the last irradiation period on a single straw residue.

Total dissolved solids increase rapidly during the first 25 hours of photooxidation and then begin to level off. After 75 hours of photooxidation the concentration of dissolved solids declined slowly in a linear fashion. A similar but more accelerated pattern was observed with the differential photooxidative system. A rapid increase occurred initially then approached fifty percent of the sustained photooxidation concentration. The decline occurred much earlier, 5 hours after photooxidation, and at an increased rate before approaching a slow, linear decline at 50 hours irradiation.

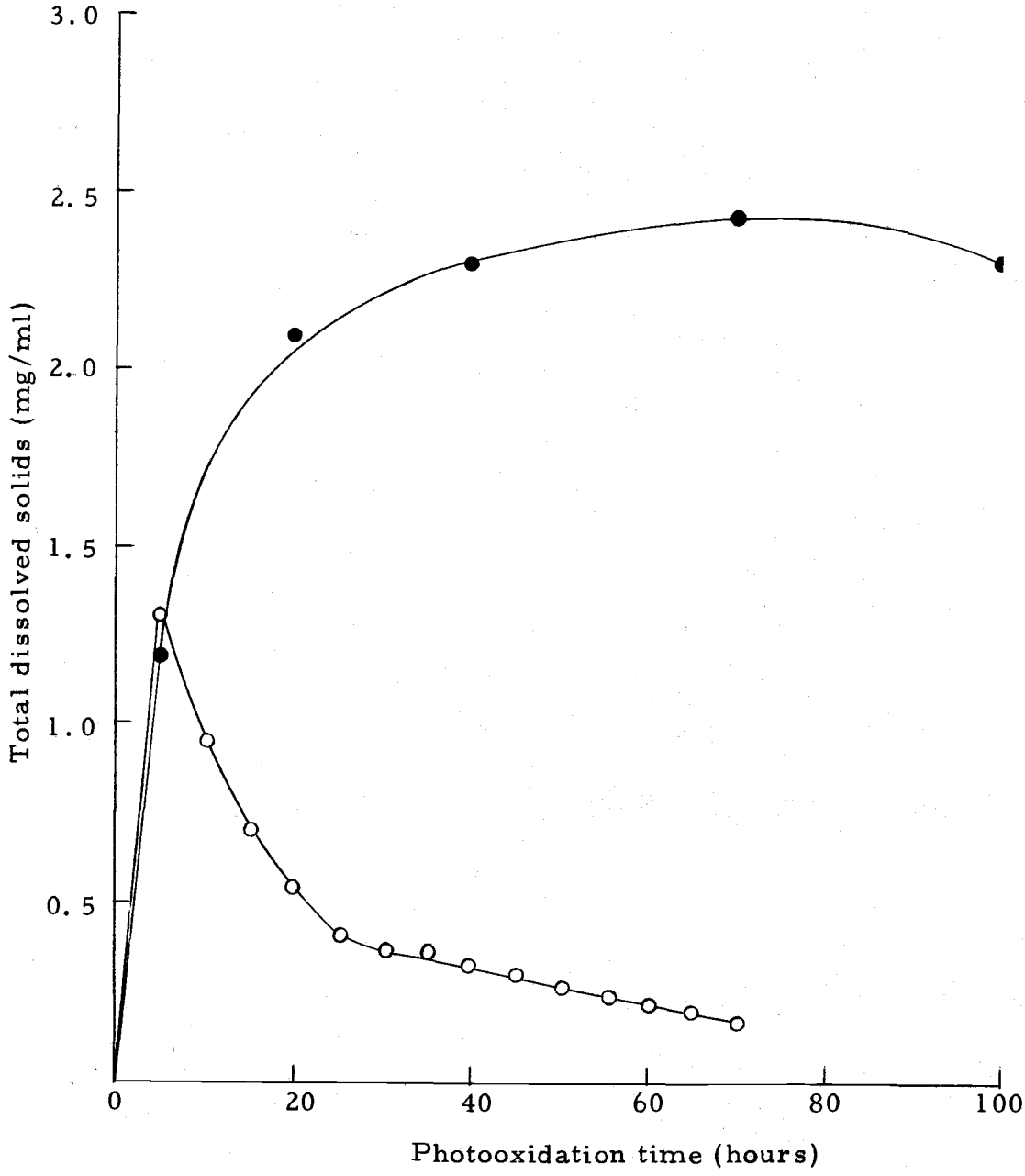


Figure 3. Total dissolved solids released during sustained (●—●) and differential (○—○) photooxidation of H_2SO_4 hydrolyzed ryegrass straw at pH 2 with 10 ml/min of oxygen purging the reaction chamber.

Total Carbon

Total carbon values obtained from the photolyzates of both sustained and differential straw photooxidations with respect to irradiation time are shown in Figure 4. The overall response for both systems closely resemble the dissolved solids data. A gradual decrease in total carbon is seen after 25 hours irradiation in the sustained photooxidation system.

Total carbon values obtained from differential photooxidation do not exceed 560 μg/ml. The total carbon decreased at a very rapid rate after 5 hours photooxidation to approximately 80 μg/ml by 30 hours. The rate with which total carbon is released is very low after 30 hours irradiation.

Carbohydrate Release

Gas-Liquid Chromatography (GLC) combined with Mass Spectrometry data were used to delineate photodecomposition products, which were mainly carbohydrates. Table 1 shows carbohydrates detected by these methods and their respective GLC retention times. Both sustained and differential photooxidation conditions produced peaks with the same retention times. With the exception of the unidentified peak having a retention time of 9.8 minutes all spectra were compared to commercial alditol-acetate standards by adding

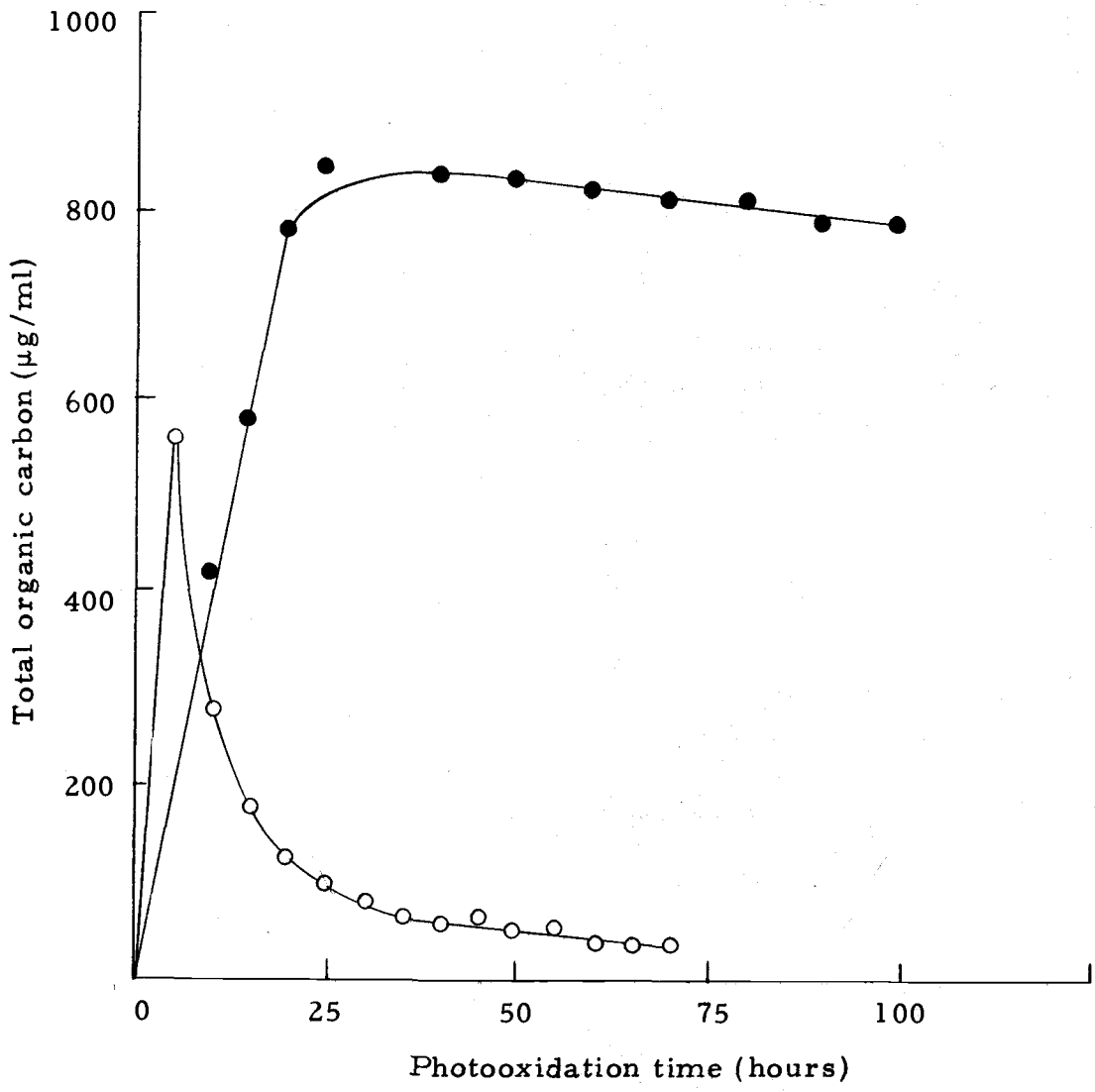


Figure 4. Total organic carbon remaining in solution after sustained (●—●) and differential (○—○) photooxidation of a H_2SO_4 hydrolyzed straw at pH 2 with 10 ml/min oxygen purging the reaction chamber.

Table 1. Retention times obtained from alditol-acetate derivatives of carbohydrates isolated in all straw photolyzates.

Programmed on GLC: 2°/min @ 120°-185°C

Carbohydrate	Retention Time (min)
Glyceraldehyde	4.6
Unknown Triose	9.8
Erythrose	16.8
Threose	19.2
Rhamnose	25.6
Arabinose	31.2
Xylose	35.4
Mannose	43.1
Glucose	46.9
Myo-inositol (internal standard)	51.8

a standard to the test solution and noting increased peak heights. All standards were found to correlate well with both GLC and mass spectroscopy data. The unidentified compound produced a spectra resembling a triose but could not be positively identified.

The concentrations of the nine carbohydrates isolated at various differential photooxidation periods are shown in Figures 5 and 6. The points shown all represent an average of several runs under standard conditions. The deviation from the mean of all runs never exceeded 20 μg/ml. All carbohydrates with exception of the unknown are released from the straw most efficiently between 10 and 20 hours of photooxidation. Xylose and mannose are apparently released very early in the photooxidation period at a rate much greater than photodecomposition of these sugars in solution occurs. The ratio of xylose and mannose release rate to photodecomposition rate decreases after 5 hours irradiation so that xylose and mannose, reach a concentration of less than 20 μg/ml by 35 hours photooxidation. Rhamnose also reaches its maximum ratio of release to photodecomposition rate at 5 hours irradiation, however, the rhamnose concentration is much lower than xylose or mannose and decreases to zero by 15 hours irradiation. Glyceraldehyde attains a maximum of 185 μg/ml by 5 hours irradiation and maintains this level through 10 hours before decreasing to the level characteristic of all the carbohydrates except rhamnose at 35 hours.

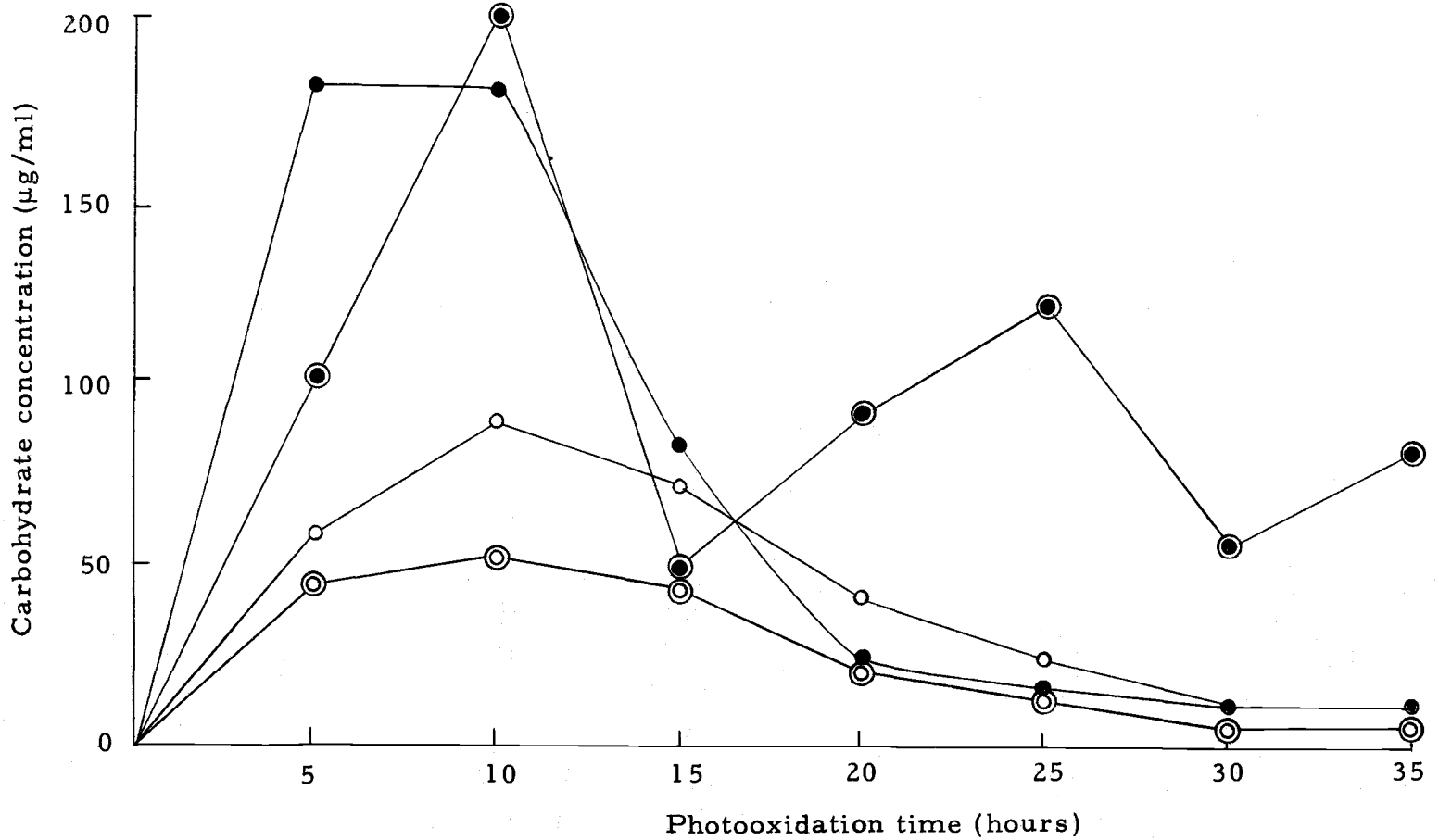


Figure 5. Concentrations of glyceraldehyde (●—●), threose (○—○), erythrose (◎—◎) and an unknown triose (⊙—⊙) released during differential photooxidation as determined by GLC analysis.

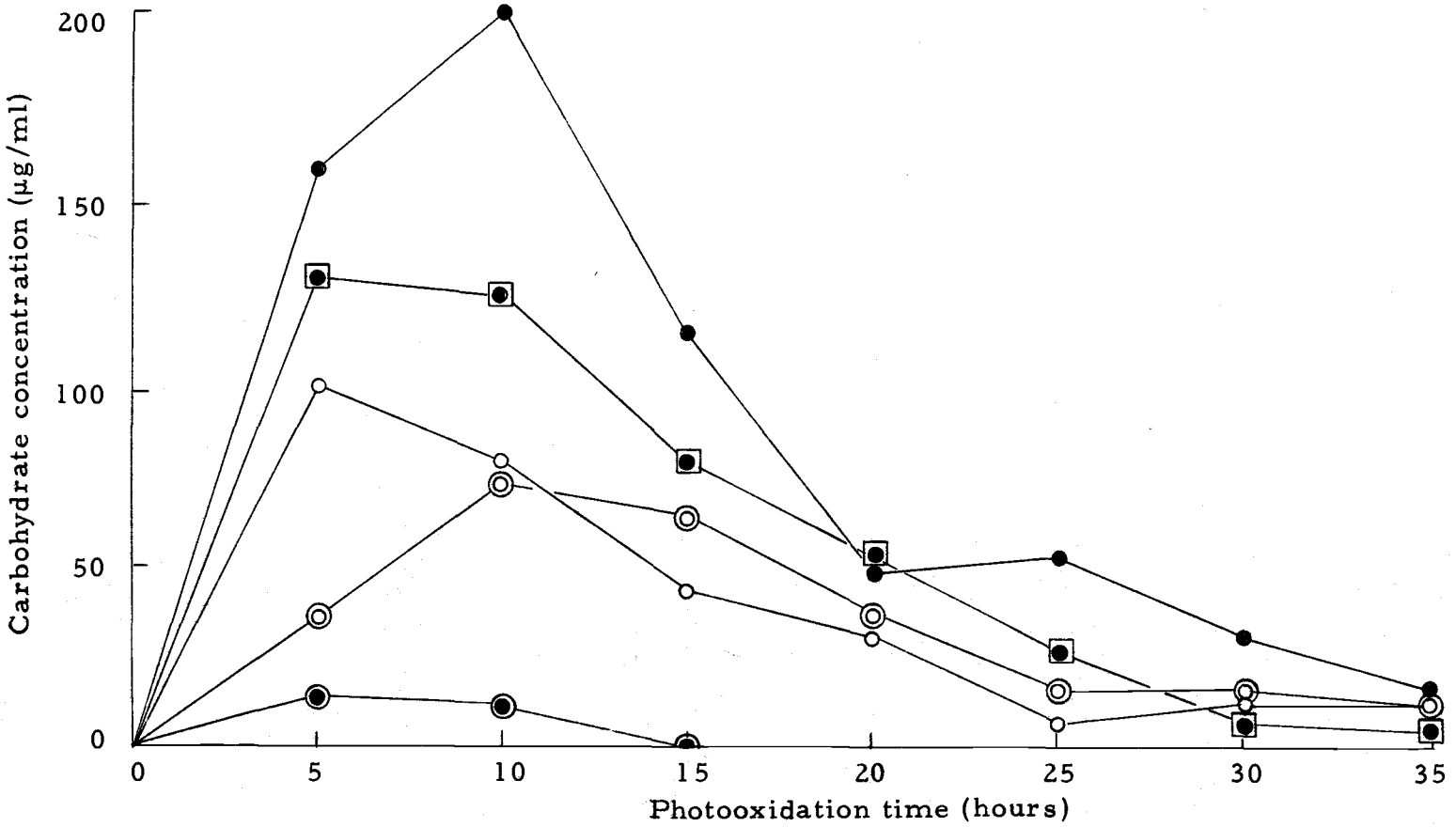


Figure 6. Concentrations of arabinose (●—●), xylose (▣—▣), mannose (○—○), glucose (◎—◎) and rhamnose (⊙—⊙) released during differential photooxidation as determined by GLC analysis.

The rate of increasing concentration changes somewhat for threose, arabinose, erythrose and glucose during photolysis. Threose, erythrose and glucose reach their maximum levels at 10 hours irradiation at slower rates and decrease in a similar fashion until the 35 hour level is reached. Arabinose and the unknown triose are both released in relatively large quantities during the first 10 hours photooxidation and then decrease at irradic rates after 20 and 15 hours irradiation respectively.

Aromatic Nuclei and Phenolic Hydroxyl Formation

Figure 7 shows the highest concentration of aromatic nuclei occurred at 5 hours photooxidation. The percent aromatic nuclei released by irradiation rapidly decreased after 5 hours irradiation to the very low level indicated at 15 hours. After 10 and 15 hours irradiation, 62 and 6 percent respectively of the remaining total aromatic nuclei has been released. Photooxidation longer than 15 hours did not change the percent released significantly.

A maximum of 0.43 percent phenolic hydroxyl occurred at 10 hours photooxidation. This peak is in close agreement with the aromatic nuclei peak. A rapid decline occurred beyond 10 hours irradiation reaching essentially the same release level as the aromatic nuclei by 25 hours photooxidation.

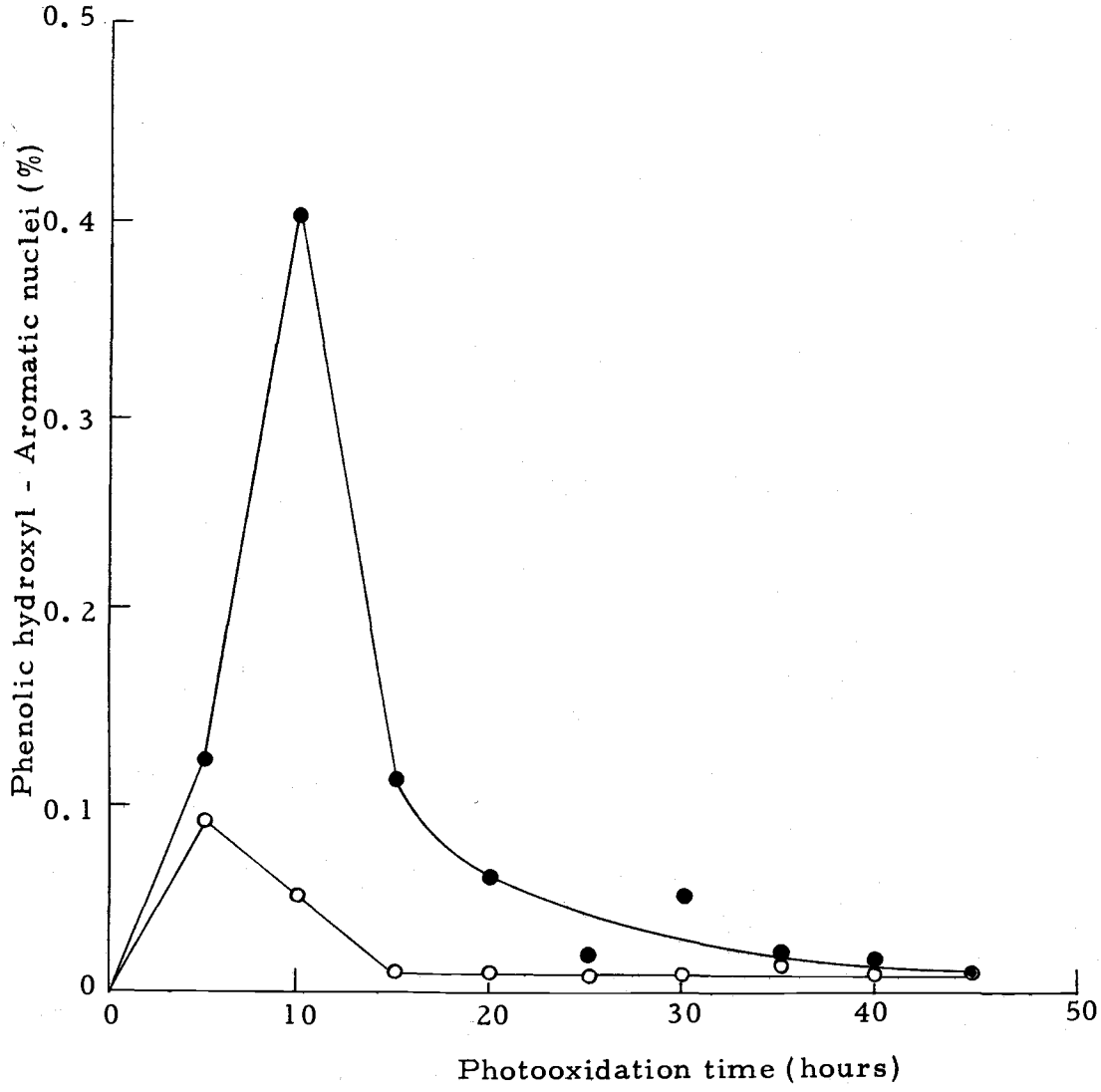


Figure 7. Effect of photooxidation time on the release of phenolic hydroxyl groups (●—●) and aromatic nuclei (○—○) into straw photolyzates at pH 2 and a 10 ml/min oxygen flow rate.

Lignin Changes

The percent lignin remaining in the straw residue after photo-oxidation is shown in Figure 8. The sulfuric acid hydrolyzed straw used in this study had a lignin content of 33.6 percent prior to photo-oxidation. For the first 15 hours there was a rapid decline in lignin followed by a gradual decrease to a level of 9 percent at 40 hours (Figure 8).

The characteristic dark brown color noted with Klason lignin in non-irradiated straw turned colorless after 10 hours irradiation and then a dark grey at 15 hours. The grey color persisted throughout the remainder of the irradiation period. These color changes correspond to the rapid decrease in the lignin content. After 15 hrs the decomposition approached a constant rate. The residue after approximately 5 hours irradiation became very finely dispersed in the photolyzate and required one to three hours standing to settle out. Once the residue was filtered and dried it became very rigid and tough and could not be resuspended in water. The texture resembled paper or cardboard, and had to be pulverized in a Wiley mill before the lignin content could be accurately determined.

Growth Response to Straw Photolyzates

The growth response of Rhodotorula mucilaginosa to the straw differential photolyzates is shown in Figure 9. Rhodotorula growth

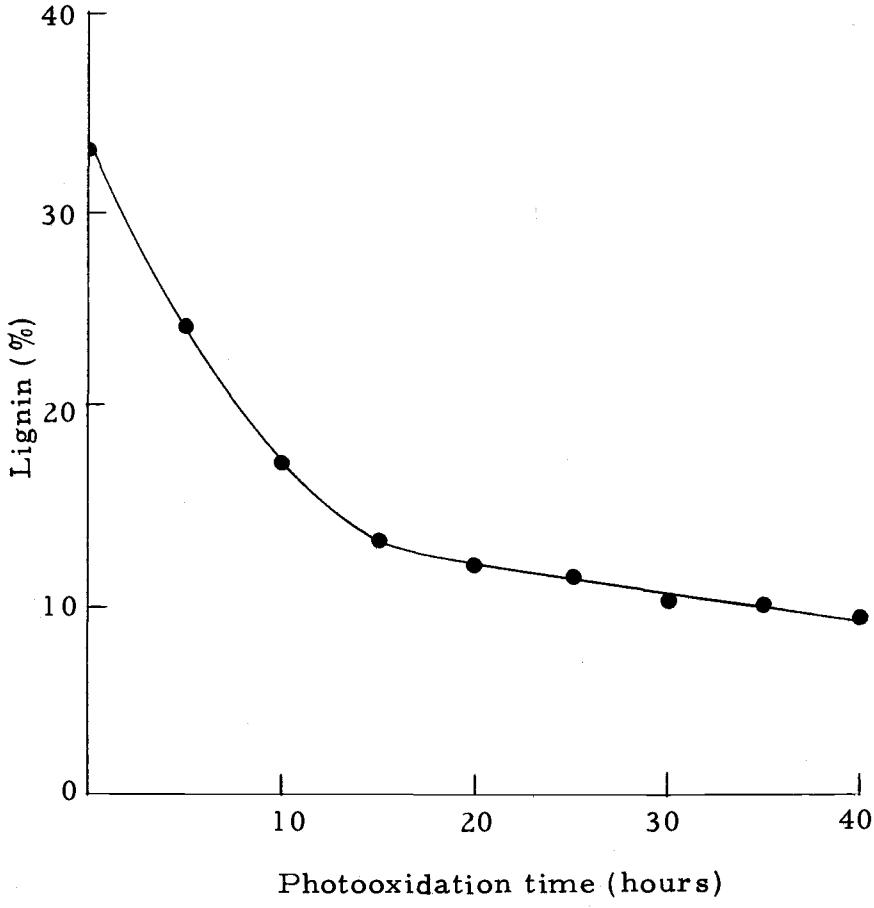


Figure 8. Effect of photooxidation time on the dissolution of straw-lignin at pH 2 and a 10 ml/min oxygen flow rate.

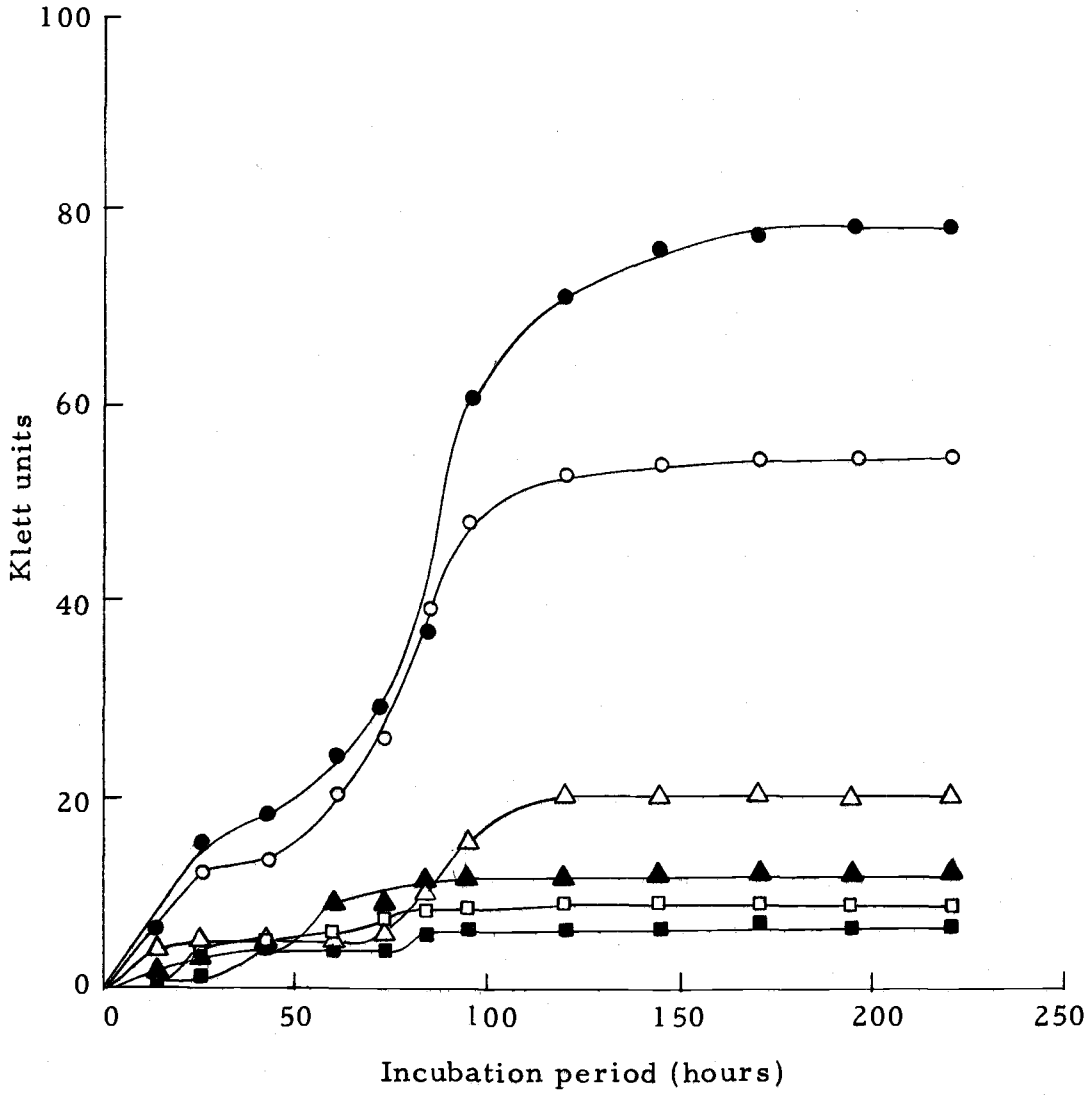


Figure 9. Growth response of Rhodotorula mucilaginosa to photolyzates from 5 (●—●), 10 (○—○), 15 (△—△), 20 (▲—▲), 25 (□—□) and 30 (■—■) hour differential photooxidations of a 1% w/v ryegrass straw.

in all photolyzates was evident after 15 hours incubation. After 25 hours incubation growth in the 15, 20, 25 and 30 hour photolyzates exhibited the beginning of a 50 hour lag period. The lag appearing in the 5 and 10 hour growth systems was not as pronounced as in the others but instead exhibited a decreased growth rate for approximately 25 hours followed by a rapid increase to maximum values of 78 and 55 Klett units respectively.

Maximum growth response decreased very rapidly with increasing photooxidation time for the 5, 10, and 15 hour samples. The 20, 25, and 30 hour samples supported growth to a much lesser degree and were fairly uniform in their maximum response. As photooxidation time increased, the incubation time required for maximum growth response decreased. The 5, 10, and 15 hour growth systems reached a maximum response after 170, 145, and 120 hours incubation, respectively. The 20, 25, and 30 hour growth systems had essentially reached a maximum level by 95 hours incubation. Growth responses to photolyzates from straw treated longer than 30 hours were not great enough to give reliable data.

The reducing groups present in the differential photolyzates before and after Rhodotorula growth are shown in Figure 10. The maximum production of reducing groups (2.06 mg/ml) from photolysis occurred at 5 hours and corresponds with the maximum growth response exhibited in Figure 9. Reducing group production decreased

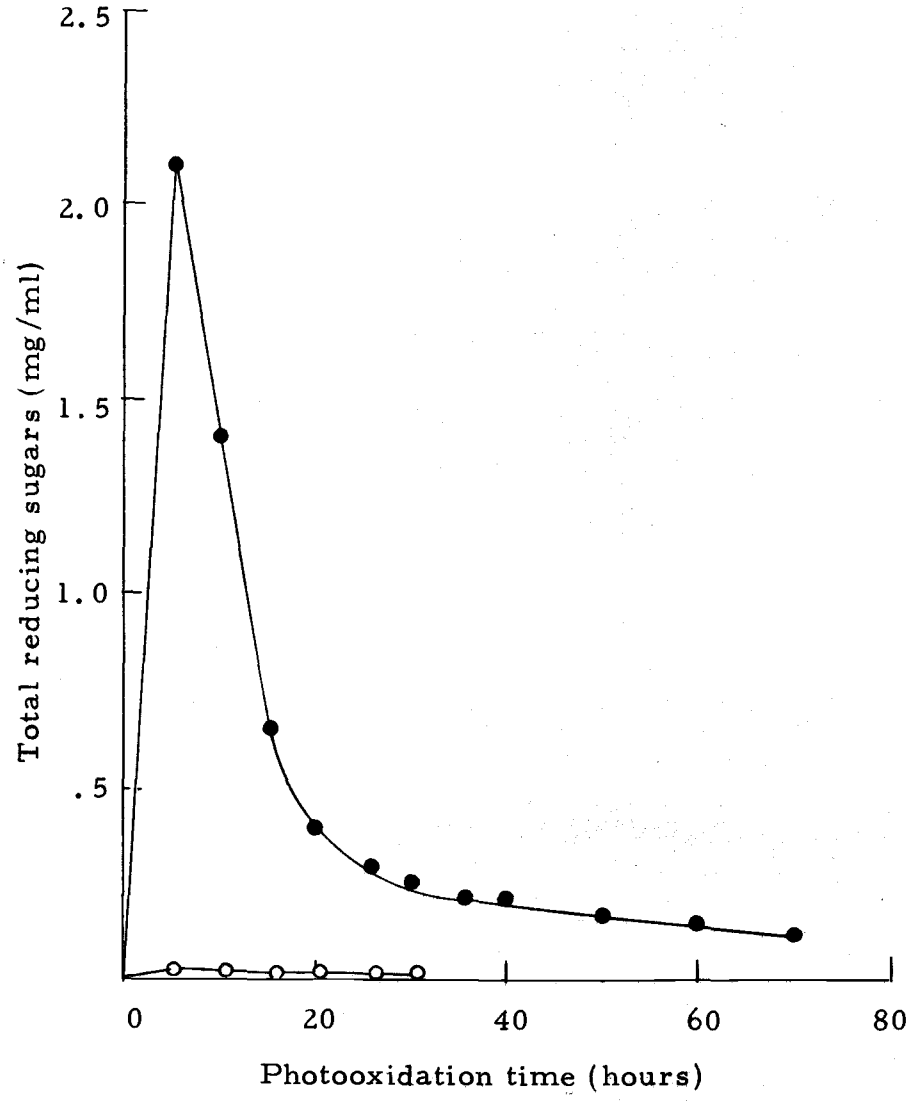


Figure 10. Total reducing sugars present in straw photolyzates before growth (●—●) and after growth (○—○) with Rhodotorula mucilaginosa.

rapidly after reaching the maximum level and diminishing to approximately 150 μg/ml after 60 hours photooxidation.

Growth responses of Rhodotorula mucilaginosa to straw photolyzates after sustained photooxidation are shown in Figure 11. Growth was fairly rapid in all photolyzates tested at 15 hours incubation. After 50 hours incubation, growth in the 20 hour photolyzate decreased producing a slight lag before a second response occurred after approximately 100 hours incubation. The second response reached a maximum of 88 Klett units after 160 hours incubation. The 40 hour sample exhibited a similar pattern but with an increased initial response followed by a lag in growth after 50-60 hours incubation. Growth appeared minimal or non-existent during the lag period in this sample. The secondary response was also rapid in this sample, reaching a maximum of 66 Klett units by 140-150 hours incubation. The 70 hour photolyzate response was less pronounced in the early stages and entered the lag phase somewhat later (80 hours) than in the other photolyzates. The secondary response was reduced considerably and only increased 8 Klett units after the lag. The secondary responses uniformly decreased in the photolyzates as photooxidation time increased.

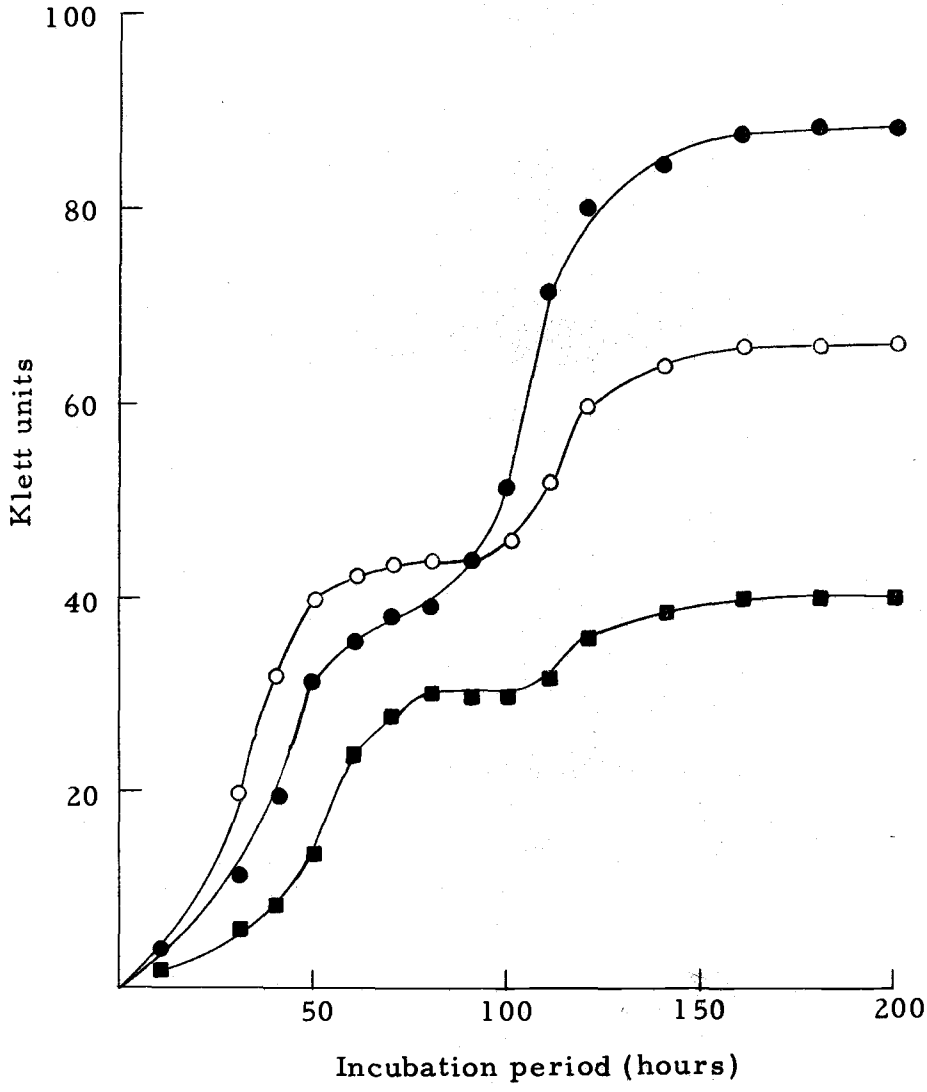


Figure 11. Growth response of Rhodotorula mucilaginosa to photolyzates from 20 (●—●), 40 (○—○) and 70 (■—■) hour sustained photooxidations of a 1% w/v ryegrass straw.

DISCUSSION

One of the major spectral lines emitted from the mercury vapor lamp used in the study is at 280.3 nm (49). The calculated quantum energy of this spectrum (56), would be sufficient to cleave the common C-C (60 Kcal/mole), C-O (70 Kcal/mole) and C-H bonds (90 Kcal/mole) found in the lignin, cellulose and hemicellulose molecules.

The energy distribution of the lamp is reported to have 6.9 percent of its spectral output at wavelengths between 180 and 280 nm (49). Many of the longer wavelengths, even though they contain lower energy, still have sufficient energy to slowly degrade lignin, cellulose and hemicellulose molecules if an electron acceptor is present. This should not be overlooked when evaluating the photooxidation process. Oxygen is activated by an energy level of 34.9 Kcal/mole and is readily influenced by wavelengths as long as 819 nm. The mercury vapor lamp used in this study provided many wavelengths containing sufficient energy to activate the oxygen present in the system and bring about a photooxidative process (52).

Most of the energy produced by the lamp existed at wavelengths longer than 280 nm which can produce some direct C-C, C-O and C-H bond cleavage. However, most of the energy is dissipated as heat or utilized to activate oxygen which in turn causes bond cleavage by secondary reactions. The power output at wavelengths shorter

than 219 nm is only 0.53 percent of the total available. Most of the direct bond cleavage, however, may occur as a result of these shorter wavelengths after which many secondary reactions with activated oxygen could occur degrading the complex lignin and polysaccharide molecules to aliphatic acids and monosaccharides.

The purpose of this study was to better understand the role of ultraviolet light in the photodecomposition of straw and subsequent microbial utilization of the compounds released. It was deemed valuable to evaluate not only normal long term photooxidation but also short term differential photooxidation. From differential photooxidation data one is able to determine types of compounds released at given time intervals and therefore, more reliably evaluate the photodecomposition mechanism.

Before beginning the photooxidation study the straws were extracted with hot water to reduce the presence of water soluble hemicelluloses. These compounds would not have resulted from photooxidation and could have confused the results.

The very early release of large quantities of soluble organic compounds in the aqueous photolyzates is one important fact quite evident in most of the data presented. Data in Figure 3 demonstrate the release of dissolved solids at a rapid rate during the first 20 hours of photooxidation. Photooxidation of dissolved solids undoubtedly occurs at the same time, producing carbon dioxide and water.

This apparently occurs at a much slower rate than the release of photooxidative products from the complex water insoluble structures. After approximately 25 hours of photooxidation the decomposition to carbon dioxide and water slightly exceeds the formation and release of organic molecules. This is indicated by gradual decline in concentration at each time interval examined.

The data obtained by Gas-Liquid-Chromatography and Mass Spectroscopy analyses showed most compounds released were carbohydrate structures consisting of trioses, tetroses, pentoses and hexoses. The total concentration of each carbohydrate isolated with respect to photooxidation time correlated closely with the total carbon values obtained (Figure 4). A value of 40 percent carbon was used for carbohydrates. This suggests very few other non-carbohydrate compounds were released to the aqueous phase during photooxidation. After 5 hours differential photooxidation the total concentration of the nine released carbohydrates was approximately 830 μg/ml. The calculated carbon per organic molecule present is approximately 67% when compared to the total carbon value of 560 μg/ml. The total carbon values per organic molecule for the 10, 20, 30 and 40 hour photooxidation periods were calculated to be 45, 42, 40 and 40 percent respectively. This suggests that most, if not all, of the organic structures in the aqueous phase were carbohydrates by 30 hours photooxidation. Gas-liquid-chromatography data of the

alditol-acetate derivatives obtained from these early irradiation periods show many small peaks with concentrations too low to be analyzed by mass spectroscopy. These occur primarily in the 5 hours photolyzates having a carbon content of 67% suggesting the calculated percentage is not correct with respect to carbohydrate concentration since all of these organic structures were not accounted for in the nine carbohydrates reported. It would seem improbable that only carbohydrates would be present in the photolyzates when lignin is also decomposed by ultraviolet light (17, 32, 38). The total carbon, which exceeded the 40% level, may be from the production of aromatic constituents which are non-reducing and would not appear in results from the alditol-acetate procedures used for GLC. Data in Figure 17 supports this concept by showing the maximum aromatic nuclei and phenolic hydroxyl content occurs at 5 and 10 hours of photooxidation. After 10 hours exposure the decline in both aromatic nuclei and phenolic hydroxyl groups is rapid and corresponds to the decrease in percent total carbon per molecule in the aqueous phase. The disappearance of these minor peaks after 20 hours of photooxidation and the very low concentration of aromatic nuclei and phenolic hydroxyl groups places the significance of the total carbon increase in question and supports the suggestion that most of the carbon present represents carbohydrates. Fragments containing 2-3 carbon atoms would also be altered photolytically and gain hydroxyl groups in their

structure. These compounds would be activated and appear in the GLC analysis. Most of the low concentration peaks found in the 5 and 10 hour photooxidation GLC analyses were not identified or reported among the nine isolated in this study. These compounds had low retention times on the column similar to two and three carbon structures.

The lignin content of the straw before photooxidation determined by the standard 72% sulfuric acid treatment was found to be 33.6 percent (Figure 8). Photooxidation in the presence of oxygen had a very pronounced effect on the apparent lignin concentration which rapidly decreased to approximately 14 percent after 15 hours photooxidation and finally 9.6 percent by 40 hours photooxidation. With this rapid decrease in lignin concentration one would expect to see a rapid increase in the aromatic nuclei and phenolic hydroxyl concentrations reaching maximums around 15 to 20 hours photooxidation. This does not occur and the data suggests the lignin phenylpropanoid structure may have been fragmented to 2, 3 or 4 carbon units. If this type of reaction occurred in the pH 2 photolyzates with ultraviolet activation, one would expect formation of hydroxylated carbon atoms. These would be subject to acetylation by the alditol-acetate procedure and appear in fairly large quantities in the GLC chromatograms. This evidently happens to some extent since glyceraldehyde and the unidentified triose are present in fairly high concentrations (Figure 7). The

high concentration of glyceraldehyde and the triose could explain some of the lignin loss if photooxidation of the C_3-C_6 phenylpropanoid structure occurred.

The decrease in lignin concentration could also be accounted for by an increased solubility in sulfuric acid due to changes in the lignin structure resulting from photooxidation. This type of change has been noted to some degree by Forman (17). When wood meal was exposed to ultraviolet light for 170 hours in air, he noted a lignin decrease from 27.5 percent to 18.0 percent, as determined by the 72 percent sulfuric acid method. Forman suggested the increased lignin solubility in sulfuric acid was due to loss of methoxyl groups from the aromatic ring of the phenylpropanoid structure. As the methoxyl loss continued the material yellowed and finally became bleached. This color change was also noted in this study when the straw residue first became light brown or yellow and finally bleached between 10 and 15 hours photooxidation. This also corresponds to the change in slope on the lignin concentration curve in Figure 6. This change of slope which corresponds to the bleaching of the straw suggests a methoxyl loss could be responsible for increased lignin solubility in sulfuric acid. When the material becomes bleached most of the demethoxylation has occurred thus reducing the rate with which lignin solubility was increased.

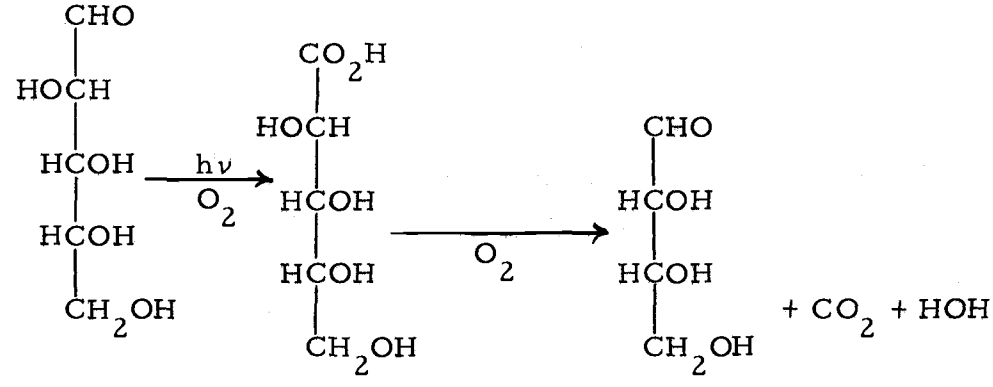
It is interesting to note that when lignin was prepared by the

72 percent sulfuric acid procedure, a procedure commonly used to prepare "pure" Klason lignin, followed by photooxidation using the procedures of this study, all of the sugars found in the straw residues were also found in Klason lignin with the exception of rhamnose. The unidentified triose was also present. These data suggest cellulose and hemicelluloses are closely linked to the lignin macromolecule and are probably bonded to it throughout many points of its structure.

The sugars commonly found in the hemicellulose portion of grasses (3), i.e. xylose, arabinose, glucose, rhamnose, mannose, and galactose, were also found in this study with the exception of galactose as is shown in Figure 6. In most species of grass, sugars occur in similar proportions with an approximate ratio of 1:2:3:12 for galactose, arabinose, glucose and xylose, respectively. Generally speaking xylose is the most abundant and galactose the least (4). Rhamnose has been reported in small amounts as a component sugar to the basic units reported above. In this study, rhamnose also occurs and at a much lower concentration than the other sugars (Figure 6). Galactose was not detected, indicating it was not present to any appreciable extent in the annual ryegrass used in this study or it had been removed by the previous sulfuric acid hydrolysis and hot water extraction. Earlier studies (4) report over 90 percent of the total hemicelluloses are extractable from grasses by hot

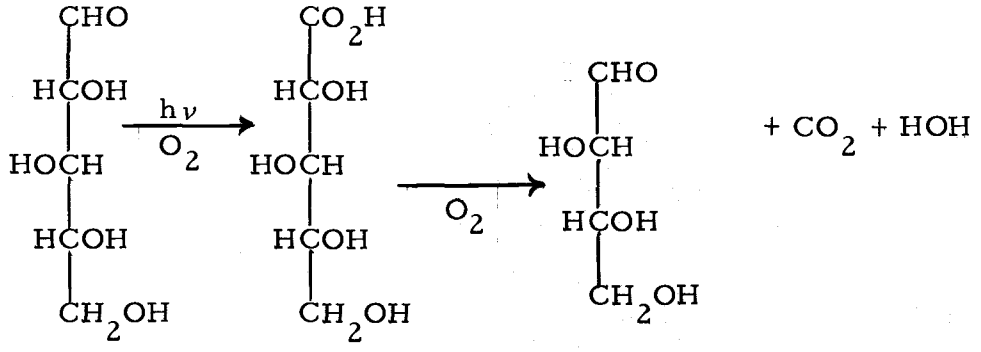
0.01 N HCl. The straw used in this study was hydrolyzed with 3 percent sulfuric acid at 120° C and then extracted 24 hours with hot water at the low pH of the residual acid. This treatment would remove many of the hemicelluloses and may explain why galactose was not detected.

The sugars shown in Figure 5 are unique since they have not been reported present in grasses. These sugars appear to be photo-oxidative fragmentations of existing sugars. As reported earlier, glyceraldehyde and the unidentified triose could have resulted from photolysis of the aliphatic three carbon structure on the basic lignin phenylpropanoid unit. The occurrence of the other sugars in Figure 5 and possibly some of the glyceraldehyde, could be due to cleavage of the number one carbon group of the more abundant sugars, arabinose and xylose, leading to the formation of erythrose and threose.



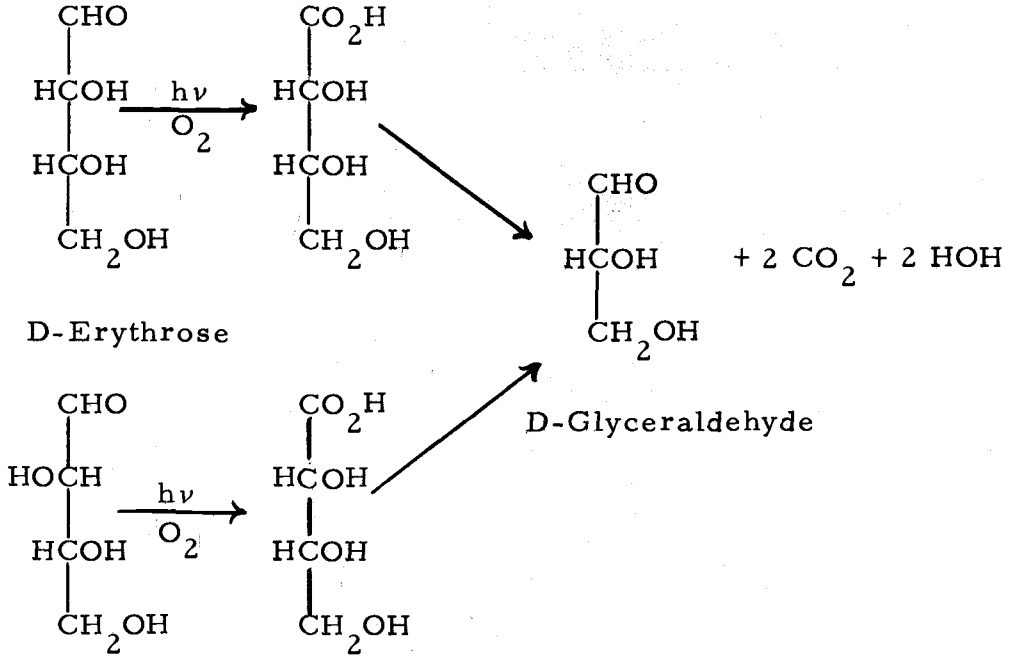
D-Arabinose

D-Erythrose



D-Xylose D-Threose

Further, C_1 degradation of erythrose and threose would lead to glyceraldehyde.

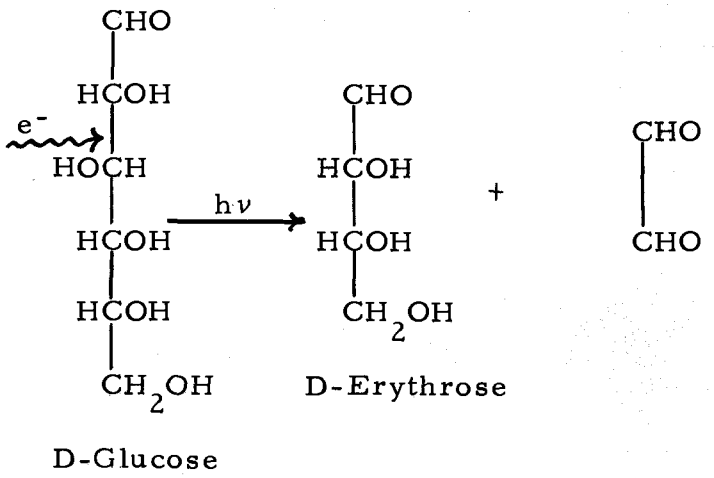


D-Threose

Xylose and arabinose are normally found in concentrations higher than mannose, glucose or rhamnose. Since xylose and arabinose were found at concentrations of the same magnitude as

mannose, glucose and rhamnose suggest these sugars are being selectively degraded to erythrose and threose and finally to glyceraldehyde. The increased concentration of glyceraldehyde is probably the summation of this process coupled with that production resulting from the lignin macromolecule cleavage.

The fact that the concentration of arabinose exceeds that of xylose which is an inversion of what is normally found in grasses, suggests that the C_1 group is being photolytically cleaved from glucose, which would be plentiful in the cellulose portions of grass, leading to arabinose. This type of cleavage is quite common with glucose photooxidation in the presence of oxygen. The mechanism is the same as shown earlier for arabinose and xylose and generally leads to arabinose as one of the photolytic by-products as well as xylose, 2 and 3 carbon aldehydic fragments and glyceraldehyde (47, 48). Erythrose has also been reported as a product of glucose photooxidation apparently occurring in the following manner (58).



D-Glucose D-Erythrose

The original concept that a lignin containing material, such as straw, could be modified to structures dissimable by microorganisms (15, 35, 45, 52) was further supported by this study. The degree photooxidation influences the growth response of Rhodotorula mucilaginosa was dependent upon the time exposure of straw to ultraviolet light in the presence of oxygen (Figure 9).

Rhodotorula mucilaginosa is capable of metabolizing a wide variety of substrates including many aromatic and carbohydrate structures. The only major sugar which is not metabolized by this species is lactose (13). It is not surprising to see a growth response as demonstrated in Figure 9 with essentially complete utilization of all carbohydrates present (Figure 10). Early in the incubation period growth response is quite pronounced on 5 and 15 hour photolyzates and to some extent in all the other samples. The early growth response undoubtedly corresponds to hexose metabolism which is typically utilized preferentially before pentoses by most yeast strains via the glycolytic pathway.

While no specific references were found relating to the induction of enzymes for the utilization of pentoses by Rhodotorula mucilaginosa it has been reported that many yeasts including other Rhodotorula strains (27) selectively metabolize glucose and mannose before attacking pentoses, resulting in a diauxic response. This type of response is quite evident in the 15 through 30 hour

photolyzates and suggests a repression of enzyme synthesis necessary for pentose utilization during hexose metabolism. After induction, which is quite prolonged for these samples, the pentoses are then metabolized, probably via the hexose monophosphate pathway. The initial responses for the 15 through 30 hour samples are considerably lower than the 5 and 10 hour photolyzate responses. This occurs because the concentration of hexoses has decreased considerably by 15 hours photooxidation (Figure 8) and is rapidly depleted with very little increase in cell mass before the lag period required for pentose enzyme induction is initiated.

The responses after 20 hours incubation for the 5 and 10 hour photolyzates do not exhibit a true diauxic response as do the other samples. It has been suggested by Horecker (27) that a semi-induction of pentose enzymes can occur while glucose is being metabolized. However, even though the induction of enzymes for pentose metabolism is taking place the pentoses are not metabolized while glucose is present. When the glucose is depleted the metabolism of the pentoses begins immediately since the enzyme system has already been partially activated. Generally, little or no diauxic response is seen when this phenomenon occurs. A similar type response is seen in the 5 hour photolyzate and to a lesser degree in the 10 hour photolyzate. In both samples the lag phase is very short and growth is not completely terminated, indicating the presence of a partially induced

enzyme system for pentose metabolism. The levels of response on low hexose concentrations in the other samples evidently does not allow sufficient time for this induction to occur prior to hexose depletion resulting in an extended lag phase. Also, the low hexose and pentose levels after 15 hours photooxidation may be only slightly above maintenance levels required for Rhodotorula resulting in a decreased enzyme synthesis rate which is paralleled by an increased lag period.

A second possibility, which would account for the diauxic effect, could be the utilization of phenolic structures present. After carbohydrate utilization the diauxic lag would allow induction of the enzymatic system necessary for aromatic structure utilization (52) followed by microbial dissimilation. The types of aromatic fragments would probably be similar to the basic lignin monomeric phenolic precursor, since Rhodotorula cannot utilize dimeric and larger structures (12). Since the secondary response is much larger than the initial response and the concentration of phenolic structures is considerably lower than the carbohydrates present it is doubtful that the diauxic response can be accounted for by enzyme induction for aromatic structure utilization but is instead a carbohydrate response.

As for the carbohydrates shown in Figure 5, glyceraldehyde and erythrose can be phosphorylated and metabolized readily via the pentose phosphate pathway. The other carbohydrate structures

present are probably utilized by this pathway or the glycolytic pathway since these are predominate pathways for carbohydrate dissimilation in yeast.

Similar responses to the photolyzates obtained during sustained photooxidation are seen in Figure 11. The initial response, which is probably due to hexose utilization, is followed by a diauxic type response in all photolyzates. As photooxidation continues, a photolytic decomposition of the carbohydrates responsible for the secondary response occurs resulting in decreased growth as irradiation time increases.

The initial response to the 40 hour photolyzate is somewhat greater than the 20 hour photolyzate. This may be due to the reduced hexose release rates seen in the early periods of photooxidation (Figure 6). The 20 hour photolyzate probably does not have as great a hexose concentration as the 40 hour sample due to this reduced release rate resulting in a lesser growth response. All other aspects of the growth study on the sustained photolyzates correlates very well with the growth responses observed in the differential photolyzates. This is expected since the sustained system should be the summation of the differential results minus the losses due to photodecomposition from extended exposure to photooxidation.

A rapid photooxidative release of carbohydrates has been demonstrated by this study on sulfuric acid hydrolyzed straw suggesting a

method whereby large quantities of carbohydrates may be collected and utilized by food yeasts from normal non-hydrolyzed field straw. A more complete understanding of photooxidative methods and electron acceptors as well as more efficient, inexpensive light sources could lead to more economical systems for the production of single cell protein from grass straws.

SUMMARY

Annual ryegrass straw was hydrolyzed with 3% sulfuric acid, homogenized and extracted with hot water. The homogenate was exposed to ultraviolet light in the presence of gaseous oxygen at pH 2 as a 1% (w/v) water mixture. The photooxidative by-products were used to elucidate the effects of ultraviolet light on straw decomposition and to determine the feasibility of treating straw in this manner to provide readily metabolizable substrates supporting growth of a Rhodotorula mucilaginosa strain.

The carbohydrate components of straw as well as the lignin structure appear to be quite susceptible to photooxidation with ultraviolet light in the presence of oxygen. Within the first ten hours of photooxidation maximum levels of total dissolved solids, total carbon and carbohydrates were observed. These results were quite noticeable in the differential photooxidation studies.

The maximum carbohydrate levels obtained by ten hours photooxidation can probably be attributed to a combination of rapid carbohydrate release coupled with slower photodecomposition of these structures. As irradiation time increased the rate of carbohydrate release apparently decreased while photodecomposition of the carbohydrates in solution remained fairly constant. The combination of these two reactions produced an additive effect on substrate

loss as is evidenced by the rapid decrease in the concentration of soluble organic compounds, total carbon and carbohydrates in the differential photooxidative system.

All nine of the polyhydroxylated structures detected by acetylation with GLC were aldoses, eight of which were identified as glyceraldehyde, erythrose, threose, arabinose, xylose, mannose, rhamnose and glucose. The ninth structure was tentatively identified as a triose but further identification was not possible under the conditions used. Three of the aldoses, glyceraldehyde, erythrose and threose have not been reported as being present in the normal straw cellulose or hemicellulose structure. However, glyceraldehyde and erythrose have been reported by some authors as appearing in trace amounts after exposure to high-energy irradiation. It has been postulated in this study that the erythrose and threose present could have occurred from arabinose and xylose C_1 photodecomposition. Glyceraldehyde would be present as a result of both erythrose and threose C_1 photodecomposition as well as photooxidative cleavage of the C_3-side chain found in the C_3-C_6 lignin phenylpropanoid structure.

Lignin solubility in 72% sulfuric acid was also increased by photooxidation by ultraviolet light in the presence of oxygen. The level of lignin detected by the 72% sulfuric acid method decreased from 33% to 12% within 12 hours of photooxidation. The straw residue also became bleached in this same period of time. This

loss in percent lignin concentration may be due to methoxyl group loss from photooxidation which has been reported to occur with lignified woody materials. A similar bleaching effect has also been reported with wood and paper and has been attributed to photolysis of the methoxyl groups on the lignin phenylpropanoid structure.

Rhodotorula mucilaginosa growth responses were varied on the photolyzates. All growth studies exhibited some degree of diauxic response resulting from an apparent pentose enzyme induction requirement. The greater growth occurred in the 5 hour differential and 20 hour sustained photolyzates. The initial growth response on the 5 and 10 hour differential and 20 and 40 hour sustained photolyzates reached a peak by 25 and 50 hours respectively, before entering the lag period followed by further growth which terminated at approximately 125 hours. The initial growth was attributed to hexoses present in the photolyzates followed by the diauxic lag necessary for pentose dissimilatory enzyme induction. All carbohydrates present were essentially utilized as evidenced by the decrease in total reducing sugars from 2.1 mg/ml to less than 50 μg/ml in all photolyzates. GLC tracing after growth did not reveal any acetylated structures.

BIBLIOGRAPHY

1. Adler, E., Pepper, J. M. and Erikson, E. Action of mineral acid on lignin and model substances of guaiacyl-glycerol-β-aryl ether type. Industrial and Engineering Chemistry 49: 1391-1392. 1957.

2. Albersheim, P., Nevins, D. J., English, P. D. and Karr, K. A. A method for the analysis of sugars in plant cell-wall polysaccharides by Gas-Liquid Chromatography. Carbohydrate Research 5:340-345. 1967.

3. Anonymous. Carbohydrate studies on grasses, Regional Pasture Research Laboratory Report U. S. D. A. No. 25-30:14-16. 1957.

4. Anonymous. Carbohydrate studies on grasses. Regional Pasture Research Laboratory Report. U. S. D. A. No. 31-35:20-22. 1958.

5. Anonymous. Chlorine-UV oxidized organic pollutants. Chemical and Engineering News 27 (29):98-99. 1969.

6. Armstrong, F. A. J., Williams, P. M. and Strickland, J. D. H. Photo-oxidation of organic matter in sea water by ultraviolet radiation, analytical and other applications. Nature 211:481-483. 1966.

7. Berl, E. and Koerber, W. Fermentation of cellulose and cellulose humic acid and lignin and lignin humic acid. Journal of the American Chemical Society 60:1596-1598. 1938.

8. Brauns, F. E. The chemistry of lignin. New York, Academic Press, Inc. 1952. p. 15.

9. Browning, B. L. The chemistry of wood. New York, Interscience Pub. 1963. p. 258-260.

10. Browning, B. L. Methods of wood chemistry. New York, Wiley. 1967. p. 717.

11. Bulla, C. D. III and Edgerly, E., Jr. Photochemical degradation of refractory organic compounds. Journal of Water Pollution Control Federation 49(4):546-556. 1968.

12. Cain, R. B., Bilton, R. F. and Darrah, J. A. The metabolism of aromatic acids by microorganisms. Biochemical Journal 108:797-828. 1968.

13. Cook, A. H. The chemistry and biology of yeasts. Academic Press, Inc., New York. 1958. p. 278.

14. Eldridge, J. P. and Klein, D. A. Biodegradation of photolytically treated ligninsulfonate by a soil Pseudomonas sp. Bacteriological Proceedings GP97. 1970.

15. Eldridge, J. P. Utilization of products derived from photolytically treated ligninsulfonate by Pseudomonas putida. Ph. D. Thesis, Oregon State University, Corvallis, Oregon. 1972. 135 numbered leaves.

16. Erdtman, H. The chemical nature of wood. Tappi 32(2):71-74. 1949.

17. Forman, L. V. Action of UV light on lignin. Paper Trade Journal III (1):34-40. 1940.

18. Freudenberg, K. and Richtzenhain, H. Enzymatische versuche zur entstehung des lignins. Chemische Berichte 76:997-1006. 1943.

19. Freudenberg, K. Biogenesis and constitution of lignin. Pure and Applied Chemistry 5:9-20. 1962.

20. Freudenberg, K. Constitution and biosynthesis of lignin. Molecular Biology, Biochemistry and Biophysics 2:47-122. 1968.

21. Goldschmid, O. Determination of phenolic hydroxyl content of lignin preparations by ultraviolet spectrophotometry. Analytical Chemistry 26:1421-1423. 1954.

22. Gottlieb, S. and Pelczar, M. J. Microbiological aspects of lignin degradation. Phytopathology 15:55-56. 1951.

23. Harris, E. E., Eilanni, J. and Adkins, H. Reactions of hardwood lignin with hydrogen. Journal of the American Chemical Society 60:1467-1470. 1938.

24. Henderson, M. E. K. Isolation, identification of growth of some soil hyphomycetes and yeast-like fungi which utilize aromatic compounds related to lignin. Journal of General Microbiology 26:149-154. 1961.

25. Henderson, M. E. K. The metabolism of aromatic compounds related to lignin by some hyphomycetes and yeast-like fungi of soil. Journal of General Microbiology 26:155-165. 1961.

26. Higuchi, T., Kawamura, I. and Kawamura, H. Properties of lignin in decayed wood. Journal of Japan Forest Society 37: 298-302. 1955.

27. Horecker, B. C. Pentose metabolism in bacteria. New York, Wiley, 1962. p. 65-96.

28. Ishakawa, H., Schubert, J. and Nord, F. F. On the enzymatic degradation of isolated soft-wood lignin. Life Sciences 8:365-371. 1962.

29. Ishakawa, H. and Oko, T. The oxidative decomposition of lignin. I. The enzymatic degradation of softwood lignin and related aromatic compounds by peroxidase. Journal of Japan Wood Research Society 10:207-213. 1964.

30. Ishakawa, H., Schubert, W. J. and Nord, F. F. Investigations of lignins and lignification. XXVII. The enzymic degradation of softwood lignin by white-rot fungi. Archives of Biochemistry and Biophysics 100:131-139. 1963.

31. Johnson, M. J., Borkowski, J. and Engblom, C. Steam sterilizable probe for dissolved oxygen measurement. Biotechnology and Bioengineering 6:457-468. 1964.

32. Kalnins, M. S., Steelink, C. and Tarbow, H. Light-induced free radicals in wood. United States Forest Service Research Paper - FPL 58. 1966.

33. Klason, P. Beitrage zur konstitution des fichtenholz-lignins, VI: uber die identitat von 2-lignin-hydrosulfonsaure und coniferyl-aldehydrosulfonsaure. Chemische Berichte 61: 171-176. 1928.

34. Klason, P. Beitrage zur konstitution des fichternholzlignins, VIII: untersuchung des nahrungssaftes des fichte. Chemische Berichte 62:635-639. 1929.

35. Klein, D., Rockhill, R. C., Eldridge, J. P. and Park, J. E. Fungal and bacterial responses to photooxidized lignin sulfonate substrates. Tappi 53(8):1469-1472. 1970.

36. Kujirai, C. Studies on the photodegradation of cellulose. III. Ultraviolet absorption spectra of irradiated samples. Sen-I Gakkaishi 29(5):260-266. 1965.

37. Kujirai, C. Studies on the photodegradation of cellulose. VII. Mechanism and quantum yield of photodegradation of cellulose. Sen. I Gakkaishi 22(2):84-89. 1966.

38. Leary, G. J. The yellowing of wood by light. Park I. Tappi 50(1):17-19. 1967.

39. Lekprayoon, C. Production of Candida utilis from annual ryegrass straw hydrolyzate. M. S. Thesis, Oregon State University, Corvallis, Oregon. 1973. 52 numbered leaves.

40. Lewis, H. F and Fronmuller, D. The "Fading" of groundwood by light. III. The changes taking place in the nature of the compounds present in groundwood as the result of fading. Paper Trade Journal 121(14):25-28. 1945.

41. Lewis, H. F., Reineck, E. A., and Fronmuller, D. The "Fading" of groundwood by light. I. A study of the relation between variables in sheet formation and fading. Paper Trade Journal 121(8):44-48. 1945.

42. Minear, R. A. M. S. Thesis, University of Washington, Seattle, Washington. A method for evaluating lignin sulfonate biodegradation. 1966. 95 numb. leaves.

43. Neckers, D. C. Mechanistic organic photochemistry. New York, Reinhold, 1965, Chapter 2.

44. Norman, A. G. Biological decomposition of lignin. Science Progress 119(30):442-456. 1936.

45. Park, J. E. Soil and rumen microbial responses to photo-oxidized grass straw. Oregon State University, M. S. Thesis. 1970. 58 numbered leaves.

46. Pearl, I. A. The chemistry of lignin. New York, Marcel Dekker, Inc. 1967. p. 67.

47. Phillips, G. O. Radiation chemistry of carbohydrates. Advances in Carbohydrate Chemistry. New York, Academic Press, Inc. Vol. 16, 1961. pp. 13-58.

48. Phillips, G. O. Photochemistry of carbohydrates. Advances in Carbohydrate Chemistry. New York, Academic Press, Inc. Vol. 18, 1963. p. 23.

49. Pitts, J. N., Jr. Relations between molecular structure and photodecompostion modes. Journal of Chemical Education 34: 112-121. 1957.

50. Raabe, E. W. Biochemical oxygen demand and degradation of lignin in natural waters. Journal Water Pollution Control Federation 49(5):145-150. 1968.

51. Robinson, T. The organic constituents of higher plants, 2d ed. Minnesota, Burgess, 1967. p. 69-74.

52. Rockhill, R. C. Fungal growth responses to a photooxidized sodium ligninsulfonate. M. S. Thesis, Oregon State University, Corvallis, Oregon. 1970. 70 numbered leaves.

53. Sandermann, W. and Schlumborn, F. Photometrische und chromatographische untersuchungen an holzmehlen. Holz Roh-Werkstoff 20:245-252. 1962.

54. Sarkanen, K. V. and Suzuki, J. Delignification by peracetic acid; studies on oxidative delignification mechanisms. Tappi 48(8):459-464. 1965.

55. Sarkanen, K. V. and Ludwig, C. H. Lignins occurrence, formation, structure and reactions. New York, Wiley, 1971. p. 1-3.

56. Seliger, H. H. and McElroy, W. D. Light: physical and biological action. New York, Academic Press, 1965, 417 p.

57. Smith, F. B. and Brown, P. E. The decomposition of lignin and other organic constituents by certain soil fungi. Journal of American Society of Agronomy 27:109-119. 1935.

58. Swallow, A. J. Radiation chemistry of organic compounds. Pergamon Press, Inc. New York, New York. 1960. Vol. 2 p. 196-199.