

MICROBIAL TRANSFORMATION OF LIGNIN

by

Kiran L. Kadam

Dissertation submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Chemical Engineering

APPROVED:

S. W. Drew, Chairman

D. L. Michelsen

P. R. Rony

G. W. Claus

P. L. Hall

March, 1979

Blacksburg, Virginia

ACKNOWLEDGEMENTS

My first and foremost indebtedness is to my Guru, Dr. Stephen W. Drew, who gave me an opportunity to work under his tutelage and helped me brave the trials and tribulations of the doctoral program. Not unlike my predecessors, I had my share of maxima, minima and singularities during this academic odyssey. Whenever the going got tough during this arduous endeavor, my mentor always managed to uplift my sunken spirits by his idyllic pep talks which were strewn with metaphors of the most vivid kind. If at all I have metamorphosed into a professional biochemical engineer, I owe it mostly to his incessant encouragement, healthy criticism and scientific counsel.

I am thankful to all members of "Drew's Crew" for their cooperation and camaraderie. Their wit and humor afforded a welcome escape and breathed freshness into an otherwise sterile atmosphere of the Biochemical Engineering laboratory. Special thanks are due Mr. Charlie Edwards for technical advice, skilled assistance, stimulating discussions and friendly support. Drs. Philip Hall and Wolfgang Glasser and their associates are appreciated for providing information which appears elsewhere in this dissertation. I am grateful to Ms. Charlotte Barnett for complying with, at times untimely, requests for ^{14}C -labeled lignin, to Dr. Lance Kress for generously supplying loblolly pine seedlings and to Prof. Samuel Bingham for sharing his greenhouse.

I am also greatly appreciative of Prof. Henry McGee, Jr., Head, Chemical Engineering Department for allowing me to pursue the Ph.D. degree at Virginia Tech; of the National Science Foundation for providing me with financial support; and of members of my advisory committee for the review and critique of this investigation.

Last but not the least is my gratitude toward my parents who always offered their love, understanding and unwavering support and without whom I would not have accomplished much.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
LIST OF TABLES	ix
LIST OF FIGURES	x
 I. INTRODUCTION	 1
A. Source and Function of Lignin	1
B. Nature of Lignin	2
C. Structure of Softwood Lignin	4
D. Kraft Lignin	4
1. Effect of Pulping on Lignin	7
2. Molecular Weight of Kraft Lignins	8
3. Kraft Lignin as a Substrate	11
E. Chemical Treatment of Lignin	13
F. Microbial Treatment of Lignin	14
1. Lignin-degrading Microorganisms	14
2. Fermentation Medium	14
3. Operating Mode	15
4. Type of Catalysis	15
5. Operating Conditions	15
6. Scale-up and Economics	15
 II. LITERATURE REVIEW	 16
A. Microbial Aspects of Lignin Degradation	16
1. White-Rot Fungi	18
2. Brown-Rot Fungi	22
3. Soft-Rot Fungi	23
4. Bacteria	24

	Page
B. Enzymology of Lignin Degradation	27
1. Monooxygenases	29
2. Dioxygenases	31
3. Phenol Oxidases	31
C. Lignin Fermentation Parameters	39
1. Oxygen	39
2. Agitation	40
3. pH	41
4. Carbohydrates	41
5. Nutrient Nitrogen	42
6. Trace Nutrients	42
D. Results of Kirk and Coworkers	43
E. Lignin Bioprocessing	44
1. Processes	44
2. Products	45
3. Economics	46
III. MATERIALS AND METHODS	47
A. Materials	47
1. Cultures	47
2. Chemicals	47
3. Media	48
a. Inoculum Media	48
b. Induction Media	48
c. Production Media	49
4. CO ₂ Trapping Solution	50
5. Scintillation Cocktails	50
B. Methods	50
1. Sterilization of Equipment and Materials	50
2. Inocula Preparation	51
3. Purification of Kraft Lignin	52
4. Preparation of ¹⁴ C-Labeled Kraft Lignin	53

	Page
5. Measurement and Control of Operating Parameters	54
a. Temperature	54
b. pH	54
c. Agitation	55
d. Aeration	55
6. Measurement of $^{14}\text{CO}_2$	56
7. Determination of Total CO_2	59
8. Multi-Stage Fermentations ²	59
9. Harvesting of Deep-Tank Fermentations	60
10. Dry Cell Weight Determination	61
a. Hydrolysis	61
b. Glucosamine Assay	62
11. Glucose Oxidase Assay for Glucose	63
12. Starch Determination	64
13. Nutrient Nitrogen Determination	64
14. Dissolved Oxygen Measurement	65
15. Reduced Data and Statistical Significance	66
IV. RESULTS AND DISCUSSION	67
A. ^{14}C -Lignin Assay	67
B. Screening of Lignin-Degrading Fungi	68
1. Primary Screening	69
2. Possible Low MW Pool	73
3. Dialysis Fermentation	74
4. Temperature Effects	79
5. Secondary Screening	79
C. Effect of Carbohydrate on Lignin Metabolism	86
D. $^{14}\text{CO}_2$ vs. Time Curves	90
E. Cell-Lignin Interactive System	92
1. Chemical Reaction with Mass Transfer	94
2. Yield of Intermediates	96
F. Kinetic Modeling for Lignin Catalysis	98
1. Development of the Model	99
2. Consequences of the Model	103

	Page
G. <u>Aspergillus fumigatus</u>	104
1. Comparison with <u>C. versicolor</u>	105
2. Lignin Derived CO ₂ /Total CO ₂	105
H. General Process Considerations	112
1. Mode of Operation	112
2. Type of Catalysis	112
I. Multi-Stage Lignin Fermentations	113
1. AspI	114
2. AspII	116
3. Lignolytic Efficiency	121
4. AspIII	125
J. Analysis of Degraded Lignins	129
1. Elemental and Functional Composition	130
2. C ₆ -C ₃ Formulae	133
3. Permanganate Oxidation	135
4. Carboxylation	139
K. Effect of Fermentation Parameters	140
1. Temperature	140
2. Nitrogen Levels	140
3. Glucose Consumption and Nitrogen Concentration	145
4. Loss of Activity of <u>A. fumigatus</u>	147
5. C/N Ratio	153
6. Glucose Catabolite Repression	163
L. Mixed and Successive Fermentations	171
1. Rationale	175
2. <u>P. placenta</u> and <u>C. versicolor</u> Fermentations	177
V. CONCLUSIONS	180
VI. RECOMMENDATIONS	183

	Page
VII. SUMMARY	187
VIII. BIBLIOGRAPHY	191
IX. VITA	198

ABSTRACT

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Representative Composition of Kraft Lignin	9
2. Calculated C ₆ -C ₃ Units in Lignins	10
3. Molecular Weights of Alkali Lignins	12
4. Investigation of Lignin-Derived Products of Low MW	76
5. Analyses of Degraded Lignins	131
6. Analyses of <u>Aspergillus</u> Lignins Based on C ₆ -C ₃ Units	134
7. Permanganate Oxidation of <u>Aspergillus</u> Lignins	137
8. Effect of Nitrogen Levels on Lignin Metabolism by <u>A. fumigatus</u>	146
9. Loss of Lignolytic Activity of <u>A. fumigatus</u>	151
10. C/N Ratio and Lignin Metabolism by <u>C.</u> <u>versicolor</u>	162

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Lignin building blocks and their relative abundance	3
2. Computer-simulated structure of softwood lignin	5
3. Computer-simulated structure of hardwood lignin	6
4. Hypothetical structure of a portion of lignin after attack by white-rot fungi	21
5. Types of dioxygenase-catalyzed cleavage of aromatic rings	32
6. Reactions of phenol oxidases	34
7. Proposed mechanism for cellobiose:quinone oxidoreductase	37
8. Schematic diagram of the setup for ^{14}C -labeled lignin experiments	57
9. Primary screening of lignin-degrading fungi: White-rot fungi	70
10. Primary screening of lignin-degrading fungi: Isolates	71
11. Dynamics of a hypothetical pool of low molecular weight products derived from lignin	75
12. Growth of <u>S. pulverulentum</u> on different lignin fractions.	78
13. Effect of temperature on lignolytic activities of <u>S. pulverulentum</u> and <u>P. chrysosporium</u>	80

<u>Figure</u>		<u>Page</u>
14.	Secondary screening of lignin-degrading fungi with cellobiose as a cosubstrate	82
15.	Secondary screening of lignin-degrading fungi with PSC as a cosubstrate	83
16.	Secondary screening of lignin-degrading fungi with Avicel as a cosubstrate	84
17.	Secondary screening of lignin-degrading fungi: White-rot fermentations during secondary screening before the breakage of dialysis bags	87
18.	Effect of carbohydrates on lignin metabolism by <u>C. versicolor</u> . I.	89
19.	Hypothetical concentration profile of a membrane-bound enzyme involved in the rate-limiting step during lignin metabolism	93
20.	Lignolytic activities of <u>A. fumigatus</u> and <u>C. versicolor</u>	106
21.	Lignin derived CO ₂ /total CO ₂ ratios during <u>A. fumigatus</u> and <u>C. versicolor</u> fermentations	108
22.	Lignin metabolism in relation to carbohydrate metabolism in an <u>A. fumigatus</u> fermentation	110
23.	Multi-stage lignin fermentation by <u>A. fumigatus</u> : Biotransformation stage of AspII	119
24.	Lignin fermentation by <u>S. pulverulentum</u> in a deep-tank fermenter	120
25.	Schematic diagram of the setup for determining lignolytic efficiencies	122
26.	Definitions of lignolytic and normalized lignolytic efficiencies	123

<u>Figure</u>		<u>Page</u>
27.	Lignolytic efficiencies during the bio-transformation stage of <u>AspIII</u>	127
28.	Monomeric and dimeric products from permanganate oxidation of lignin	136
29.	Effect of nutrient nitrogen and temperature on lignin metabolism by <u>A. fumigatus</u> : Starch as a cosubstrate	141
30.	Effect of nutrient nitrogen on growth of <u>S. pulverulentum</u>	143
31.	Effect of nutrient nitrogen on lignin metabolism by <u>A. fumigatus</u> : Glucose as a cosubstrate	144
32.	Lignolytic activity and glucose consumption during a lignin fermentation by <u>A. fumigatus</u> : 2.6 mM nitrogen	148
33.	Lignolytic activity and glucose consumption during a lignin fermentation by <u>A. fumigatus</u> : 17.4 mM nitrogen	149
34.	Lack of accumulation of lignin-derived low molecular weight products during a dialysis fermentation by <u>A. fumigatus</u>	152
35.	Effect of nitrogen concentration on lignin metabolism by <u>C. versicolor</u> : Avicel as a cosubstrate	154
36.	Effect of nitrogen concentration on lignin metabolism by <u>C. versicolor</u> : Soluble starch as a cosubstrate	155
37.	Effect of nitrogen concentration on lignin metabolism by <u>C. versicolor</u> : Glucose as a cosubstrate	156
38.	Effect of carbohydrates on lignin metabolism by <u>C. versicolor</u> . II	158
39.	Effect of C/N ratio on lignin metabolism by <u>C. versicolor</u> : Initial glucose concentration of 1 g/l	159

<u>Figure</u>	<u>Page</u>
40. Effect of C/N ratio on lignin metabolism by <u>C. versicolor</u> : Initial glucose concentration of 5 g/l	160
41. Effect of C/N ratio on lignin metabolism by <u>C. versicolor</u> : Initial glucose concentration of 10 g/l	161
42. Investigation of glucose catabolite repression in lignin metabolism by <u>C. versicolor</u> . I . . .	164
43. Investigation of glucose catabolite repression in lignin metabolism by <u>C. versicolor</u> . II . . .	165
44. Rates of $^{14}\text{CO}_2$ evolution during lignin fermentations having different initial glucose concentrations	167
45. History of organism and lignin metabolism	169
46. Lignin metabolism and free carbohydrate concentration: A hypothetical case depicting critical glucose concentrations	170
47. Parameters of a lignin fermentation by <u>C. versicolor</u> : Initial glucose concentration of 1 g/l	172
48. Parameters of a lignin fermentation by <u>C. versicolor</u> : Initial glucose concentration of 5 g/l	173
49. Parameters of a lignin fermentation by <u>C. versicolor</u> : Initial glucose concentration of 10 g/l	174
50. Formation of <u>o</u> -diphenol structures during lignin metabolism	176
51. Mixed and successive fermentations with <u>P. placenta</u> and <u>C. versicolor</u>	178

I. INTRODUCTION

A. Source and Function of Lignin

Lignocellulosics account for roughly 95% of the land-bound biomass. The composition of lignocellulosic biomass is approximately 50% cellulose, 25% hemicellulose and 25% lignin, the exact figures varying from species to species.

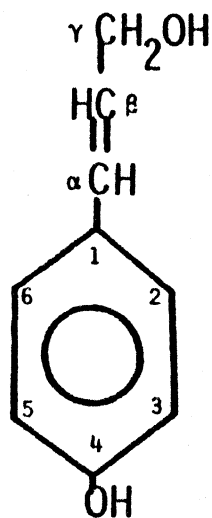
Lignin is a phenylpropanoid structural polymer characteristically found in vascular plants such as lycopods, ferns, gymnosperms and angiosperms. The function of lignin is to give plants rigidity and bind plant cells together (Higuchi, 1971, p. 208). Lignin also decreases water permeation across cell walls of xylem tissue and protects plant tissues from invasion by pathogenic microorganisms (Ander and Eriksson, 1978, p. 2). Lignin is converted to humus by different microorganisms on the death of plant tissues. The degradation of humus is a very slow process which may in part be due to oxygen deficiency in lower parts of the soil. The lifetime of humus has been calculated to be between 360 and 2860 years depending on the type of soil investigated (Christman and Oglesby, 1971, p. 784). Humic acid, which is by far the most significant fraction of humus, has the carboxyl group as its most predominant functional group and is significantly lower in methoxyl content than lignin. However, humic acid, like lignin, may also be regarded as a polyphenol.

Although lignin constitutes merely 28% of the dry mass of softwood, its fuel value of 12,700 BTU/lb comprises a full 40% of the fuel value of this lignocellulosic material (Falkehag, 1975). Hence, the pulp and paper industry burns lignin to improve the economics of its operation. In spite of the high calorific value of lignin, the heat produced by burning it is only slightly greater than that necessary to evaporate water from spent liquors (Goheen, 1971, p. 797).

B. Nature of Lignin

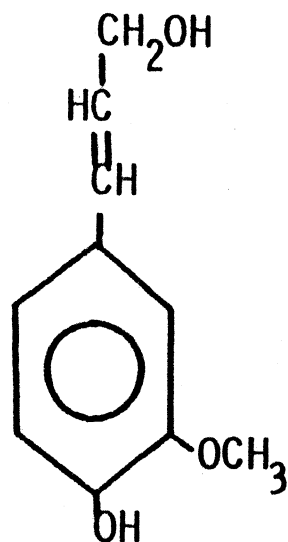
Sarkanen and Ludwig (1971, p. 2) define lignins to be polymeric natural products arising from an enzyme-initiated dehydrogenative polymerization of three primary precursors: trans-coniferyl, trans-sinapyl and trans-p-coumaryl alcohols (Fig. 1).

Most lignins can be divided into three categories: a) gymnosperms or softwood lignins, b) angiosperms or hardwood lignins and c) grass lignins. These three types of lignins are best differentiated on the basis of nitrobenzene oxidation products. While gymnosperm lignins yield mainly vanillin with some p-hydroxybenzaldehyde, both syringaldehyde and vanillin are obtained from angiosperm lignins and significant amounts of all three aldehydes are obtained from grass lignins (Sarkanen and Hergert, 1971, p. 43). A typical softwood lignin contains approximately 80% coniferyl alcohol, 14% p-coumaryl alcohol and 6% sinapyl alcohol. Hardwood lignin, on the other hand, contains similar amounts of coniferyl and sinapyl alcohols but a minor amount of p-coumaryl alcohol. In bamboo and grass lignins



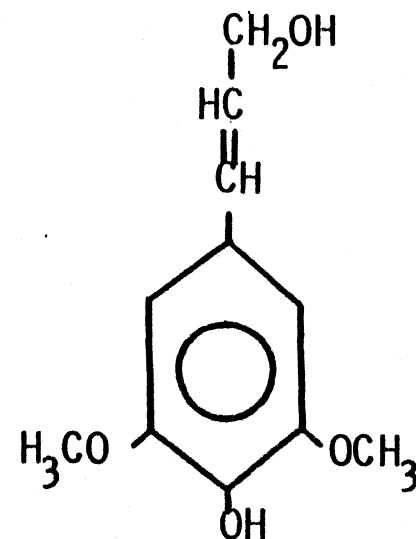
p-COUMARYL ALCOHOL

(14 %)



CONIFERYL ALCOHOL

(80 %)



SINAPYL ALCOHOL

(6 %)

Figure 1. Lignin building blocks and their relative abundance. Figures in parentheses represent approximate proportions of these monomers in softwood lignin (Ander and Eriksson, 1978).

the amount of p-coumaryl alcohol is higher than in softwood and hardwood lignin (Ander and Eriksson, 1978, p. 3).

C. Structure of Softwood Lignin

Softwood lignins have some end groups with unattached side chains. The existence of cinnamyl alcohol and cinnamaldehyde end groups in the lignin macromolecule has been well established (Lai and Sarkanen, 1971, pp. 195-196).

Units with free phenolic hydroxyl groups as they occur in conifer lignin may be divided into those possessing an unsubstituted 5-carbon and others in which the position 5 carries an aryl, aryloxy or alkyl substituents. These units are often referred to as "uncondensed" and "condensed" phenolic units, respectively.

Structures in lignin in which the side chain is connected with the next unit through a β -O-4 linkage have been commonly called "arylglycerol- β -ether" structures. These linkages are the most abundant in lignin. Other types of structures found in lignin are β -1 lignols, 4-O-1 and 5-1 linkages, β -5 lignols, β - β lignols, 5-5 and 5-O-4 lignols, etc. Glasser and Glasser (1978) have proposed computer-simulated structures for softwood lignin (Fig. 2) and hardwood lignin (Fig. 3).

D. Kraft Lignin

In the sulfate process for pulp production, a mixture of sodium hydroxide and sodium sulfide is used to delignify both softwoods and

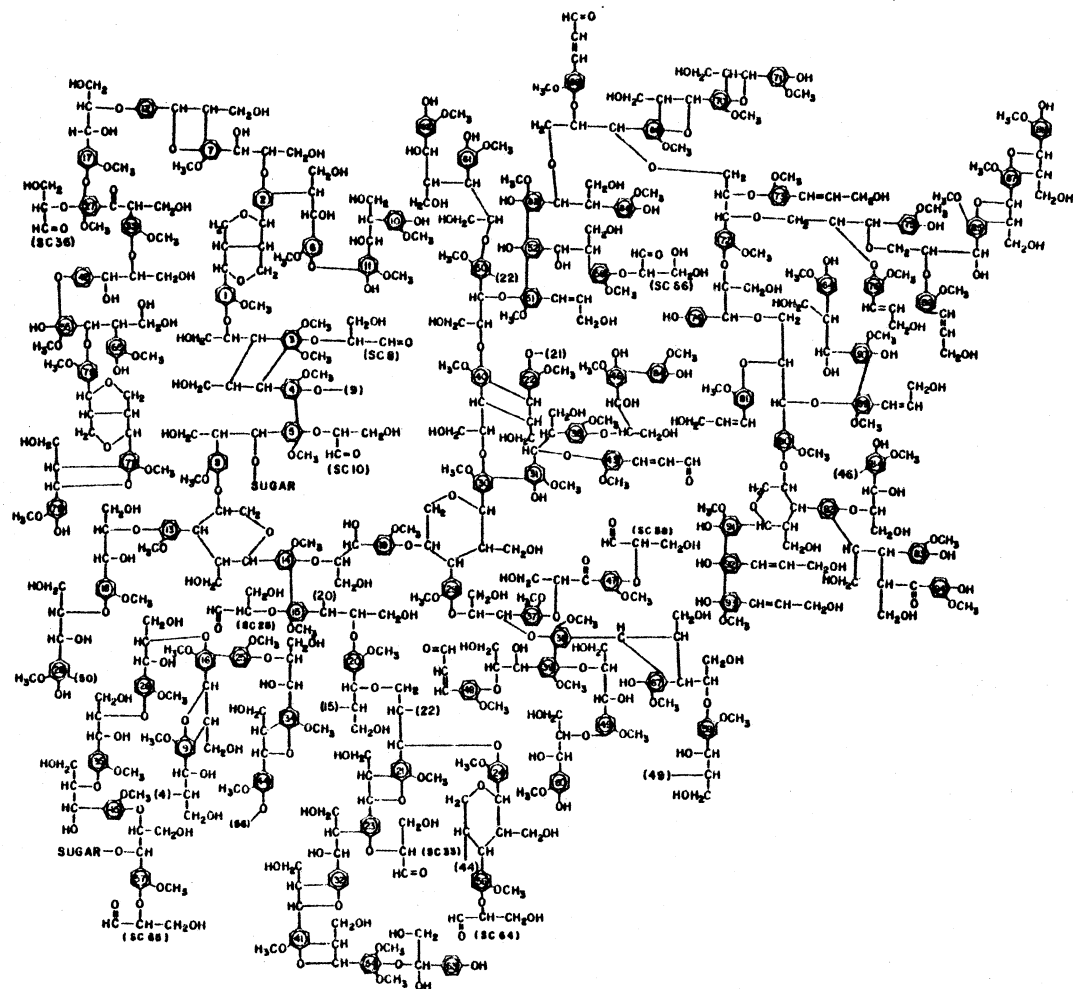


Figure 2. Computer-simulated structure of softwood lignin.
(Glasser and Glasser, 1978.)

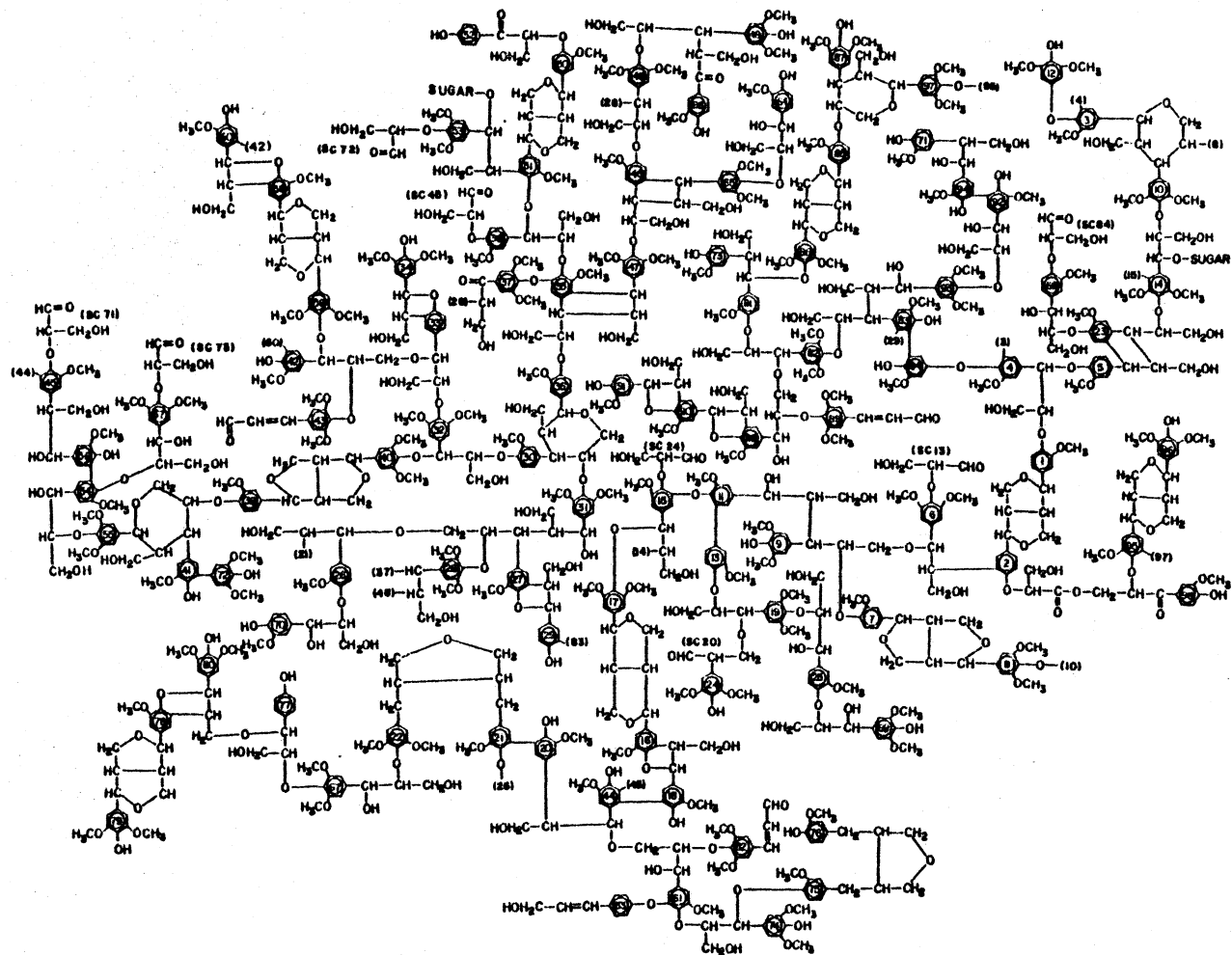


Figure 3. Computer-simulated structure of hardwood lignin.
(Glasser and Glasser, 1978.)

hardwoods. This process is commonly referred to as the "kraft" (meaning strength in German) process and the lignin resulting from this process is called kraft lignin.

1. Effect of Pulping on Lignin: The essential step of delignification is the fragmentation of the lignin macromolecule by the action of alkali and other nucleophiles. With some exceptions, it is generally true that carbon-to-carbon bonds are stable in alkali but carbon-to-oxygen linkages can be cleaved under pulping conditions. The most significant reaction of this type is the liberation of new phenolic hydroxyl groups by the cleavage of aryl alkyl ether bonds. The mechanism of various alkaline cleavage reactions can be viewed from a common starting point by assuming that in phenolic units the hydrolysis proceeds through the intermediate formation of reactive p-quinone methide structures. Formation of o-quinone methide structures is also possible, e.g., from certain α -5 linked structures. Quinone methides are formed only from units containing a free phenolic hydroxyl group thus phenolic units constitute the most reactive structures in alkaline pulping. The nonphenolic structures are, in general, resistant to alkaline attack but may under specific circumstances, undergo cleavage of alkyl aryl ether bonds (Marton, 1971, pp. 650-651).

Wood source, pulping conditions and the method of isolation influence the chemical properties of isolated kraft lignins. Hardwood lignins especially are different in many important structural details.

Tables 1 and 2 give structural information on kraft lignins which were isolated from commercial batch pulpings of southern pine wood (mostly loblolly pine) and a mixture of hardwoods (oak and gum).

The comparison of C_6-C_3 formulae of milled wood and kraft lignins are not exact since in reality the composition of an average unit in kraft lignin is probably closer to a C_6-C_2 than to a C_6-C_3 unit as a consequence of the release of formaldehyde from side chains during pulping. The average unit in the model for kraft lignin proposed by Marton (1971, p. 679) has the composition of $C_{8.35}H_{6.4}O_{2.1}(OCH_3)_{0.78}$, the unit weight being 168. The most apparent changes brought about in the analytical composition of lignin by kraft pulping are losses of hydrogen, oxygen and some methoxyl groups, and the introduction of sulfur into about every tenth unit.

2. Molecular Weight of Kraft Lignins: Due to the polydispersity of the lignin polymer, there exists a certain degree of ambiguity in ascribing a single value to its molecular weight. The number average

$$\bar{M}_n = \sum_i w_i / \sum_i \frac{w_i}{M_i}$$

$$\bar{M}_w = \sum_i w_i M_i / \sum_i w_i$$

w_i and M_i are weight and molecular weight, respectively, of fraction i .

molecular weight, $\bar{M}_n$ may be an order of magnitude smaller than the weight average molecular weight, $\bar{M}_w$. A useful procedure is to measure both

Table 1
Representative Composition of Kraft Lignins^a

%	Pine	Hardwood	Kraft MWL ^b Spruce
C	65.87	65.43	65.81
H	5.82	5.77	6.20
O	25.90	25.31	
S	1.56	1.50	1.44
OCH ₃	14.04	19.24	15.7
Ash	0.96	0.50	1.4

^aMarton, 1971, p. 671

^bMilled wood lignin

Table 2
Calculated C₆-C₃ Units in Lignins^a

Lignin	C ₆ -C ₃ Formula	Unit Weight
MWL spruce ^b	C ₉ H _{8.8} O _{2.37} (OCH ₃) _{0.96}	185
MWL birch ^c	C ₉ H _{8.24} O _{2.95} (OCH ₃) _{1.38}	206
Kraft pine	C ₉ H _{7.9} O _{2.1} S _{0.1} (OCH ₃) _{0.82}	178
Kraft hardwood	C ₉ H _{7.2} O _{1.8} S _{0.1} (OCH ₃) _{1.15}	183
Alkali spruce	C ₉ H _{7.3} O _{2.2} (OCH ₃) _{0.9}	179
Kraft MWL spruce	C ₉ H _{7.3} O _{2.2} S _{0.1} (OCH ₃) _{0.9}	182

^aMarton, 1971, p. 672

^bCorrected for carbohydrates

^cNot corrected for carbohydrates; OCH₃ content is probably higher.

$\bar{M}_n$ and $\bar{M}_w$ by appropriate methods and then to regard the ratio, $\bar{M}_w / \bar{M}_n$ as a measure of polydispersity. For a monodisperse polymer, $\bar{M}_w / \bar{M}_n = 1$ (Goring, 1971, p. 699). Table 3 compares molecular weights of alkali lignins.

3. Kraft Lignin as a Substrate: Kraft lignin was chosen as the substrate for all the studies on lignin biotransformation that follow. Several million tons of Kraft lignin are produced each year in the United States as a byproduct of the paper mills. If not recovered from the black liquor and subsequently burned, lignin poses a serious disposal problem. Furthermore, research performed with kraft lignin should have sufficient relevance to the bioprocessing of lignocellulosics and "uncooked" lignins. Lastly, kraft lignin is readily available as a commercial product whereas an elaborate separation scheme is necessary to recover lignin from wood. ^{14}C -labeled kraft lignin was prepared by subjecting labeled loblolly pine seedlings to a laboratory-scale kraft cook. The loblolly pine is a gymnosperm and the lignin derived from it falls into the category of softwood lignins.

Table 3

Molecular Weights of Alkali Lignins^a

Lignin Preparation	Acetone Solubility, %	$\bar{M}_n^b$	$\bar{M}_w^b$	$\frac{\bar{M}_w}{\bar{M}_n}$	MW by Gel Filtration
Kraft pine	65-75	1600	3500	2.2	
Kraft pine, acetone soluble fraction	100	1000			
Kraft pine, mildly oxidized in alkaline solution	15-25	3000-4300			
Kraft hardwood	90	1050	2900	2.8	
Sulfate, spruce	46		5600		10,000
Soda, spruce	51		82,000		20,000

^aMarton, 1971, p. 685.

^bThe number average molecular weight, $\bar{M}_n$ of pine and hardwood kraft lignins were determined by the thermoelectric method, and the weight average molecular weight, $\bar{M}_w$ by equilibrium sedimentation using an ultracentrifuge.

E. Chemical Treatment of Lignin

Attempts to create an industry based on spent liquors have not been very successful. Basically, it is not that chemicals are difficult to make from lignin, but that it is hard for them to economically compete with the same chemicals produced from other sources. Energy requirements are the key to the problem. During the eons of earth's geological history, natural forces and energies of the earth's crust acted upon deposits of plant remains to convert much of their organic matter into simple molecules found in natural gas and petroleum. To accomplish the same results from lignins, an astronomical amount of energy must be supplied. Chemical engineers appear to be better able to build up from relatively small compounds such as the ones present in petroleum and natural gas than to achieve a controlled breakdown of large, natural polymers like lignin. Thus, the petrochemical industry has a naturally built-in advantage over chemicals from lignin (Goheen, 1971, pp. 797-798).

Production of vanillin from lignin has been by far the most successful process of lignin utilization. Organic sulfur chemicals such as dimethyl sulfide and methyl mercaptan, and phenolics such as phenol, *p*-cresol, catechol, etc., can also be produced by chemical treatment of lignin. The lack of commercial processes for lignin utilization by chemical treatment is due to the high energy requirement and the high cost of product recovery.

F. Microbial Treatment of Lignin

Though the kinetics of microbial treatment of lignin is rather slow, its energy requirement is much lower than that of chemical treatment. Since microbial attack on lignin is fairly specific, it may be possible that the cost of product recovery will not be as high as that in the case of the chemical treatment.

Very limited knowledge exists regarding the basics of microbial catabolism of lignin. Virtually nothing is known about the intricacies and nuances which one would expect to exist in the complex metabolic pathways needed for the microbial breakdown of a refractory, crosslinked biopolymer such as lignin. Thus, the concept of lignin utilization through biotransformation is in its embryonic stage and a lot of fundamental information has to be amassed before a really effective process development is possible. The task of a biochemical engineer then is to develop an efficient lignin fermentation system using the available knowledge.

Following are some of the topics of concern to a biochemical engineer striving to develop a lignin fermentation:

1. Lignin-degrading Microorganisms: The choice of a suitable organism will depend on the objectives of the fermentation, e.g., the production of low molecular weight products, lignolytic enzymes or a modified lignin polymer.

2. Fermentation Medium: Various medium components can significantly affect the efficiency of a given fermentation.

3. Operating Mode: The mode of operation can be either batch, semibatch (fed-batch) or continuous depending on the kinetics and other factors affecting the fermentation.

4. Type of Catalysis: Lignin catalysis can be achieved either with viable cells or a cell-free enzyme preparation, the choice being dependent on the location and retrievability of enzymes.

5. Operating Conditions: Environmental parameters such as temperature, pH, dissolved oxygen levels, mixing characteristics are obvious candidates for controlling the fermentation performance. Regulatory phenomena like catabolite repression, induction and cell differentiation can also dictate the efficacy of the fermentation.

6. Scale-up and Economics: The laboratory-scale data should serve as a basis for possible pilot plant studies which, in turn, should yield sufficient information for economic feasibility evaluation.

II. LITERATURE REVIEW

A. Microbial Aspects of Lignin Degradation

The contrast between lignin and other common biopolymers such as proteins, polysaccharides, nucleic acids, etc. as to structure and mode of formation should be apparent from the brief review of lignin chemistry presented in the Introduction. The rate at which lignin is metabolized, as compared to other biopolymers, is extremely slow. The diversity of intermonomer linkages and monomer structures in lignin as well as the plethora of various functional groups provide an opportunity for the organism to bring about a variety of limited changes in lignin without necessarily effecting significant degradation, i.e., conversion to CO_2 , H_2O and other products. Research to date indicates that the capacity for complete degradation of lignin by a single microorganism may be limited to fungi that cause white-rot type wood decay and to the related litter-decomposing Basidiomycetes (Kirk, 1971). This observation, though rather disconcerting, is not totally unexpected since lignin degradation in nature is mostly the result of mixed or successive attacks by two or more organisms. Hence, only a few organisms, acting alone, can be expected to degrade lignin completely.

The biological degradation of the lignocellulose cell walls represents one of the essential links in the natural process of

carbon circulation. The decayed lignified plant tissues are attacked on the surface of the soil or in its upper layers, first of all, by fungi which thus become the instigators of the decomposition of this comparatively intractable material. This primary attack, which often transforms to a great extent the original substrate, is naturally amplified by the action of other components of soil microflora. Under conditions favorable to the soil microflora, they contribute toward further mineralization. Which of the fungal species, at a given moment, will occupy the decomposing lignocellulose depends above all on the character and degree of changes brought about in the substrate by the foregoing partner. These changes, whether physical or chemical, must correspond to the demands of the organism next in succession (Tichý, 1970). A distinction has to be made between in vitro studies of wood decay and studies made in the natural habitat. If we wish to mimic nature in its efficient lignin degradation, we should realize that the decomposition of wood in nature is generally brought about by several species of fungi and other microorganisms, which are inhabitants of wood, acting in succession or conjunction. Each of the organisms contributes, on its own, to the final transformation of the substrate attacked, suppressing organisms that had attacked the substrate before or being itself influenced by their activity (Tichý, 1975).

There are three major types of fungi which degrade one or more of the wood components: white-rot fungi, brown-rot fungi and

soft-rot fungi. Litter-decomposing fungi, which are Basidiomycetes, are also able to degrade lignin found in leaves, twigs, etc. that comprise forest litter. White-rot and brown-rot fungi both are Hymenomycetes (Holobasidiomycetes) and soft-rot fungi are Ascomycetes or Fungi Imperfecti.

1. White-Rot Fungi: The ability of white-rot fungi to degrade lignin in wood has been recognized for years. Cowling (1961) showed that Polyporus versicolor can degrade over 97% of the lignin in sweetgum wood. It thus can be concluded that, under suitable conditions, white-rot fungi can totally degrade lignin in wood though the rates of decay are very slow.

Though it has been reported that phenylpropane (C_6-C_3) compounds are intermediates in lignin degradation by white-rot fungi (Fukuzumi, 1970; Ishikawa et al., 1963), the evidence is not overwhelming. The small amounts of such intermediates detected by these investigators could have been formed from "side groups" or minor structural elements and not from the bulk of the phenylpropane units of the polymer itself (Kirk, 1971). A scheme showing the release of C_6-C_3 compounds from lignin by cleavage of arylglycerol- β -aryl ether bonds in lignin has been proposed by Ishikawa et al. (1963) and Schubert (1965, p. 116). Despite the abundance and importance of these linkages, evidence indicates that their cleavage can release only a small amount of compounds containing single aromatic rings. Thus far there is no compelling evidence to believe that substantial

portion of the lignin polymer is degraded by white-rot fungi via intermediates containing single aromatic rings (Kirk, 1971).

In view of the multiplicity of intermonomer linkages in lignin, the complete degradation may not involve a direct attack on each of these linkages. Such an attack would result in free, low molecular weight phenolic compounds. Alternatively, a direct attack on common structural elements of the polymer, i.e., aromatic rings, may occur and the cleavage of bonds linking the phenylpropane units may follow indirectly (Kirk, 1971). Kirk and Chang (1975) have postulated that white-rot fungi oxidatively attack the aromatic nuclei as well as the side chains and that phenolic elements, while still in the polymer, are the site of attack.

Kirk et al. (1977, pp. 377-378) have presented an overview of lignin degradation by white-rot fungi: Phenolic units in various parts of the polymer are converted to o-diphenolic units by demethylation and perhaps by aromatic hydroxylation. The o-diphenolic units are cleaved to produce aliphatic, carboxy-rich residues. These are then degraded, probably via both hydrolytic and oxidative reactions, to release low molecular weight aliphatic products which enter the fungal hyphae. New phenolic units are gradually freed for further attack by these reactions. In the process, some low molecular weight aromatics are released and metabolized intracellularly. While this attack on aromatic regions proceeds, some terminal side chains are oxidized to aromatic carboxyl (C_6-C_2) residues and other side chains are oxidized in the α -position. The structures of ring cleavage fragments, however, are not known.

During fungal degradation, lignin appears to undergo both oxidation and demethoxylation. Hence, in many cases, the extent of lignin degradation has been estimated by the decrease in methoxyl content. Lignin from decayed wood gives lower yields of vanillin than lignin from sound wood. This result could be interpreted to mean that the fungal attack takes place preferentially on the "open" elements of lignin thus depleting vanillin-yielding moieties in decayed wood lignin and concomitantly increasing the relative proportion of moieties that do not yield vanillin (Schubert, 1965, pp. 88-89). In one study, analysis of milled wood decayed by Polyporus versicolor revealed that the fungal attack had not led to any accumulation of low molecular weight phenolic compounds (Kirk and Lundquist, 1970).

Known consequences of fungal attack include decreases in H ; OCH_3 ; aromaticity; phenolic, aliphatic and total hydroxyl; yield of aromatic products on chemical degradation and increases in $COOH$; O ; $C = O$; α , β -unsaturated $COOH$; and aryl $COOH$ (Kirk and Chang, 1974 & 1975). Although these and similar studies of lignin degraded by white-rot fungi provide valuable insights into the gross changes that take place in the polymer, no specific structures resulting from fungal attack have been elucidated. Kirk et al. (1977, p. 378), however, have proposed a hypothetical structure of a portion of lignin after attack by white-rot fungi (Fig. 4). Though this hypothetical structure shows a muconic acid residue, such residues are

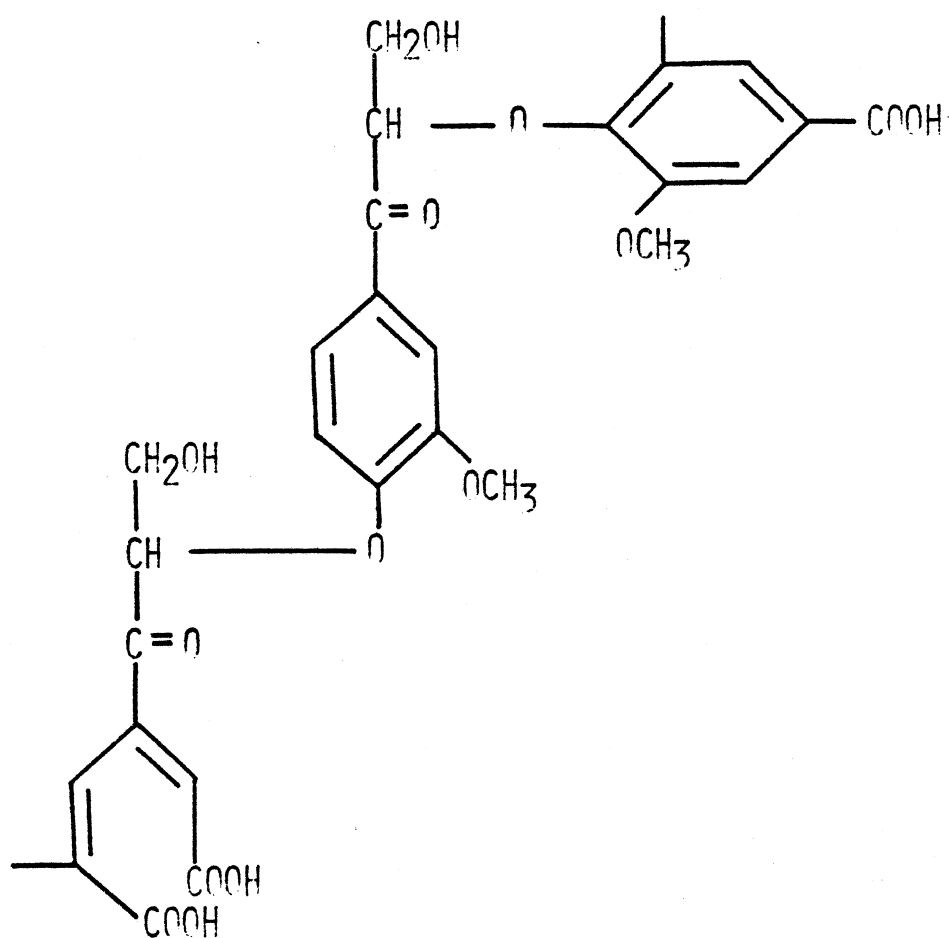


Figure 4. Hypothetical structure of a portion of lignin after attack by white-rot fungi (Kirk et al., 1977).

apparently not formed or, if they are formed, do not persist during the fermentation (Kirk et al., 1977, p. 378).

Though the picture of events during lignin breakdown is not very lucid, some headway has been made in understanding the metabolism of compounds related to lignin. Schubert (1965, p. 116) has proposed a scheme for the degradation of guaiacylglycerol- β -coniferyl ether units present in softwood lignin by white-rot fungi. Higuchi (1971, p. 276) has presented a possible pathway for the degradation of o-diphenol and phenylpropane units by white-rot fungi.

Though it is questionable whether an absolutely specific attack on lignin can be achieved with naturally occurring white-rot fungi, they are probably destined to be an important part of any scenario involving lignin bioprocessing.

2. Brown-Rot Fungi: Brown-rot fungi, unlike white-rot fungi which decompose both carbohydrates and lignin in wood, degrade only the carbohydrates without bringing about any appreciable decrease in the lignin content of wood. One of the main differences in the modes of attack by brown-rot and white-rot fungi seems to be that brown-rot fungi lack an efficient ring-cleavage system, although this has not been substantially documented (Kirk, 1975). Brown-rot fungi may also lack the ability to further metabolize ring-cleavage fragments if they are formed. Neither do brown-rot fungi lead to oxidative shortening of side chains in terminal phenylpropane units as do white-rot fungi. In contrast to white-rot fungi, brown-rot fungi do not possess the enzyme, cellobiose:quinone oxidoreductase (Ander and Eriksson, 1978).

The most noticeable change in lignin as a result of attack by brown-rot fungi is the decrease in methoxyl content. As a result of decay of sweetgum by Lenzites trabea, methoxyl-deficient units containing phenolic hydroxyl groups were formed in the lignin skeleton (Kirk and Adler, 1970). The brown color of brown-rotted wood may be the result of both spontaneous and phenol oxidase catalyzed oxidation of catechol (o-diphenol) units to quinoid or melanin type chromophores. Intracellular phenol oxidases are probably released during the autolysis of old fungal mycelia (Kirk, 1971).

Brown-rot fungi, by themselves, are not considered to be of significance in lignin degradation. However, considering the possible symbiosis that may exist between brown-rot and white-rot fungi in wood decay, a role for brown-rot fungi in lignin bioprocessing should not be ruled out.

3. Soft-Rot Fungi: Under very moist conditions, certain Ascomycetes and Fungi Imperfecti can cause substantial wood decay (Kirk, 1971). Eslyn et al. (1975) studied the decay of red alder, balsam poplar and western white pine by six soft-rot fungi. Western white pine was not decayed significantly by three fungi and was decayed only to a moderate extent by the remaining three fungi. In red alder and balsam poplar, carbohydrates were depleted faster than lignin.

Haider and Trojanowski (1975) presented conclusive evidence to prove that soft-rot fungi do really degrade lignin. They grew six soft-rot fungi and two white-rot fungi in the presence of ^{14}C -labeled DHP (dehydrogenative polymer of coniferyl alcohol) and followed the

evolution of $^{14}\text{CO}_2$. $^{14}\text{CO}_2$ was released from side chains and aromatic rings earlier than from methoxyl groups. Later during the incubation, however, more $^{14}\text{CO}_2$ was released from methoxyl groups than from side chains and aromatic rings. These results led the authors to the conclusion that soft-rot fungi can attack methoxyl groups, β -ether linkages of side chains, and also aromatic ring structures of DHP and hence of lignin. The levels of $^{14}\text{CO}_2$ released from ^{14}C -labeled DHP were comparable for soft-rot and white-rot fungi. An interesting aspect of this investigation by Haider and Trojanowski (1975) was that no laccase activity could be detected in soft-rot fungi and the peroxidase activity was lower than that in white-rot fungi. Thus, soft-rot fungi may be as important as white-rot fungi in lignin bioprocessing.

4. Bacteria: Examination of reports pertaining to bacterial degradation of lignin reveals that in many instances weaknesses in experimental techniques have led to erroneous or questionable conclusions. Kirk (1971) has enumerated these drawbacks. Despite the said uncertainties, enough evidence seems to have accumulated to lend credence to the claim that bacteria do cause certain amounts of degradation in lignin.

Trojanowski et al. (1977) provided the first concrete evidence for the ability of Nocardia sp., a gram-positive bacterium, to degrade lignin. They reported that Nocardia sp. could release $^{14}\text{CO}_2$ from ^{14}C -labeled methoxyl groups, side chains and ring carbons of DHP.

More recently, Gradziel et al. (1978) claimed that Nocardia sp. was more active in producing $^{14}\text{CO}_2$ from ^{14}C -labeled plant lignin and DHP than soft-rot and white-rot fungi tested by Haider and Trojanowski (1975).

Haider et al. (1978) also employed ^{14}C -labeled lignins to screen several species of Nocardia and Pseudomonas for their lignin degrading capacity. The Nocardia spp. were better in lignin degradation than Pseudomonas spp.

Cartwright and Holdom (1973) found only one bacterium, namely Arthrobacter sp. which survived repeated subculture on the lignin substrate. Their claim that Arthrobacter sp. is capable of utilizing lignin as a sole carbon source is rather dubious since their observations are highly qualitative and in contradiction with the current notion that a growth substrate is absolutely essential for lignin biodegradation.

Odier and Monties (1978) reported that a Xanthomonas isolated from decomposing wheat straw was able to utilize dioxane lignin as a sole carbon source. Their observations are quite unique: After 15 days of incubation in a mineral medium, 77% of the initial lignin was degraded but in the presence of glucose only 23% degradation was observed. Condensation of the residual lignin occurred during the first 24 hours of incubation and was followed by depolymerization. Ether-extractible phenols were produced during the condensation phase, two of which contained keto and carboxyl groups. These substances disappeared upon prolonged incubation while other phenols were produced.

Xanthomonas 23 was also able to degrade wheat dioxane lignin in the absence of oxygen and in the presence of NO_3^- and glucose. Under anaerobic conditions two phenolic acids with a carbonyl group were produced. The extremely high rate of lignin degradation and the ability of Xanthomonas 23 to utilize lignin as the sole carbon source are astounding observations. However, wheat dioxane lignin may be a special type of low molecular weight lignin. Furthermore, Odier and Morties (1978) used UV absorbance at 315 nm as their assay for lignin, which is not exactly the most reliable assay for lignin.

Very recently, Crawford (1978) provided an unequivocal proof for the fact that Streptomyces can degrade lignin. Streptomyces sp. was able to release $^{14}\text{CO}_2$ from lignin labeled (^{14}C) lignocellulose and ^{14}C -labeled milled-wood lignin. Kawakami (1976) has shown that Pseudomonas ovalis degrades a large portion of high molecular weight milled wood lignin. Forney and Reddy (1978) reported that a mixed culture of 22 strains of pseudomonads degraded 42% of high molecular weight (>1500) kraft lignin in 52 days. Bacillus megaterium has also been proven to possess the ability to degrade lignin (Robinson and Crawford, 1978).

Crawford and Olson (1978) observed a novel conversion of vanillate to guaicol by Bacillus sp. and Streptomyces sp. This catabolic capability may be a fairly common taxonomic characteristic of bacteria. These observations indicate that an alternative ring-fission substrate, catechol, may be used by certain micororganisms to dissimilate

vanillin (or veratrate). Crawford and Olson (1978) also state that the classic separation of catabolic pathways leading to specific ring-fission substrates such as protocatechuate and catechol are often interconnectable by single-enzyme transformations. They further suggest that catabolism of aromatic compounds by microorganisms is better represented by a web of interconnected sequences rather than a list of separate, distinct pathways.

The research activity in the field of bacterial degradation of lignin has been less vigorous than that in fungal degradation of lignin. Nevertheless, pathways for metabolism of aromatic compounds have been delineated mostly in the case of bacteria. Thus, even though bacteria may not feature prominently in lignin bioprocessing, they may be instrumental in the search for lignin degradation pathways.

B. Enzymology of Lignin Degradation

Very little is known about the lignin-degrading enzyme system. Though no pathways for lignin degradation have been postulated, a few speculations regarding the nature of enzymes can be made from the well-established pathways for degradation of aromatic compounds. A thorough knowledge of the constituents of the enzyme system and the delineation of degradation pathways are crucial to the concept of lignin bioprocessing.

Zeller was among the first to investigate enzymes of a wood-rotting fungus (Schubert, 1965, pp. 79-80). In 1919 he recorded the

following enzymes in Lenzites sepiaria: esterases, maltase, invertase, raffinase, emulsin, tannase, diastase, cellulase, hemicellulase, pectinase, inulinase, urease, hippuricase, nuclease, protease, erepsin, trypsin, rennet, catalase, tyrosinase and "oxidase". With the exception of the last three, he found greater amounts of these enzymes present in the vegetative mycelium than in the sporophore tissue.

More recently, Dagley (1971) has proposed possible types of reactions that could be important in lignin degradation. He argues that if lignin metabolism is analogous to the metabolism of low molecular weight aromatics, the following types of reactions can be envisioned: lactonization, delactonization, isomerization, hydrolyses, transfers, dehydrogenation and decarboxylation. Each reaction presumably would require a separate enzyme.

In nature, lignin is present in a solid form and the wood-rotting fungi must alter and/or degrade lignin before reaching cellulose. The extensively cross-linked polymer structure of intact lignin makes it highly unlikely that lignin as such could enter the microorganism. For these reasons, the lignin-degrading organisms must possess extracellular enzymes, at least to facilitate the initial enzymatic attack on the biopolymer. Furthermore, lignin contains few hydrolyzable bonds and is very polydisperse. Hence, hydrolytic cleavage is not possible as is in the case of almost all other biopolymers.

Drawing an analogy between the metabolism of lignin and lignin-related aromatic compounds, we can say that the most important enzymes in lignin degradation are monooxygenases, dioxygenases and phenol oxidases.

1. Monooxygenases: Monooxygenases, along with dioxygenases, play a primary role in the catabolism of aromatic compounds. Cleavage of aromatic rings in lignin would require two steps: a) preparation of rings for cleavage and b) cleavage itself. To serve as a substrate for oxidative cleavage by microorganisms, aromatic rings must have two hydroxyl groups either ortho or para to each other. The proper aromatic structure can be realized in lignin by demethylation of methoxyl groups and/or by aromatic hydroxylation.

In their studies of fungus-degraded lignins, Kirk and Chang (1975) found no evidence for either type of reaction. Despite a low methoxyl content, neither demethylated structures nor hydroxylated aromatic rings were detected. This can be interpreted to mean that the rate of preparation of rings for cleavage is much slower than the rate of ring cleavage (Kirk, 1975). In other words, in the catabolism of aromatic compounds, the preparation of rings for cleavage may be the rate limiting step. Another interpretation, not suggested by Kirk (1975), may be that "prepared rings" are susceptible to competing side reactions.

Though it has not been demonstrated how aromatic rings are prepared for cleavage in lignin metabolism, both demethylation and

aromatic hydroxylation have been reported to occur in the degradation of vanillic acid (4-hydroxy-3-methoxybenzoic acid) by different white-rot fungi in liquid cultures. Flaig and Haider (1961) and Cain et al. (1968) observed demethylation of vanillic acid to protocatechuic acid by Coriolus versicolor. Kirk and Lorenz (1973) showed that vanillic acid is oxidatively decarboxylated to yield methoxyhydroquinone.

Enzymes responsible for preparation of ring for cleavage by hydroxylation and/or demethylation are of the monooxygenase type (Gibson, 1968). These enzymes are also called mixed function oxygenases (Wood et al., 1977). Monooxygenases which catalyze aromatic hydroxylation are called flavoproteins. Monooxygenases catalyze the incorporation of one atom of molecular oxygen into the substrate and the other atom into H_2O , and they require a reducing coenzyme such as NADH or NADPH for activity.

It is pertinent to mention that demethylation of methoxyl groups and hydroxylation of specific positions in aromatic rings of high molecular weight lignin have been shown to accompany attack of wood by brown-rot fungi (Kirk and Adler, 1970 & Kirk et al., 1970). These Holobasidiomycetes degrade cellulose in wood and leave behind essentially all lignin but in a modified form. In fact the major degradative mode is demethylation of methoxyl groups. This observation suggests that extracellular monooxygenases needed to effect demethylation and hydroxylation of high molecular weight lignin occur in higher Holobasidiomycetes.

2. Dioxygenases: Aerobic cleavage of aromatic rings by microorganisms is catalyzed by enzymes of the dioxygenase type. These enzymes are non-heme iron-proteins and require no coenzymes (Wood et al., 1977 & Kirk, 1975). Both atoms of molecular oxygen are incorporated into the aromatic ring to yield an aliphatic acid. All ring cleaving dioxygenases so far encountered in fungi have been of the intradiol type, i.e., they act between ortho substituted hydroxyl groups. Fig. 5 depicts three types of dioxygenases-catalyzed aromatic ring cleavage mechanisms.

Only intradiol type cleavage has been demonstrated in fungal degradation of aromatic compounds. Thus, fungally degraded lignin should show a decrease in hydroxyl groups and an increase in carboxyl groups. This has, in fact, been observed with lignin degraded by fungi (Kirk et al., 1977). Though only intradiol type cleavage by fungi has been demonstrated, Cain et al. (1968) have provided an indirect evidence for extradiol type cleavage by extracts of Penicillium sp. They speculate that fungi may turn out to possess no less diverse modes of aromatic ring cleavage than bacteria. Gentisic acid type cleavage has not yet been reported in the case of fungi. Aliphatic carboxylic acids produced by the action of dioxygenases are capable of entering the TCA cycle.

3. Phenol Oxidases: Phenol oxidases have been detected in lignin fermentation broths by many investigators (Ander and Eriksson, 1976; Blaich and Esser, 1975; Selin et al., 1975; Kirk and Kelman, 1965). These enzymes [laccase (O_2 : p-diphenol oxidoreductase,

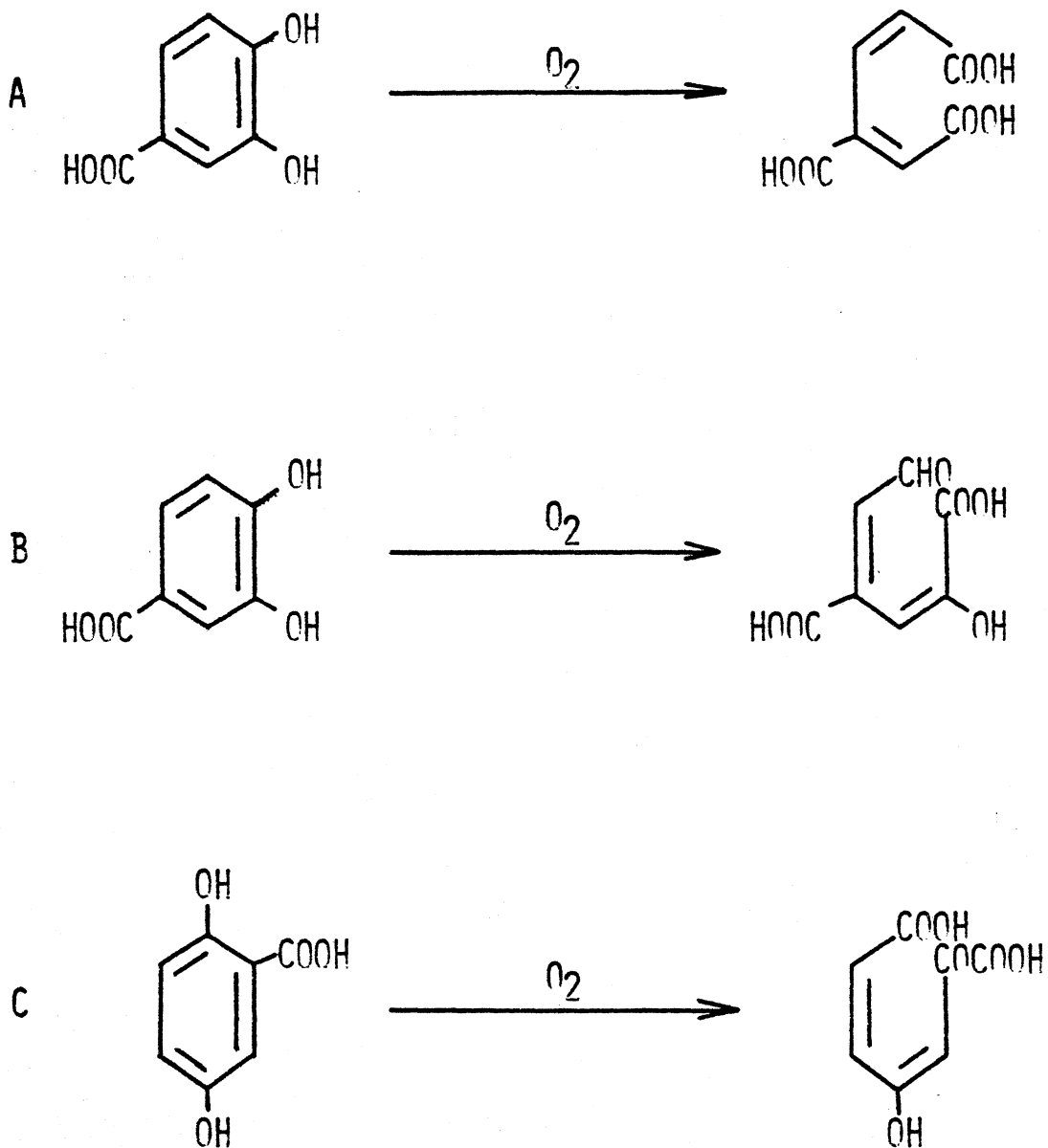


Figure 5. Types of dioxygenase-catalyzed cleavage of aromatic rings. (A) intradiol, (B) extradiol and (C) gentisic acid-type (Kirk, 1975).

E.C. 1.10.3.2), tyrosinase (O_2 :o-diphenol oxidoreductase, E.C. 1.10.3.1) and peroxidase (donor: H_2O_2 oxidoreductase, E.C. 1.11.1.7)] are copper-containing proteins which have the common property of catalyzing the direct oxidation of their respective substrates by atmospheric oxygen, i.e., they catalyze the abstraction of electrons from substrate phenols. With laccase and peroxidase, one reducing equivalent is considered to be removed whereas with tyrosinase, two reducing equivalents are considered to be removed simultaneously from the substrate (Mason, 1965). Phenol oxidases do not function anaerobically, i.e., they do not bring about the oxidation of their substrates via methylene blue or similar dyes and their action does not result in the formation of hydrogen peroxide. Hence, they are classified as oxidases rather than as dehydrogenases (Schubert, 1965, p. 81).

Enzymes of the laccase type probably catalyze the color reactions that led to the early detection of phenol oxidizing enzymes in most white-rot fungi (Higuchi, 1953). Tyrosinase may be distinguished from laccase and other oxidases through its ability to catalyze two essentially different oxidations, namely, the introduction of a hydroxyl group into monohydric phenols, ortho to the one already present (Eq. A, Fig. 6), and the oxidation of o-dihydric phenols to the corresponding o-quinones (Eq. B, Fig. 6). Laccase, on the other hand, can directly accomplish the oxidation to quinones of o-dihydric phenols (Eq. B, Fig. 6), p-dihydric phenols

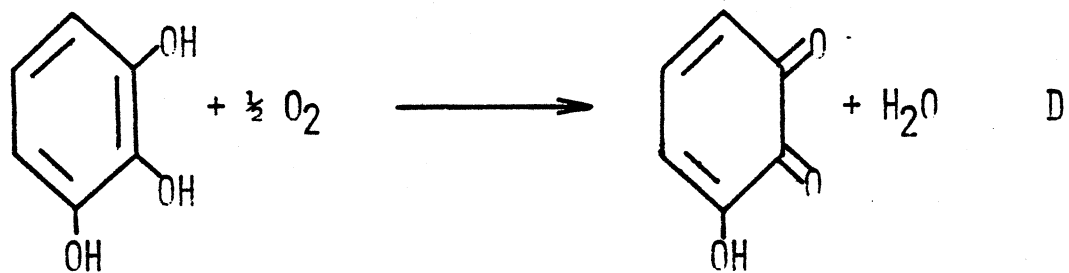
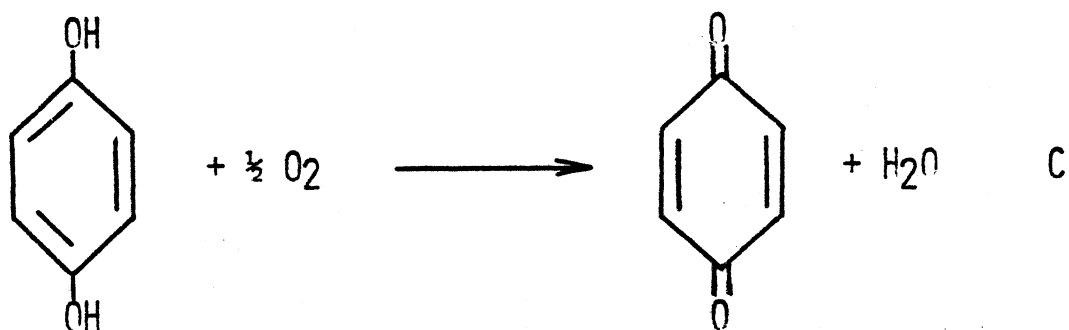
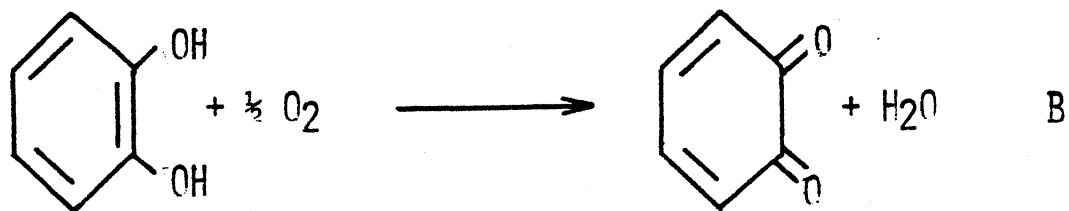
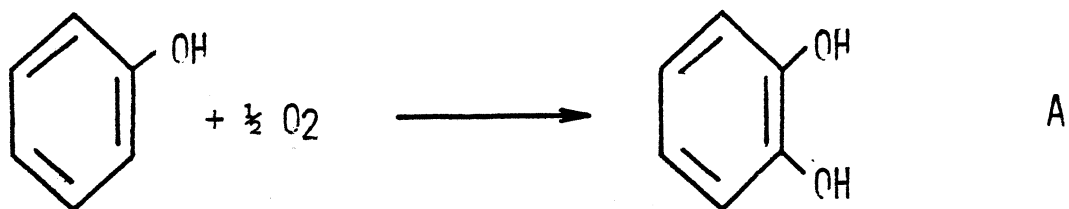


Figure 6. Reactions of phenol oxidases (Schubert, 1965).

(Eq. C, Fig. 6) and polyhydric phenols (Eq. D, Fig. 6), but it is without any effect on monohydric phenols.

There appear to be three main reasons why phenol oxidases have been considered important in lignin metabolism: 1) the fact that white-rot fungi which degrade lignin possess extracellular phenol oxidizing enzymes, whereas the taxonomically similar brown-rot fungi, which only alter lignin but do not degrade it, apparently do not produce these enzymes; 2) the fact that lignin is a phenolic material and should, therefore, be a substrate for phenol oxidizing enzymes, which it is; and 3) the realization that lignin degradation is primarily an oxidative process and hence phenol oxidases act "in the right direction" (Kirk, 1971). Though phenol oxidases can theoretically bring about some oxidation and degradation of lignin, the extent and exact nature of their role in lignin degradation is not clear. In any event, phenol oxidases can only be a small fraction of the complex enzyme system that must be required for lignin metabolism.

An indirect role for phenoloxidases, in detoxifying toxic phenolic compounds that may be released during lignin fermentation, has been proposed (Gadd, 1957). Lyr (1962) has suggested that peroxidases would be particularly appropriate detoxifying agents for fungi inhabiting the heartwood of trees since they do not compete with intracellular substrate oxidations for oxygen. Green (1977) has postulated that glucose oxidase may promote the oxidative degradation of lignin through an efficient laccase-glucose: quinone oxidoreductase

cycle without accumulation of toxic quinoid byproducts. Selin et al. (1975) reported that ligno-sulfonates, even in conditions which did not support fungal growth, underwent strong polymerization when fermented by phenol oxidase producing white-rot fungi. On the other hand, Polyporus dichrous, an atypical white-rot fungus which does not produce extracellular phenol oxidases, did not cause polymerization of lignosulfonates.

Westermarck and Eriksson (1974) reported a new wood-degrading enzyme from white-rot fungi. The enzyme, cellobiose: quinone oxidoreductase (cellobiose dehydrogenase) had a high specificity for the oxidation of cellobiose but was able to reduce both ortho and para quinones. They also suggested that cellobiose dehydrogenase may be involved in the enzymic degradation of both cellulose and lignin in wood. Their proposed mechanism for cellobiose dehydrogenase is shown in Fig. 7. Ander and Eriksson (1978), however, have recently conceded that this enzyme is not entirely necessary for lignin degradation.

Ander and Eriksson (1976) had proposed that phenol oxidases function in regulating the production of both lignin- and polysaccharide-degrading enzymes by oxidation of lignin and lignin-related phenols when Sporotrichum pulverulentum is growing on wood. Their hypothesis was based on following observations: The phenol oxidase-less mutant was unable to degrade kraft lignin or wood. On the addition of highly purified laccase to kraft lignin agar plates, the phenol oxidase-less mutant could degrade kraft lignin. The phenol

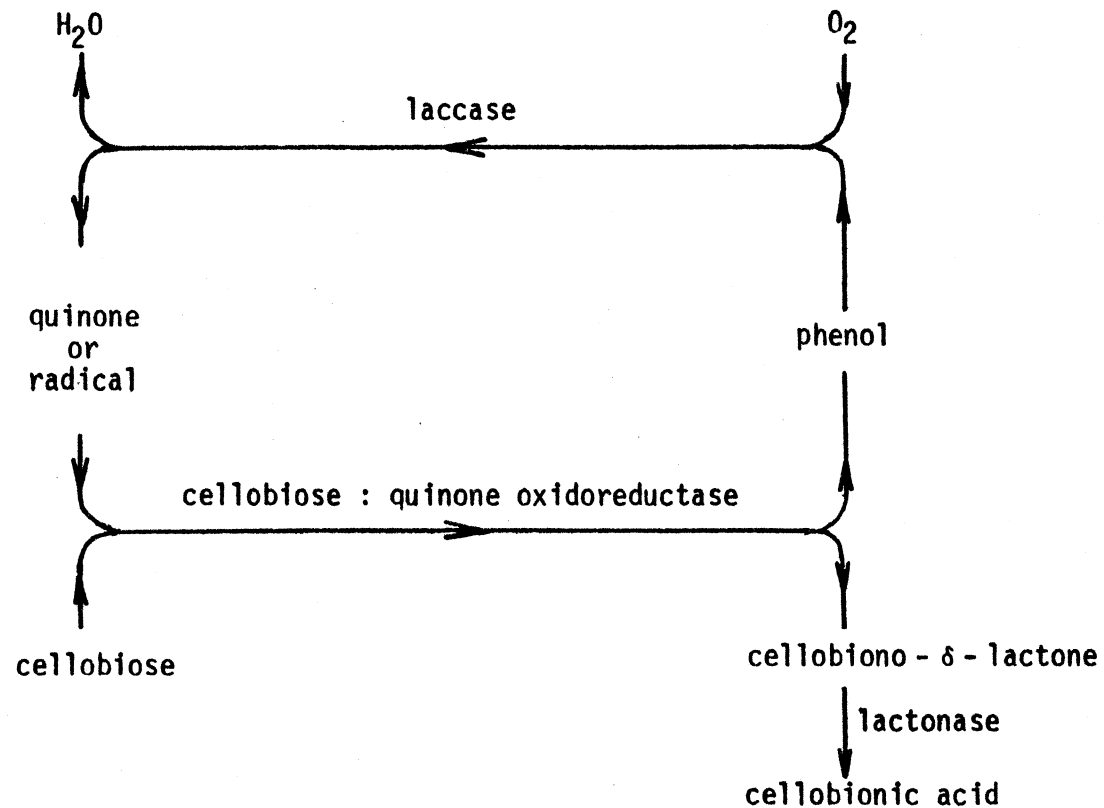


Figure 7. Proposed mechanism for cellobiose:quinone oxidoreductase. (Westermarck and Eriksson, 1974.)

oxidase-positive revertant of this mutant regained the ability of the wild type to degrade kraft lignin and all major components of wood.

The above hypothesis which features phenol oxidases as regulatory agents in lignin metabolism is highly tenuous since it is based mostly on circumstantial evidence. Results of Ander and Eriksson (1976) can be well explained by the fact that laccase does detoxify uncoupling agents of oxidative phosphorylation. Thus, the inability of the phenol oxidase-less mutant to degrade kraft lignin could be due to a build-up of toxic phenolic compounds. There is abundant speculation in the literature regarding the importance of phenol oxidases in lignin degradation. In all likelihood, phenol oxidases play only a secondary role in lignin metabolism, the role being that of scavengers of toxic compounds.

Phenol oxidases may also be involved in demethylation of lignin and lignin phenols (Harkin and Obst, 1974; Ishihara and Miyazaki, 1974; Trojanowski and Leonowicz, 1969). The extent of demethylation by phenol oxidases would be expected to be much smaller than that by monooxygenases. Kirk and colleagues work with a special strain of Phanerochaete chrysosporium which produces barely detectable amounts of phenol oxidizing enzymes thus minimizing unwanted oxidative coupling reactions of phenolic compounds (Kirk, 1979).

It is apparent that the current understanding of enzymology of lignin degradation leaves much to be desired. This is a major

hurdle in the way of biochemical engineers desirous of developing processes and designing reactor schemes for lignin utilization.

C. Lignin Fermentation Parameters

In the development of a lignin bioconversion process, the optimization of fermentation parameters is a highly desirable engineering pursuit. A fermentation can be oriented toward the maximization of products such as lignolytic enzymes, polymeric lignin with desired modifications or low molecular weight chemicals. The nature of an optimal lignin fermentation will depend on specific goals and the available basic knowledge. Since very sparse knowledge of the lignin degrading enzyme system exists, studies on the effect of process parameters on specific enzyme activities are not currently possible. However, evolution of $^{14}\text{CO}_2$ from ^{14}C -labeled lignin, which is the only sensitive microassay available for lignin breakdown, has been used to study the effect of various factors on gross lignin metabolism. Other lignin assays are either unreliable or require large samples.

1. Oxygen: Kirk et al. (1978) studied the effect of oxygen on $^{14}\text{CO}_2$ evolution from ^{14}C -labeled DHP by Phanerochaete chrysosporium. They found that growth during the first 3 days of incubation was similar at all oxygen concentrations employed, namely, 5%, 21% and 100% O_2 . Lignin decomposition to $^{14}\text{CO}_2$ began after the initial growth period under 21% and 100% O_2 but not under 5% O_2 .

Rates and levels of $^{14}\text{CO}_2$ evolution were higher at 100% O_2 than at 21% O_2 . The effect of oxygen concentration on lignin degradation was similar with glucose, cellulose or xylose as a growth substrate. Fahraeus and Lindeburg (1953), however, had earlier reported that less aerated cultures of white-rot fungi degraded lignin to a greater extent than well aerated cultures. Since lignin degradation is mostly an oxidative process, one would expect improvement in degradation as oxygen concentration is increased. This may be true only to a certain oxygen concentration. On the other hand, white-rot fungi may well be oxygen-limited in their natural habitat and hence well adapted to an oxygen deficient environment. In nature, oxygen has to overcome resistances of bulk diffusion to the hyphae, diffusion through the boundary layer and transport through the cell membrane before reaching the site of action. Thus, intuitively, one would expect the white-rot fungi to be less susceptible to low oxygen levels as compared to other fungi.

2. Agitation: Ishikawa et al. (1963) observed that the decomposition of lignin by F. fomentatarius and P. versicolor was more rapid with than without mechanical agitation. They also found that if mycelial pellets were used for inoculation and the resulting culture was incubated without shaking, the amount of products was increased 3 to 4 fold when compared with the amount accumulated with shaking for the same length of time. Haider and Trojanowski (1975) noticed that DHP was better metabolized by soft-rot fungi in shaking cultures

while white-rot fungi degraded DHP more effectively in stationary cultures.

Kirk et al. (1978) reported that constant shaking from the start suppressed lignin decomposition by P. chrysosporium. At 21% O_2 , cultures that were shaken after 9 days of still incubation decomposed lignin faster than cultures continuously shaken, and at a rate similar to that in stationary cultures. Cultures continuously shaken under 100% O_2 did not degrade lignin, although growth appeared to be equivalent to that at 21%. Also, at 100% O_2 , shaking after a 9 day stationary period greatly slowed lignin decomposition in comparison to stationary cultures. The investigators could not offer any explanation for these discrepancies observed under 100% O_2 . One possible explanation could be that when cultures are shaken under 100% O_2 , the dissolved oxygen concentration reaches a high level at which oxygen starts to inhibit lignin metabolism. Presumably, the dissolved oxygen concentration in stationary cultures under 100% O_2 is below the inhibitory level.

3. pH: Kirk et al. (1978) reported that DHP degradation by P. chrysosporium was maximal at pH 4.5, with substantial suppression of decomposition below pH 3.5 and above pH 5.5. The optimum pH for growth of P. chrysosporium was somewhat higher than that for lignin decomposition.

4. Carbohydrates: Kirk et al. (1976) demonstrated an absolute requirement for a growth substrate during decomposition by white-rot fungi. This requirement may also be common to other lignin-degrading

fungi and bacteria. Carbohydrates such as cellulose, cellobiose, xylose, glucose etc. can serve as growth substrates during lignin fermentation. The reason for the requirement of a growth substrate may be that the energy recovered from lignin metabolism is not adequate to support growth.

5. Nutrient Nitrogen: Kirk et al. (1978) observed that although the source of nitrogen was not critical, the concentration of nitrogen greatly influenced lignin decomposition by P. chrysosporium. The extent and rate of lignin decomposition was lower in the medium containing 2.4mM nitrogen than in the one containing 17.4mM nitrogen. They offered the following scenario to explain the adverse effect of high nitrogen concentration on lignin metabolism: High nitrogen concentration promotes rapid depletion of growth substrate known to be essential for lignin metabolism. The concomitant decrease in pH further exacerbates the situation. Nitrogen metabolism possibly competes with lignin metabolism through requirements for same cofactors. Nitrogen probably regulates the synthesis of one or more components of the lignin-degrading enzyme system.

6. Trace Nutrients: Kirk et al. (1978) found that among several vitamin supplements examined only thiamine was necessary for the growth of and lignin degradation by P. chrysosporium.

D. Results of Kirk and Coworkers

In all their work with radioactive lignin, Kirk and colleagues have employed ^{14}C -labeled DHP. DHP's are "synthetic lignins" which are prepared by peroxidase-aided polymerization of coniferyl alcohol. Though DHP's used by Kirk and coworkers and spruce lignin are qualitatively similar, they differ in relative proportions of coniferyl alcohol end groups and three normal substructures (Kirk et al., 1975). Furthermore, roughly 80% of a softwood lignin structure can be attributed to coniferyl alcohol, the rest is made up of p-coumaryl alcohol and sinapyl alcohol. Also, lignin-carbohydrate bonds present in natural lignin are absent in synthetic lignins. DHP's are also alleged to be of lower molecular weights than natural lignins.

About 50% of the total ^{14}C -label in lignin is reported to have been converted to $^{14}\text{CO}_2$ in 20 days by p. chrysosporium (Kirk et al., 1978). This is an extremely high extent and rate of lignin degradation. The extents of $^{14}\text{CO}_2$ from ^{14}C -labeled, natural lignin reported in the literature have not been greater than 20%. The results of Kirk et al. with ^{14}C -labeled DHP are certainly indicative of phenomena that might be important in natural lignin metabolism but, in view of their limitations, a direct extrapolation of such data to the degradation of natural lignin should be done with some caution.

It is worth pointing out that Kirk et al. (1978) have made a mistake in their calculation of residual lignin (Table 3, p. 282).

Implicit in their estimation is the assumption that the yield of CO_2 /unit weight of lignin is unity, which is certainly not true. Their second implicit assumption of a constant value for the yield of CO_2 /unit weight of lignin is less serious though not very sound.

E. Lignin Bioprocessing

The chemical treatment of lignin has been investigated and the production of vanillin from lignin is a viable industrial process. Due to the energy intensive nature of the chemical treatment of lignin and the difficulty of product recovery, most of the international research efforts have focused on the biological treatment of lignin. Stern and Gasner (1974) have proposed a combined chemical and biological treatment of lignin. They ozonated lignin to partially depolymerize it before subjecting it to microbial degradation. Ozonated lignin was degraded faster by Aspergillus niger, Penicillium chrysogenum and others than was untreated lignin.

1. Processes: For obvious reasons process-oriented literature on lignin biodegradation is virtually nonexistent. All the research has invariably been carried out in either shake flasks or petri plates. The only reference I came across where a deep-tank fermenter was used was a publication by Tricart et al. (1978). They studied the biodegradation of high-yield spent bisulphite liquor in two lab-scale reactors operating in series. For a given feed and residence times longer than 24 hours, their results showed that the performance of the two-stage reactor was superior to that of a single-stage reactor.

Wilke and Mitra (1975) have proposed an elaborate flowsheet for enzymatically converting cellulosic wastes to reducing sugars. They also provided process design calculations and economic evaluations to illuminate the economic feasibility of the process. At present, such an exercise is impossible in the case of enzymatic treatment of lignin.

2. Products: The production of aromatic hydroxy acids has been demonstrated in lignin fermentations. Such acids can serve as monomeric raw materials for the polymer industry. It is, however, doubtful that the degradation of lignin and its derivatives will lead to aromatic hydroxy acids with the high purity required by the polymerization processes and the high yield demanded by economics (Elias, 1976).

Several lignin degradation products have been identified, but total amounts and yields of these products have been minuscule. Schubert (1965, pp. 94-95) has detected the following products in lignin degradation by fungi: vanillic acid, p-hydroxybenzoic acid, 3-methoxy-4-hydroxyphenylpyruvic acid, p-hydroxycinnamic acid, guaiacylglycerol, vanillin, coniferaldehyde and guaiacylglycerol- β -coniferyl ether. Lignin fermentation products such as syringic acids, syringaldehyde, ferulic acid, p-coumaric acid, p-hydroxycinnamic acid, p-coumaraldehyde, dehydrodivanillin have also been reported (Henderson, 1955; Ishikawa et al., 1963; Ishikawa et al., 1963a; Sopko, 1967).

3. Economics: The economic feasibility of any process purporting to convert lignin into marketable commodities would ultimately depend on the following factors: kinetics of lignin fermentation, yield of desired products, product recovery and, most importantly, ability to compete with existing processes for the same products. The kind of detailed economic forecast presented by Wilke and Mitra (1975) for enzymatic treatment of cellulosics is not possible for the case of lignin bioprocessing. However, given the current extent of the know-how, lignin utilization through biotransformation is economically unattractive at present. This situation may change in the future as fossil fuel reserves are depleted and petroleum-based feedstocks become more expensive.

III. MATERIALS AND METHODS

A. Materials

1. Cultures: Sporotrichum pulverulentum Novobranova was obtained from Dr. K.-E. Eriksson of the Swedish Forest Products Research Laboratory, Stockholm, Sweden. Phanerochaete chrysosporium Burdsall (ATCC No. 34541) and Coriolus versicolor (ATCC No. 12679) were obtained from Dr. T. K. Kirk of the Forest Products Laboratory, USDA, Madison, WI. The two Penicillium cultures, the white isolate and Aspergillus fumigatus (VPI 887) were isolated in this laboratory.

2. Chemicals: Indulin ATR C1, softwood kraft lignin, was obtained from Westvaco, North Charleston, SC and was purified as described later before use. D-cellobiose and Sigmacell Type 20 were purchased from Sigma Chemical Co., St. Louis, MO. Avicel PH-105 and Solka Floc were received gratis from FMC Corporation, Philadelphia, PA and Biochemistry Dept., VPI & SU, respectively. Soluble starch, PPO (2,5-diphenyloxazole), POPOP (1,4-bis[2-(5-phenyloxazolyl)] benzene), Scinti-Verse cocktail, phenethylamine, scintillation grade methanol and toluene, p-dimethylaminobenzaldehyde, bromcresol green and phenolphthalein were obtained from Fisher Scientific Co., Fairlawn, NJ. ReadySolv HP and MembraneSolv cocktails were purchased from Beckman Instruments, Inc., Fullerton, CA. Vanillin was obtained from Sterwin Chemicals, Inc., New York, NY and vanillyl alcohol was

synthesized in the laboratory of Dr. W. G. Glasser of the Forest Products Dept., VPI & SU. Malt broth extract and malt extract agar were brought from Difco Laboratories, Detroit, MI. Cellulose acetate and ^{14}C -labeled compounds (L-phenylalanine, toluene and D-glucose) were obtained from Eastman Kodak Company, Rochester, NY and New England Nuclear, Boston, MA, respectively. Celite (analytical filter-aid) was purchased from Johns-Manville Products Corp., Lompoc, CA.

All the other chemicals were of reagent grade and were procured from Fisher Scientific Co., Fairlawn, NJ.

3. Media: All media were prepared with distilled, deionized water and the pH of each medium was adjusted to a value appropriate for a particular organism.

a. Inoculum media.

Inocula for shake-flask experiments were cultivated in seed media containing malt extract (15 g malt broth extract/l). Inoculum media for the growth stages of multi-stage fermentations had the following composition, unless otherwise mentioned: D-glucose, 50 g/l; $\text{NH}_4\text{H}_2\text{PO}_4$, 10 g/l; KH_2PO_4 , 0.6 g/l; K_2HPO_4 , 0.4 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 75 mg/l.

b. Induction media.

These media were used only for the induction stages of multi-stage fermentations. Induction media had the following composition, unless otherwise mentioned: Soluble starch, 10 g/l; $\text{NH}_4\text{H}_2\text{PO}_4$, 5 g/l;

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g/l and inducers (variable). Vanillin and vanillyl alcohol, when used as possible inducers, were added in the concentrations of 0.25 g/l and 0.125 g/l, respectively. When a low molecular weight lignin fraction was used as a possible inducer, 2800 ml of a dialysate were added at the beginning of the induction stage. Other components of the medium were so added as to yield the desired medium composition and the final operating volume of 8 l. The dialysate was prepared by dissolving 10 g lignin in 500 ml of 0.2 N KOH and dialyzing the solution against 3 l distilled water. Dialysis tubings (Arthur H. Thomas Co., Philadelphia, PA) with a 3500 molecular weight cutoff were used to dialyze lignin, the duration of dialysis being 2 days.

c. Production media.

The composition of a typical production medium was as follows, the figures being per litre of the final volume: 10 g carbohydrate, 1 g lignin, 2 g $\text{NH}_4\text{H}_2\text{PO}_4$, 0.6 g KH_2PO_4 , 0.4 g K_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 75 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 mg thiamine·HCl, 12 mg ferric citrate, 4 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 7 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 100 ml 1 M phthalate buffer.

In experiments where effects of carbohydrate and nitrogen levels were studied, concentrations of the carbohydrate and nitrogen sources were variable. These departures from the standard production medium as well as types of carbohydrates used are mentioned in the Results and Discussion section. When a nutrient nitrogen source other than

$\text{NH}_4\text{H}_2\text{PO}_4$ was used, the nature and concentration of the alternate nitrogen source are also reported in the text.

Media for the biotransformation stages of multi-stage fermentations were the same as the standard production medium.

4. CO_2 Trapping Solution: The CO_2 trapping solution was prepared by mixing 270 ml phenethylamine, 270 ml methanol (scintillation grade), 5 g PPO and 0.1 g POPOP and bringing the final volume to one l with toluene (scintillation grade).

5. Scintillation Cocktails: The toluene cocktail used to measure $^{14}\text{CO}_2$ was prepared by dissolving 5 g PPO and 0.1 g POPOP in one l toluene of scintillation grade. Commercially available scintillation cocktails were used for special purposes. ReadySolv HP, ScintiVerse and MembraneSolv cocktails were employed for measuring radioactivity of aqueous samples, nonaqueous liquid samples and filtered material adhering to filtering membranes, respectively.

B. Methods

Every phase of the experimental work implicating microorganisms, in a direct or an indirect way, was carried out with sterile materials using aseptic techniques.

1. Sterilization of Equipment and Materials: Fermenter vessels, filter-sterilization assemblies, blender jars, glass containers and the like were sterilized in a steam autoclave at 121 C for 20-50 minutes depending on the size and contents.

The production medium was sterilized in parts and sterile components were later aseptically mixed. All the trace salts were autoclaved separately as a group and so were $\text{NH}_4\text{H}_2\text{PO}_4$, KH_2PO_4 and K_2HPO_4 . The rest of the medium constituents were sterilized individually to avoid problems like precipitation of salts or nonenzymatic browning of carbohydrates. Lignin was dissolved in 0.2 N KOH (1 g lignin/50 ml 0.2 N KOH) and filter-sterilized. Compounds like vanillin, vanillyl alcohol, thiamine·HCl etc. which were suspected to be temperature-labile were also filter-sterilized. Filter-sterilization was carried out in sterile filtration assemblies using Millipore membranes of 0.2 μ pore size (Millipore Corporation, Bedford, MA).

2. Inocula Preparation: Working slants were prepared from original stock slants. A new batch of working slants was prepared at least every 2-3 months. All cultures were maintained on malt agar.

To prepare inocula for shake-flask experiments, spores or mycelia from working slants were inoculated into 500 ml of malt broth. Incubation was carried out in 2800 ml wide-mouth Fernbach flasks, the duration of incubation depending on a particular organism.

A. fumigatus seed cultures usually grew up to a large biomass in 2-3 days whereas C. versicolor seed cultures took 5-7 days to yield a reasonable amount of biomass. After adequate amount of biomass was accumulated, the broth was centrifuged at 10,000-12,000 rpm for ca. 20 minutes. A Sorvall RC-5 Superspeed Refrigerated Centrifuge (Du Pont Company, Instrument Products, Newtown, CT) and a

Sorvall GSA head were used for centrifugation. The supernatant was then decanted and the cell pellets were homogenized in a blender for 10-15 seconds after adding distilled water. No apparent shear damage to cells was discernible. To each experimental flask, a 250 ml Erlenmeyer flask, 5 ml of the homogenate were added with a wide-mouth pipette thus bringing the operating volume to 50 ml (final volume). The exact amount of dry cell mass issued per flask was determined and it differed to some extent from experiment to experiment.

Inocula for all multi-stage fermentations were grown in situ from spores.

3. Purification of Kraft Lignin*: A thick slurry was prepared by mixing 100 g Indulin ATR C1 with water and 500 ml of 2N NaOH were added to dissolve the lignin. Chloroform was added and 5 N HCl was poured in with stirring until a precipitate was formed. A color change was noticed at this time since the solution had turned acidic. The solution was filtered through a Whatman No. 1 filter paper and the filter paper was washed until the solution reached neutrality. The above procedure was repeated two more times and the resultant solution was freeze-dried to obtain purified lignin. Lignin thus prepared was mostly free of impurities and a reproducible starting material.

* Purified kraft lignin was supplied by Dr. W. G. Glasser of the Forest Products Dept., VPI & SU.

4. Preparation of ^{14}C -Labeled Kraft Lignin: The procedure of Crawford and Crawford (1976) was followed to selectively label the lignin component of loblolly pine seedlings.* L-(U- ^{14}C -) phenylalanine was used as a precursor for the synthesis of ^{14}C -labeled lignin.

The seedlings were grown in a greenhouse and were allowed to take up labeled phenylalanine for 7-10 days. Labeled seedlings which were on the average a foot tall were then stripped of needles and bark. The resulting wood was cut in approximately one inch pieces and was extracted with acetone:water::1:9 for two days. The air-dried wood was cooked for three hours at 180 C in a sealed container. Five ml of cooking liquor (0.425 g Na_2S + 1.274 g NaOH in 38 ml H_2O) were added per g of wood. The resulting pulp was extracted with 2 N NaOH and washed with water until the filtrate was clear. A sufficient amount of chloroform was then added to yield a concentration of 1% chloroform. Lignin was precipitated with the addition of 5 N HCl, filtered and washed to remove the acid. The precipitate was air-dried to yield ^{14}C -labeled kraft lignin.[†] The above procedure is based on that described by Larsson and Miksche(1971).

* Loblolly pine seedlings were generously provided by Dr. Lance Kress of the Air Pollution Laboratory, VPI & SU.

[†] ^{14}C -labeled kraft lignin was prepared by Ms. Charlotte Barnett of the Forest Products Dept., VPI & SU.

5. Measurement and Control of Operating Parameters:

a. Temperature.

The operating temperature was 28 C in all experiments involving S. pulverulentum, P. chrysosporium, C. versicolor, two Penicillium cultures and the white isolate, unless mentioned to the contrary. All the early experiments with A. fumigatus were performed at 28 C, but after confirming its exact identity and thermophilic nature, the later experiments were carried out at 37 C. The actual temperature of a particular A. fumigatus experiment is reported in the text. Operating temperatures were maintained with ± 1 C of the setpoint during both shake-flask and deep-tank fermentations.

b. pH.

S. pulverulentum, Penicillium cultures and the white isolate were cultivated at pH 5.5 whereas C. versicolor and A. fumigatus were grown at pH 5.0. The operating pH for P. chrysosporium was 4.6. The information regarding operating pH's of S. pulverulentum, C. versicolor and P. chrysosporium was obtained from Eriksson (1976) and Kirk et al. (1976). Each isolate was incubated at a pH similar to that of the environment in which it was originally encountered.

In shake-flask experiments, the medium was buffered with 0.1 M (final concentration) phthalate buffer which seemed to hold the pH within a reasonable range. In deep-tank fermentations, the pH was automatically controlled by a pH controller (New Brunswick Scientific Co., Inc., New Brunswick, NJ). The pH controller

automatically added requisite amounts of 10 N NaOH to maintain the operating pH within ± 0.1 pH unit of the setpoint. Since a high normality base was added, the change in the operating volume was infinitesimal. The pH was measured by an Ingold combination pH electrode (Ingold Electrodes, Inc., Cambridge, MA), which was coupled to the pH controller.

c. Agitation.

All shake-flask experiments were carried out in the Phychro-therm Incubator-Shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) or the Environ-Shaker (Model 3597-X, Labline Instruments, Inc., Melrose Park, IL). Each shaker had a platform with a 2" throw. All experiments with ^{14}C -labeled lignin as a substrate were conducted at 210-220 rpm and the rest at 250 rpm. The reason for the above difference was the relative instability of the special arrangement of flasks in experiments with ^{14}C -labeled lignin.

All deep-tank fermentations were agitated by a flat-blade impeller driven at 400 rpm. In multi-stage fermentations, rpm's of 600-700 were used during growth stages to effect a decrease in cell size. Rpm's higher than 400 could not be used in the presence of lignin due to the problem of foaming. No antifoam agents were used in any of the deep-tank fermentations reported.

d. Aeration.

In shake-flask experiments, flasks were either closed with cotton plugs or covered with special caps called Kaputs (Bellco,

Vineland, NJ). Both methods allowed sufficient air transfer into flasks and at the same time kept them uncontaminated. When ^{14}C -labeled lignin was used, flasks were completely sealed with rubber stoppers thus rendering the system air-tight. These flasks were flushed with air every 2-3 days. This type of a closed system has been reported not to be oxygen-limited (Kirk et al., 1978). Using 50 ml of media in 250 ml Erlenmeyer flasks supposedly allowed proper mixing and aeration.

In deep-tank fermentations the aeration rate was maintained at 1 v/v/m, i.e., one l/min air was supplied for every one l of the operating volume. For a brief period immediately after adding lignin, the aeration rate was reduced to 0.5 v/v/m or lower to alleviate foaming. After the foam had subsided the aeration rate was increased to its normal value.

6. Measurement of $^{14}\text{CO}_2$: All experiments employing ^{14}C -labeled lignin as a substrate were carried out in 250 ml Erlenmeyer flasks equipped with ports (Fig. 8). These flasks were incubated in the Psychrotherm Incubator-Shaker at 210-220 rpm and were totally concealed during incubation. Flasks were flushed every two days with CO_2 -free air. (Air was passed through 1 N KOH to trap ambient CO_2 .) Glasswool filters at both ends of the flasks maintained them sterile during flushing.

The CO_2 from flasks was trapped in two traps (Fig. 8), the first containing 5 ml 1 N KOH and the second containing 5 ml trapping

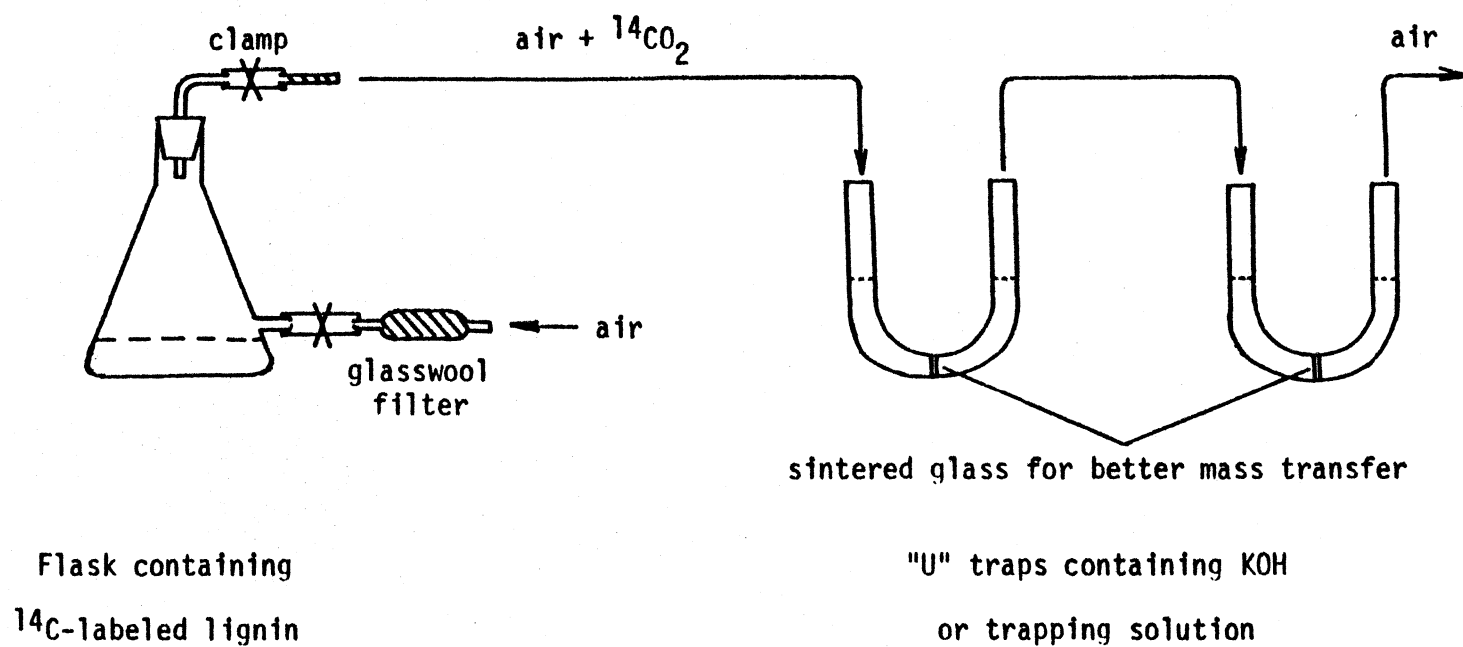


Figure 8. Schematic diagram of the setup for ^{14}C -labeled lignin experiments.

solution. The solution from the first trap was divided into two aliquots and one aliquot was assayed for total CO_2 . The second aliquot was acidified with 5 ml of 8.48 N H_2SO_4 and the CO_2 thus liberated was trapped in two additional traps containing trapping solution. Samples containing trapping solution were transferred to Poly-Q vials (Beckman Instruments, Inc., Irvine, CA) and 5 ml of the toluene cocktail were added to each vial. The radioactivity in each vial was measured in a Beckman Liquid Scintillation Counter (Model LS 3155T, Beckman Instruments, Inc., Irvine, CA). $^{14}\text{CO}_2$ data reported represent the radioactivity of an entire sample.

When total CO_2 was not determined, the first trap contained 5 ml trapping solution instead of KOH. In this case the total radioactivity of a sample was a simple addition of the radioactivity of individual traps.

Counting efficiencies were determined by establishing a plot of "external standard" vs. efficiency of counting. This plot, called a quench curve, was arrived at by using standard ^{14}C -labeled toluene or glucose samples of known radioactivity. The radioactivity of a particular sample was calculated by the following formula:

$$\text{DPM} = (\text{CPM} - \text{background reading}) / \text{efficiency of counting.}$$

where DPM: disintegrations per minute or actual radioactivity

CPM: counts per minute or radioactivity measured by the liquid scintillation counter.

7. Determination of Total CO₂: The CO₂ trapped in 1 N KOH was in the form of K₂CO₃ and/or KHCO₃. The aliquot containing K₂CO₃ and/or KHCO₃ was diluted to 50 ml with distilled water. A 25 ml aliquot from the diluted sample was titrated with 0.1 N HCl using bromocresol green as the indicator. The second 25 ml aliquot was titrated with 0.1 N HCl with phenolphthalein as the indicator. The total CO₂ was then calculated as described by Skoog and West (1963, pp. 351-353). Values reported reflect the total CO₂ of the entire sample.

8. Multi-Stage Fermentations: All deep-tank fermentations were carried out in a 14 l fermenter (Model MGF-14, New Brunswick Scientific Co., Inc., New Brunswick, NJ) which was housed in a Micro-ferm Fermenter module (New Brunswick Scientific Co., Inc., New Brunswick, NJ).

Multi-stage fermentations had three stages namely, a growth stage, an induction stage and a biotransformation stage. The details of each multi-stage fermentation are given in the text. At the end of a growth stage, air supply and agitation were stopped and cells were allowed to settle for ca. 10 minutes. The supernatant was then drained off using a peristaltic pump of the Continuous Culture Console (New Brunswick Scientific Co., Inc., New Brunswick, NJ). Air supply and agitation were then resumed and the induction medium was added to the fermenter via the same peristaltic pump. At the end of an induction stage, the same procedure was used for removing the supernatant and adding the medium for the biotransformation stage.

Some induction stages contained a low molecular weight lignin fraction as a possible inducer of lignolytic enzymes. Since the amount of lignin introduced in such an induction stage was quite small and since the induction medium was drained off before commencing the biotransformation stage, the carryover of residual lignin from the induction to the biotransformation stage was considered insignificant. Furthermore, the relatively high rate of metabolism of the low molecular weight lignin fraction would make such a carry-over even less probable.

9. Harvesting of Deep-Tank Fermentations: At the end of a lignin fermentation, air supply and agitation were stopped and the contents were emptied into large containers using a peristaltic pump. The material adhering to the sides of the fermenter was scraped off and stored frozen in a separate container. The fermentation contents were then filtered through a polyester filter cloth using a small filter-press. Nitrogen at ca. 30 psi was used to facilitate pressure filtration. Retained solids were stored frozen and later delivered to the Forest Products Dept., VPI & SU for analyses. The filtrate was passed through Whatman No. 1 filter papers and 0.2 μ Millipore membranes to render it particulate-free. The filter-press and pressurized nitrogen were again used to effect rapid filtration.

The broth, devoid of particulate matter, was stored in a refrigerator until being subjected to molecular concentration. The broth was concentrated in a Millipore Pellicon Cassette System

(Millipore Corporation, Bedford, MA) using a Pellicon ultrafiltration cassette with a 10,000 normal molecular weight cutoff (Millipore Corporation, Bedford, MA). A retentate fraction containing high molecular weight material and a filtrate fraction resulted from the ultrafiltration. Starting with 8 l of broth, it was possible to obtain a retentate fraction measuring 100 ml or less. The retentate fraction was analyzed for extracellular enzyme activities.

10. Dry Cell Weight Determination: Since lignin fermentations contained particulate matter such as lignin itself and cellulose, it was not possible to determine the dry cell weights by simple filtration. Hence, glucosamine was followed as an index of biomass. Glucosamine is a chitinous substance found in fungi and other microbes as well. This cell-wall component can be quantitatively related to the dry cell weight. The procedure used in monitoring glucosamine was that of Cochran and Vercelloti (1978) and was not impeded by the presence of residual lignin or carbohydrates.

a. Hydrolysis.

A fermentation sample containing cells, lignin and other matter was filtered through a Whatman No. 1 filter paper. Retained solids were hydrolyzed with 1 ml of 8 N HCl for 2 hours at 96-100 C. The hydrolysis was carried out in special reaction vials called Reacti-Vials (Pierce Chemical Co., Rockford, IL). Vials were then cooled and the residual acid was removed by vacuum drying. A vacuum desiccator containing KOH pellets was used for drying. The vacuum pump was protected by including KOH and dry ice-isopropanol traps in the scheme.

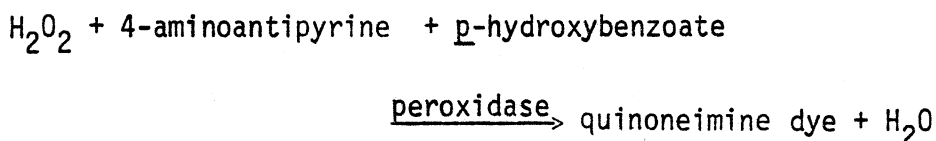
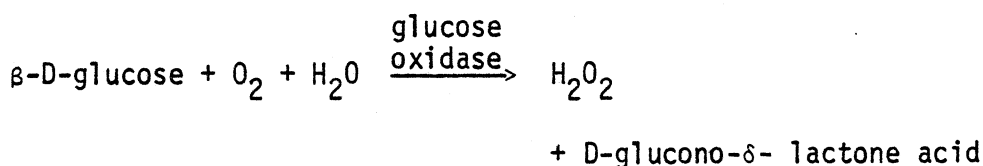
Following the addition of 2 l distilled water, the samples were filtered through a sintered glass filter containing celite as a filter-aid. The filtrate was subsequently assayed for glucosamine. In shake-flask fermentations, the entire contents of a flask were sacrificed per datum point.

b. Glucosamine assay.

The filtrate containing glucosamine extracted from the cell-wall was diluted accordingly to obtain a 0.35 ml sample. When 0.1 ml filtrate was mixed with 0.25 ml distilled water, the resulting dilute sample usually fell within the standard curve regime. The sample was allowed to stand for 6 minutes at room temperature subsequent to the addition of 0.1 ml borate buffer (1.12 M H_3BO_3 + 0.56 M KOH) and 0.05 ml ice-cold 10% acetic anhydride. After adding 0.05 ml 1 N NaOH, the sample was placed in boiling water for 9 minutes. The sample was cooled in cold water and 3 ml of Ehrlich's reagent (1 g p-dimethylaminobenzaldehyde + 1.25 ml 10 N HCl made up to 100 ml with glacial acetic acid) were added. The sample was thoroughly mixed with a vortex mixer and was incubated at 37 C for 20 min. The sample absorbance was measured at 585 nm against a reagent blank. A Varian Techtron UV-VIS spectrophotometer (Model 635, Varian Techtron, Palo Alto, CA) was used to measure sample absorbances. The same procedure was employed for establishing a standard curve which was linear in the range of 0 to 120 nmoles glucosamine.

The assay was carried out in 10 ml assay test tubes and extremely small volumes of reagents were delivered through Eppendorf pipettes (Brinkman Instruments, Inc., Westbury, NY).

11. Glucose Oxidase Assay for Glucose: This assay for glucose, called the Statzyme glucose assay, was developed by Worthington Diagnostics, Freehold, NJ. The reactions involved in the assay are as follows:



The quinoneimine chromophore has an absorbance maximum at 500 nm.

Worthington's Statzyme reagent was reconstituted with 16 or 50 ml distilled water depending on the vial size. The assay was initiated by mixing 3 ml reconstituted reagent and 30 ml sample. The above mixture was then placed in a 37 C water bath for 10 min. The sample absorbance was read at 500 nm against a reagent blank. The same procedure was followed with standard samples and the standard curve thus obtained was linear in the domain of 0 to 150 mg glucose. The assay was carried out in 10 ml assay test tubes. When sample volumes other than 30 ml were used, the sample to reagent volumetric ratio of 1:100 was still maintained.

12. Starch Determination: The sample to be assayed was placed in a 10 ml assay test tube and the volume was brought to 3 ml with distilled water. The diluted sample and 0.1 ml iodine solution (3% KI + 0.3% iodine) were thoroughly mixed. A blue color developed due to the interaction of starch and iodine. The sample absorbance was measured at 620 nm against a reagent blank. The same procedure was repeated to set up a standard curve which was linear in the region of 0 to 150 mg starch. To prepare a standard solution, soluble starch had to be dissolved by simultaneous stirring and heating.

13. Nutrient Nitrogen Determination: Since $\text{NH}_4\text{H}_2\text{PO}_4$ was used as the nutrient nitrogen source, an ammonia electrode (Model 95-10, Orion Research Inc., Cambridge, MA) was employed to follow the depletion of nitrogen during fermentations.

To minimize the loss of ammonia during measurements, square bottles (6" height, 1.5" width) were used which kept the surface area (exposed to air) to volume (of sample) ratio at a low value. Samples were magnetically stirred throughout the measurement. A radiometer PHM 63 digital pH/mV meter (Radiometer, Copenhagen, Denmark) was used to measure the mV response of each sample.

The ammonia electrode was placed in a 0.1 ppm (parts per million) N standard and one ml of 10 M NaOH was added to each 100 ml of the standard. The mV reading of the 0.1 ppm N standard was arbitrarily set at zero after enough time was allowed for the electrode response to stabilize. The same procedure was adopted for

other standard and unknown samples. The standard curve of electrode potential in mV vs. log (ppm N) was linear in the range of 0.1 to 1,000 ppm N.

14. Dissolved Oxygen Measurement: Dissolved oxygen measurements were accomplished by using a voltammetric type oxygen electrode (Model IL 530, Instrumentation Laboratory Inc., Lexington, MA). In this setup the active electrode, a platinum cathode at which oxygen is reduced, was isolated from the test media by a thin electrolyte layer and a polymeric membrane. The membrane was impermeable to ions and proteins yet was permeable to oxygen whether in a gas or liquid sample. The anode was Ag-AgCl and the electrode response time was less than 45 seconds (to reach 98% of the equilibrium value).

An amplifier (Model IL 27564, Instrumentation Laboratory Inc., Lexington, MA) was used for optimum results. The amplifier output was so calibrated that 1 mA corresponded to 1 ppm O_2 . The electrode response was linear from 0 to 8 ppm O_2 . It was possible to calibrate the electrode in ambient air since the difference in O_2 readings of stagnant and vigorously stirred samples, and of liquid samples, and gas phases in equilibrium was about 2%. The electrode was chemically sterilized before inserting into the fermentation vessel. Dissolved oxygen (DO) demand was determined by stopping the air supply and agitation for 2-5 minutes and noting the drop in the amplifier

output. The drop in mA as indicated by the amplifier was linear over the above period. Hence,

$$\begin{aligned} \text{DO demand} &= \text{change in mA reading/time elapsed in min} \\ &[=] \text{ ppm O}_2/\text{min}. \end{aligned}$$

15. Reduced Data and Statistical Significance: Data presented in Figs. 12, 18, 20, 21 and 22 represent averages of two experiments. The scatter in above experiments was approximately $\pm 10\%$. Hence,

$$X = X_0 (1 \pm 0.10),$$

where X_0 and X are observed value and statistical range of X_0 , respectively. The rest of the figures containing experimental data represent results of single experiments. The lack of duplicate experiments does not reflect a want of conscientious experimental-design but was dictated by logistical problems such as the availability of ^{14}C -labeled lignin and shaker-spaces. The statistical interpretation of single experiments may be deemed to be similar to that of duplicate experiments.

IV. RESULTS AND DISCUSSION

A. ^{14}C -Lignin Assay

Until recently no reliable, quantitative assay for monitoring lignin biodegradation was available. In 1976, Crawford and Crawford published a sensitive assay which monitored the evolution of $^{14}\text{CO}_2$ from ^{14}C -labeled lignin as an index of lignin metabolism. This assay is easy to carry out and is the only sensitive microassay at the disposal of researchers studying lignin biodegradation. Previous lignin assays either required large samples or were not sensitive enough.

The ability of a microorganism to degrade lignin is assumed to be reflected in its capacity to liberate $^{14}\text{CO}_2$ from ^{14}C -labeled lignin, though production of CO_2 from lignin is not the objective of lignin biodegradation studies. It is implicit in the above assay that an organism which produces a large amount of CO_2 from lignin can also be responsible for the production of desirable intermediates from lignin, the accumulation of such intermediates in substantial quantities being realized through genetic manipulation of the organism or proper control of metabolic pathways. However, it may be argued that either evolution of $^{14}\text{CO}_2$ from ^{14}C -labeled lignin or lack thereof may not give a true indication of an organism's potential to modify or metabolize lignin. It can be readily envisaged that an organism may evolve very little CO_2 from lignin while greatly modifying

lignin and/or producing large quantities of lignin-derived low molecular weight products. Conversely, an organism evolving a large amount of CO_2 from lignin may be doing it solely at the expense of an easily metabolized lignin fraction while bringing about neither significant changes in the bulk of the lignin micromolecule nor accumulation of lignin-derived metabolites. Thus, to complement the assay of Crawford and Crawford (1976), the possible accumulation of lignin-derived low molecular weight moieties was also studied. This was accomplished by carrying out a modified dialysis fermentation. The modified ^{14}C -lignin assay was used in most of the following studies.

Definition of "lignolytic activity": Lignolytic activity is defined as the $^{14}\text{CO}_2$ evolved from ^{14}C -labeled kraft lignin expressed as a % of the initial radioactivity.

B. Screening of Lignin-Degrading Fungi

Sporotrichum pulverulentum, a white-rot fungus, has been studied in connection with lignin and wood degradation by K.-E. Eriksson and colleagues in Sweden. Phanerochaete chrysosporium, which is considered to be the perfect state of S. pulverulentum, has been investigated by T. K. Kirk and coworkers in USA. Coriolus versicolor, also a white-rot fungus, has featured in some of the studies reported by Kirk et al. (1976).

During the early stages of this investigation, three fungi were isolated from cultures growing on a lignin-containing agar plate and a low molecular weight lignin fraction. Two of the fungi produced

green spores and were thought to be of the Penicillium genus though no attempt was made at a taxonomic classification. They were designated as Penicillium sp. 1 and Penicillium sp. 2. The third isolate produced white mycelia and was termed as the "white isolate". The above three isolates and the well-characterized white-rot fungi were included in the primary screening of lignin-degrading fungi.

1. Primary Screening: S. pulverulentum and P. chrysosporium have been reported to have 38 C as their optimum temperature for growth (Eriksson, 1976; Kirk et al., 1976). Ander and Eriksson (1975), however, have opted for 28 C as the operating temperature in studies with S. pulverulentum. Due to this discrepancy, cultures of S. pulverulentum and P. chrysosporium were incubated at both 28 C and 38 C. C. versicolor has been reported to grow best at 28 C (Kirk et al., 1976). Thus, cultures of C. versicolor and the three isolates were incubated only at 28 C. The comparison among various fungi was made only on the basis of data obtained at 28 C.

Among all fungi tested, C. versicolor was the most efficient in $^{14}\text{CO}_2$ production from ^{14}C -labeled kraft lignin (Figs. 9 and 10). As expected, S. pulverulentum and P. chrysosporium liberated about the same amount of $^{14}\text{CO}_2$ though both exhibited lignolytic activities much lower than C. versicolor. Even lower on the scale were Penicillium sp. 1 and the white isolate. Penicillium sp. 2 was slightly less effective in $^{14}\text{CO}_2$ evolution than S. pulverulentum and P. chrysosporium.

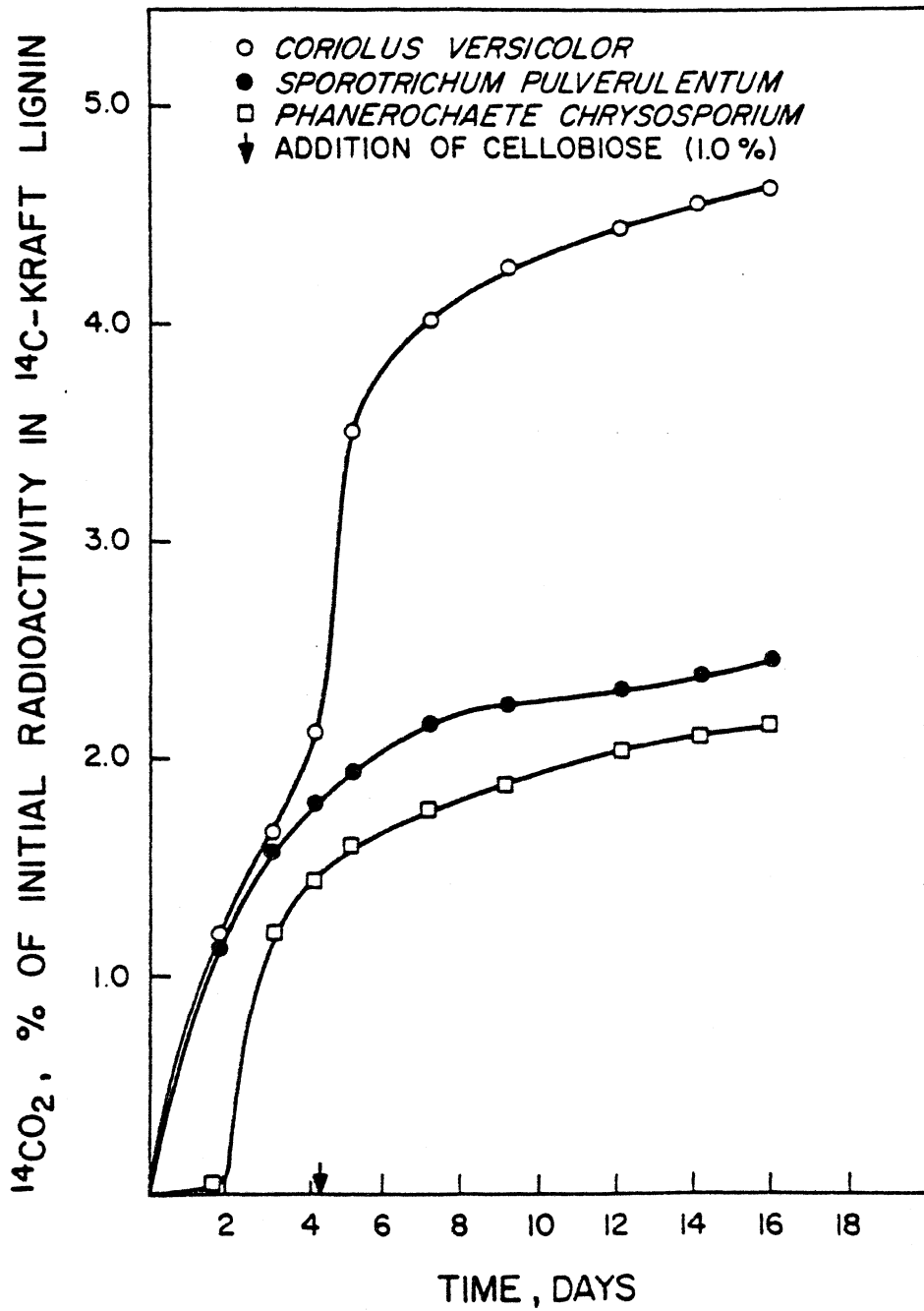


Figure 9. Primary screening of lignin-degrading fungi: White-rot fungi.

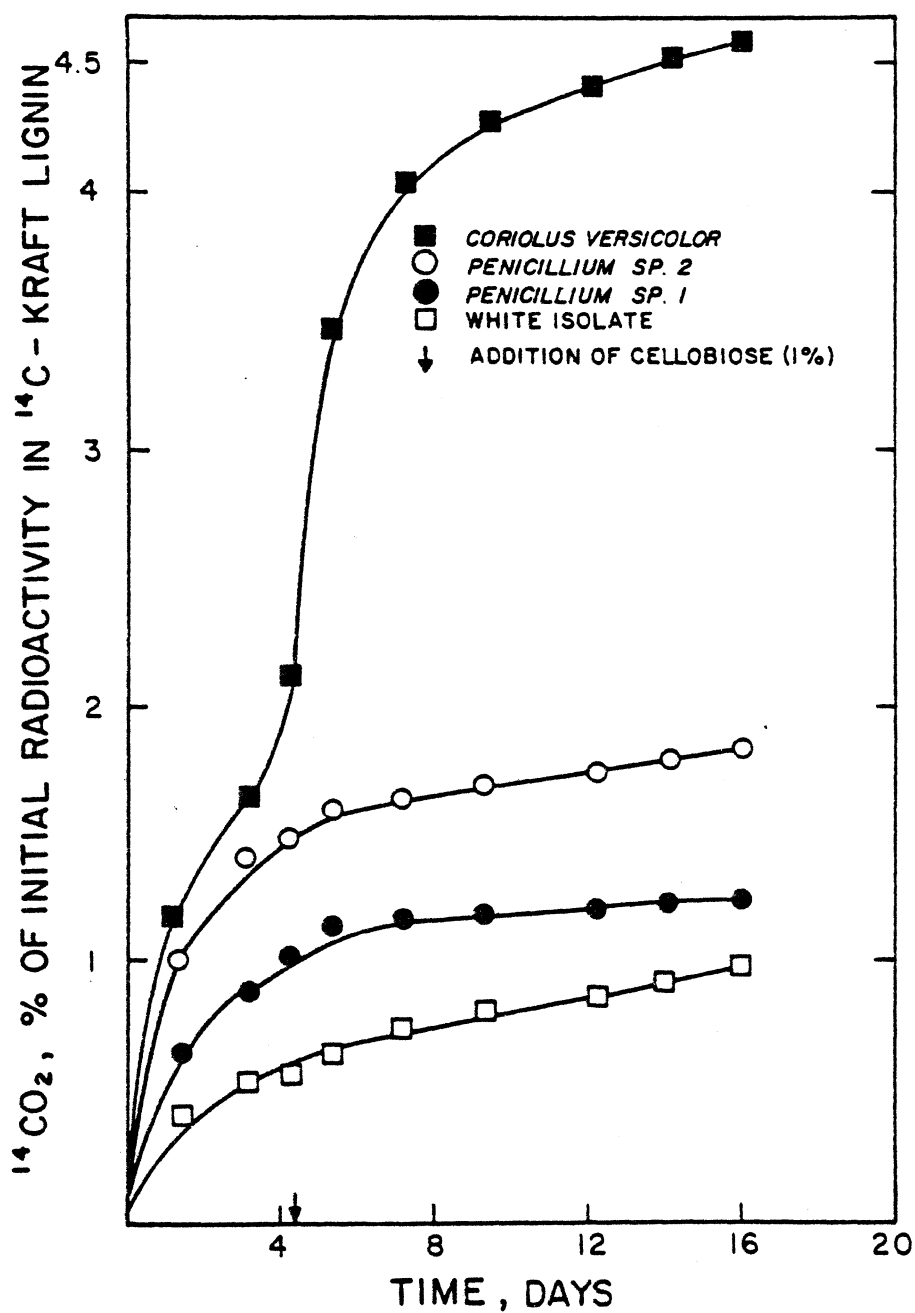


Figure 10. Primary screening of lignin-degrading fungi: Isolates.

Initially all the flasks contained 0.1% (w/v)* cellobiose as a growth substrate, but after 4.2 days, 0.5 g of sterile cellobiose was added to each flask thus bringing the cellobiose concentration to ca. 1% (w/v). The rate or extent of lignin metabolism was not altered except for C. versicolor. Soon after the addition of cellobiose, the rate of $^{14}\text{CO}_2$ evolution by C. versicolor changed dramatically. The average rate of $^{14}\text{CO}_2$ evolution for the first 1.4 days was 1775 dpm/day (disintegrations per minute/day) but it soared to 2887 dpm/day just after adding cellobiose. The rate of $^{14}\text{CO}_2$ evolution by C. versicolor dropped to a value of 624 dpm/day 2.5 days after supplemental cellobiose was added. This rate is comparable to the rate of $^{14}\text{CO}_2$ evolution seen shortly before cellobiose was added, namely 727 dpm/day.

What could be the reason that such a dramatic change in $^{14}\text{CO}_2$ evolution was seen at all and only in the case of C. versicolor and not others?[†] The initial rate of carbohydrate consumption by white-rot fungi is known to be substantial and the carbohydrate concentration drops exponentially to a low value even when the initial carbohydrate concentration is 1%. Hence, it is fair to assume that organisms become carbohydrate limited very early in the fermentation when the initial carbohydrate concentration is 0.1%. If the

*weight/volume

[†]Since these observations were not reproduced, they may very well be treated as artifactual. The ensuing explanation is, then, superfluous.

carbohydrate concentration is such that it does not supply enough energy for cellular functions and maintenance then lignin metabolism surely would suffer. Maybe when this energy deficiency is removed by supplying additional cellobiose, the organism responds with a temporarily high rate of lignin metabolism. No drastic change in the rate of $^{14}\text{CO}_2$ evolution by C. versicolor was observed when the initial cellobiose concentration was 1% and no supplemental cellobiose was added during the fermentation (Fig. 14).

The fact that other organisms did not show a similar change in $^{14}\text{CO}_2$ evolution may be explained by their relatively high growth rates. Due to their high growth rates the cellobiose concentration may have dropped much earlier and to a lower value than in the case of C. versicolor. If these cultures were energy-limited for a much longer period than C. versicolor, it may have affected their ability to fully reestablish their lignin-degrading capacity even after the energy deficiency was removed.

2. Possible Low MW pool: For a lignin biotransformation process to be successful, it is essential that the process yields products of economic potential. The production of glucose monomers from the enzymatic treatment of polymeric cellulose has proven to be a feasible process at pilot-plant scales. By analogy, lignin fermentations could be expected to accumulate low molecular weight products like phenolics or aliphatic acids.

Fig. 11 pictorially describes how a hypothetical low molecular weight pool can operate in a lignin fermentation. Cells, through the action of enzymes, can break down lignin into smaller molecules. The phenolics of low molecular weight, in turn, can be repolymerized by phenoloxidases. Cells can take up the smaller lignin-derived molecules and metabolize them intracellularly. Cells may also intracellularly synthesize new lignin-related compounds and contribute to the pool. De novo synthesis of veratryl alcohol, an aromatic compound related to lignin, from glucose by P. chrysosporium has been reported (Lundquist and Kirk, 1978). The possibility of the existence of such a pool and the levels of its constituents will depend on the relative rates of the four competing reactions described above.

3. Dialysis Fermentation: The possibility of producing low molecular weight metabolites from lignin was tested via a modified dialysis fermentation. A one inch long piece of dialysis tubing (3500 MW cutoff) tied at both ends was included in each of the culture flasks and also in the uninoculated flask. The dialysis bags initially contained only distilled water and the fermentation media contained ^{14}C -labeled lignin. At the end of the fermentation, the radioactivity of contents of dialysis bags was determined and expressed as a % of the initial radioactivity. All the dialysis bags from inoculated flasks showed lower radioactivity than the dialysis bag from the uninoculated control. Hence, there was no net accumulation of lignin-derived low molecular weight products (Table 4).

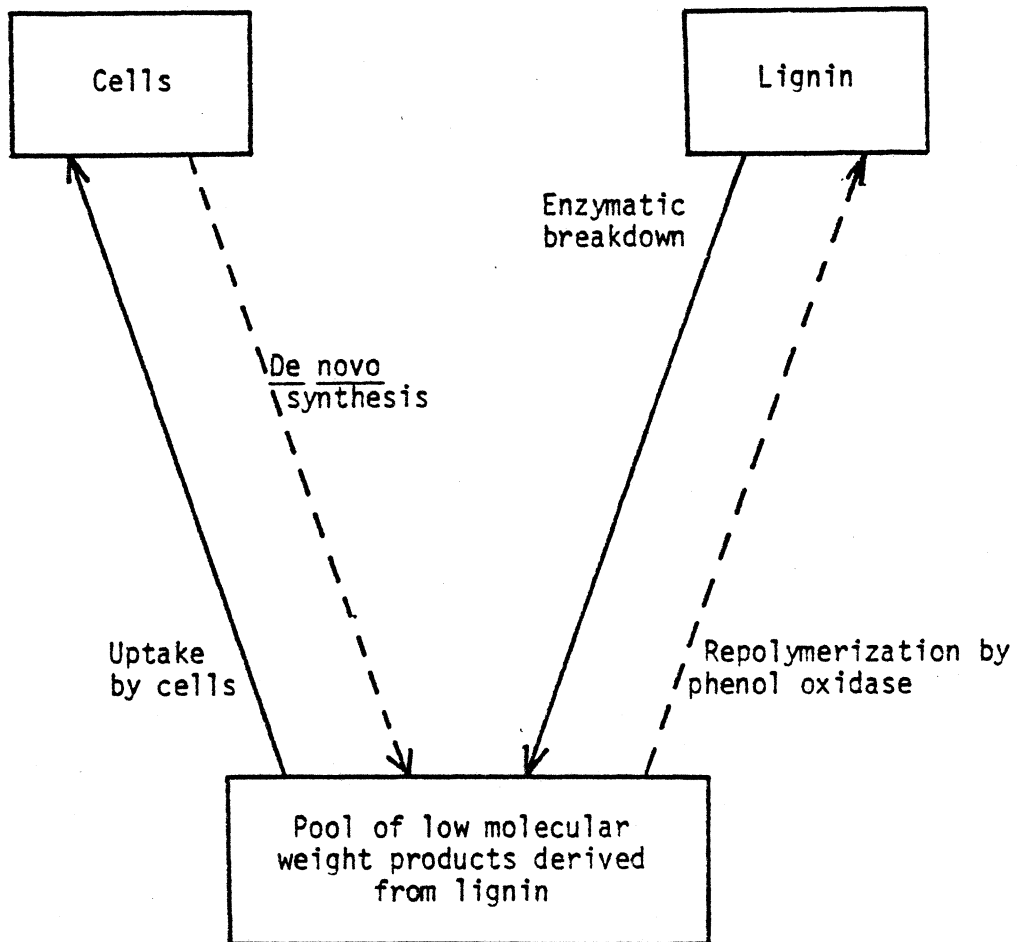


Figure 11. Dynamics of a hypothetical pool of low molecular weight products derived from lignin.

Table 4
Investigation of Lignin-Derived Products of Low MW

Organism	Temperature of Incubation, C	Dialyzable Radioactivity after 14 Days, % of Initial Radioactivity
Uninoculated Control	28	2.77
<u>S. pulverulentum</u>	28	1.75
<u>S. pulverulentum</u>	38	0.98
<u>P. chrysosporium</u>	28	1.20
<u>P. chrysosporium</u>	38	1.33
<u>C. versicolor</u>	28	0.92
<u>Penicillium</u> sp. 1	28	1.11
<u>Penicillium</u> sp. 2	28	1.32
White Isolate	28	1.28

C. versicolor which evolved the most $^{14}\text{CO}_2$ from lignin also showed the least dialyzable radioactivity.

Results of Table 4 may be explained by two alternate scenarios: i) the organisms did not release any additional low molecular weight moities from lignin but consumed some of the low molecular weight lignin already present at the time of inoculation; or ii) the organisms did indeed break down the lignin macromolecule into oligomers, but such metabolites were taken in by the organisms and further metabolized. In other words, the competing rates in Fig. 11 were such as not to allow any net accumulation of smaller metabolites. The possibility of the former scenario being true cannot be ruled out in light of the low extents of $^{14}\text{CO}_2$ evolved. It is probable that the $^{14}\text{CO}_2$ detected arose solely from the metabolism of low molecular weight lignin present initially. It is not unusual for organisms to prefer low molecular weight substrates over high molecular weight substrates for growth. This is certainly true for S. pulverulentum which demonstrated higher levels of growth on <10,000 (nominal) MW lignin fraction than on a >10,000 (nominal) MW lignin fraction or whole (unfractionated) lignin (Fig. 12).

If the latter scenario is operative then the low molecular weight pool may be harnessed by a semicontinuous dialysis fermentation where lignin-derived and presumably other low molecular weight compounds are continuously removed from the system. Since low molecular weight carbohydrates will also be dialyzed out, an optimal ratio of

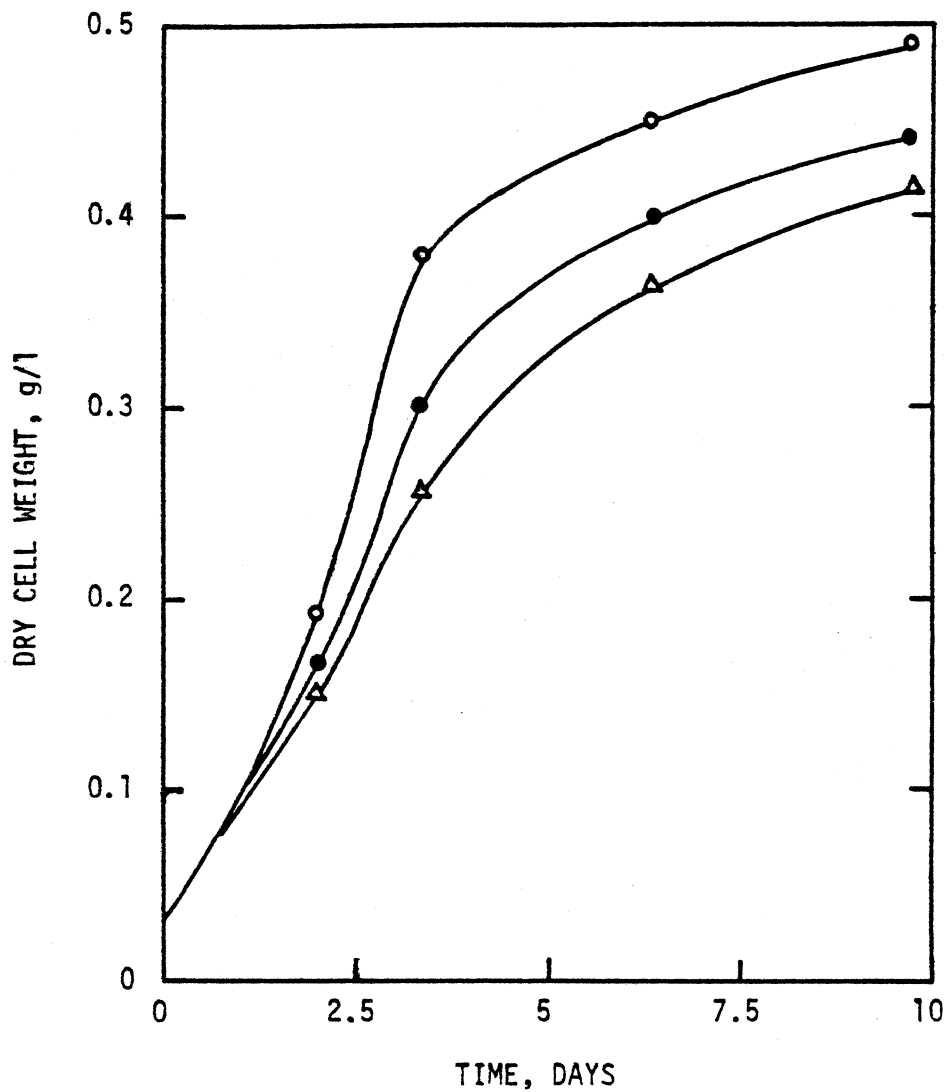


Figure 12. Growth of *S. pulverulentum* on different lignin fractions.

- <10,000 normal MW lignin fraction,
- >10,000 normal MW lignin fraction,
- △—△ whole (unfractionated) lignin.

feed rate/removal rate will have to be determined for both carbohydrate and lignin substrates. At such operating rates, the free carbohydrate and lignin concentrations will be such as to yield maximal amounts of lignin-derived low molecular weight products. This approach will not be fruitful if the desired intermediates have a very low half-life. As the lignin degradation pathways are elucidated in detail, it may become possible to create mutants which over-produce certain desirable intermediates of the pathways.

4. Temperature Effects: Both S. pulverulentum and P. chrysosporium exhibited higher lignolytic activities at 28 C than at 38 C (Fig. 13). This means that the optimum temperature for growth is not necessarily the same as the optimum temperature for lignolytic activity. Kirk et al. (1978) found that, for P. chrysosporium, the optimum pH for growth is different from the optimum pH for lignin metabolism.

5. Secondary Screening: C. versicolor, S. pulverulentum and P. chrysosporium were selected for further studies. To eliminate the possibility that the medium used in the primary screening was preferentially suited to C. versicolor and not to S. pulverulentum and P. chrysosporium, each of these three fungi was cultivated in three separate media. Eriksson and Goodell (1974) and Kirk et al. (1976) have recommended media for lignin degradation studies with S. pulverulentum and P. chrysosporium, respectively. In our

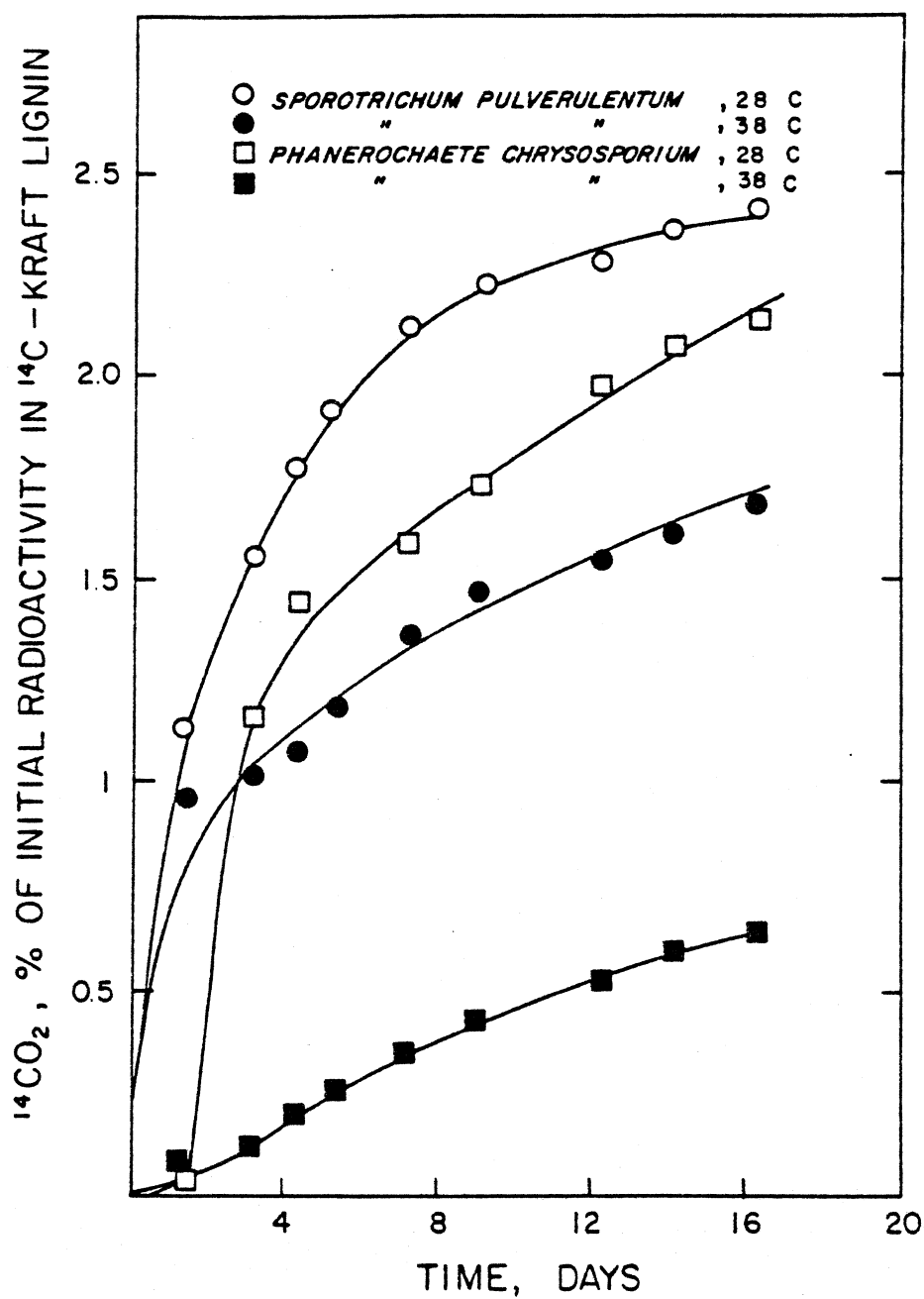


Figure 13. Effect of temperature on lignolytic activities of *S. pulverulentum* and *P. chrysosporium*.

laboratory, a medium containing phosphoric acid swollen cellulose (PSC)* was found to be suitable for C. versicolor. These media slightly differed in salt contents but the major difference was the type of carbohydrate used. Each fungus was cultivated in three different media at 28 C. Eriksson (1976) and Kirk et al. (1976) have also recommended proper operating pH's for these white-rot fungi and they are as follows: C. versicolor = pH 5.0, S. pulverulentum = pH 5.5 and P. chrysosporium = pH 4.6. Again, ^{14}C -labeled kraft lignin was used as a substrate and dialysis bags, tied at both ends, were also included in the fermentation. The initial concentration of each carbohydrate was 1% (w/v).

C. versicolor was more efficient in $^{14}\text{CO}_2$ production than S. pulverulentum or P. chrysosporium regardless of the medium used (Figs. 14, 15 and 16). For all the three cultures, Avicel, a micro-crystalline cellulose, supported the highest lignolytic activity and was followed by PSC and cellobiose. This observation can be interpreted to mean that less accessible carbohydrates tend to support higher lignolytic activity than do easily metabolized carbohydrates. Recalcitrant carbohydrates like Avicel may, due to their slow rate of hydrolysis, lead to a fairly constant but low concentration of "free" or "available" carbohydrate. Such a condition may compel the organism to utilize lignin more efficiently. This

*PSC was prepared by the method described by Walseth (1952).

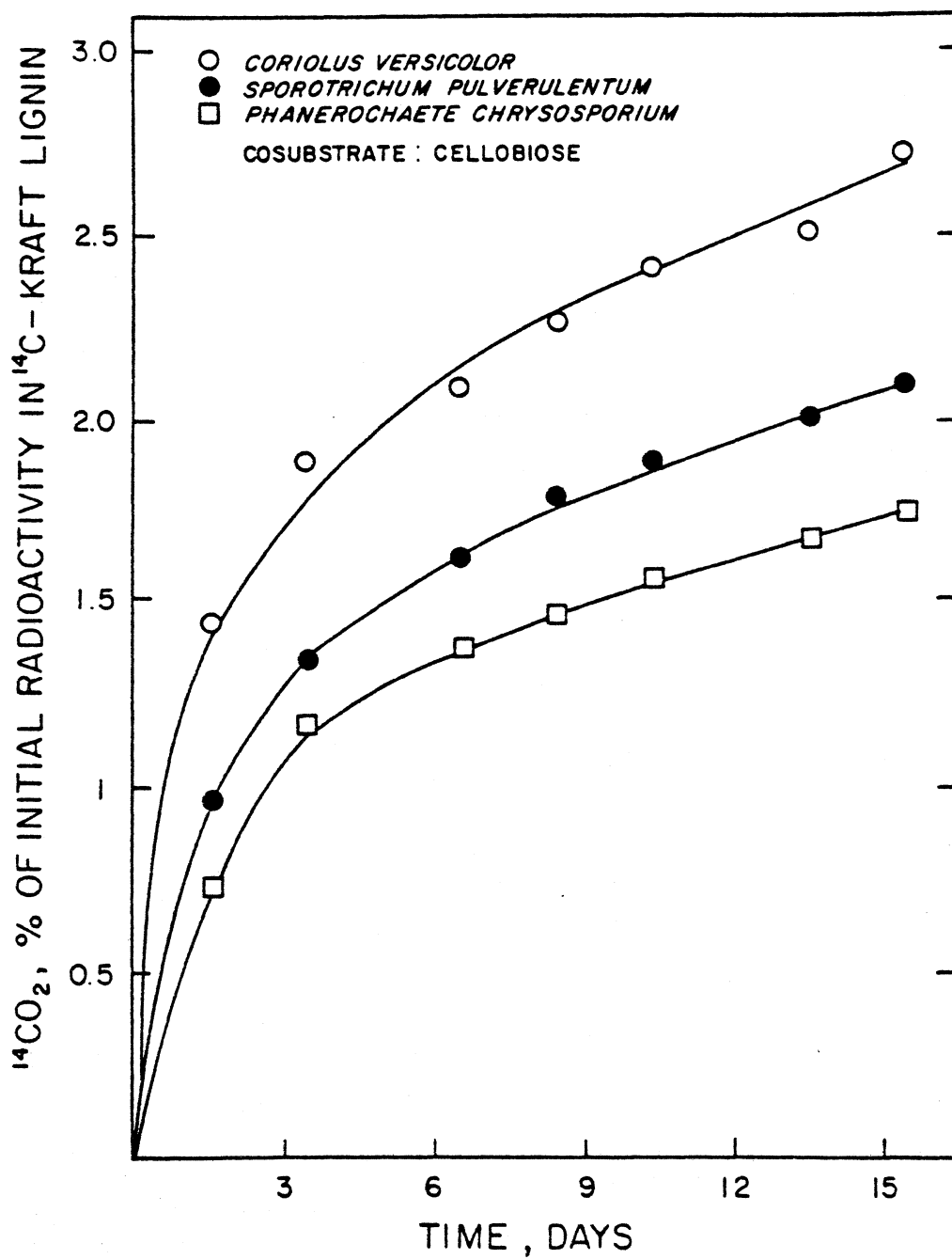


Figure 14. Secondary screening of lignin-degrading fungi with cellobiose as a cosubstrate.

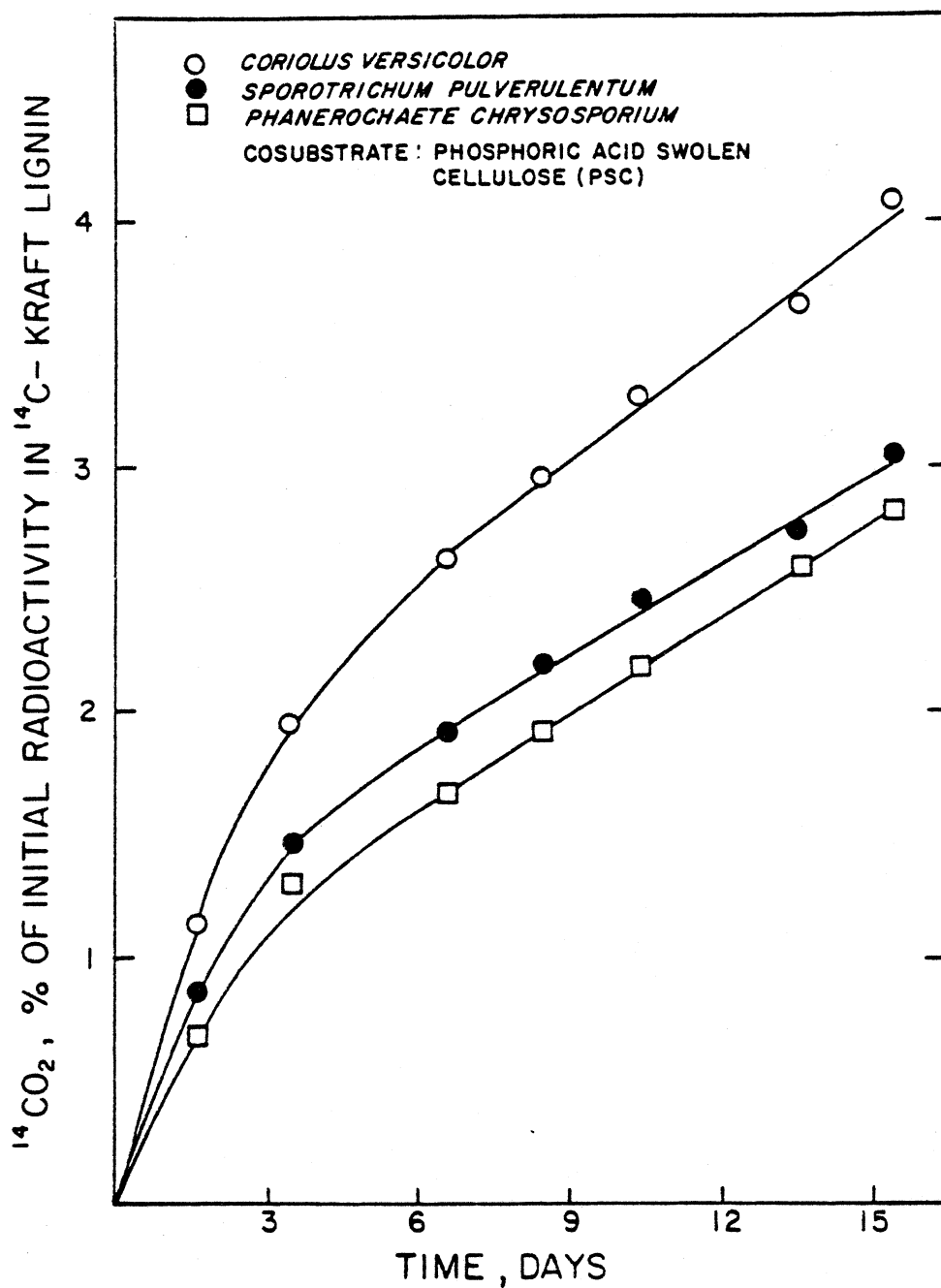


Figure 15. Secondary screening of lignin-degrading fungi with PSC as a cosubstrate.

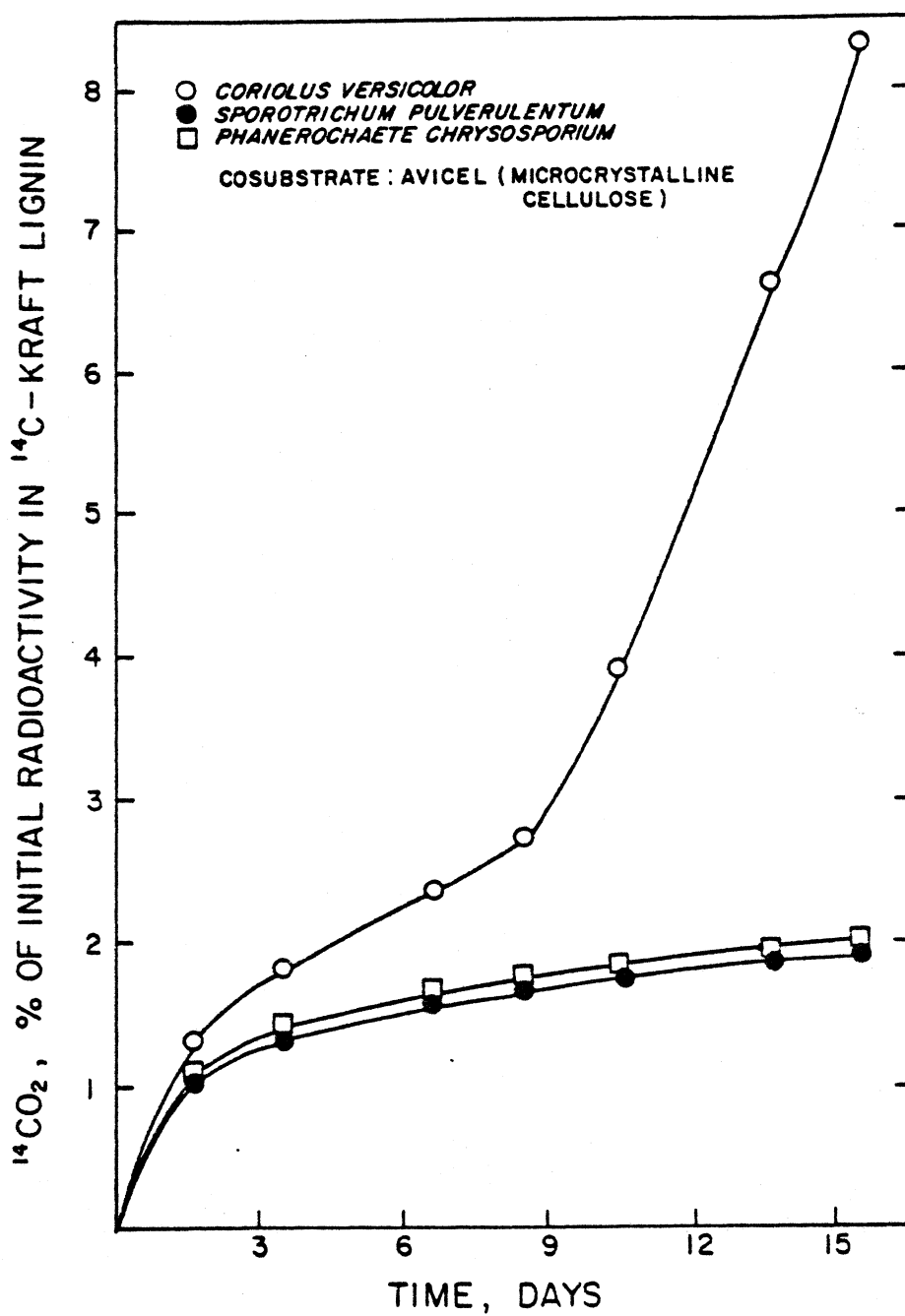


Figure 16. Secondary screening of lignin-degrading fungi with Avicel as a cosubstrate.

argument implies that lignin metabolism is controlled by carbohydrates via catabolite repression.

In all the flasks containing Avicel or PSC, the dialysis bags were disintegrated after about 7 days. This must have been due to the action of cellulases on the dialysis bag material, cellulose acetate. In the case of S. pulverulentum and P. chrysosporium, the dialysis bags were only ruptured, but the damage to the bags was severe in the presence of C. versicolor. In C. versicolor cultures, the dialysis bags suffered a relatively moderate damage when PSC was the cosubstrate, whereas they were completely shredded when Avicel was the cosubstrate. For all three cultures, the dialysis bags in flasks containing cellobiose were intact as was the case during the primary screening. A corollary to above observations is that the three white-rot fungi produce no or little cellulases constitutively.

When growing in a medium containing Avicel, C. versicolor showed a sudden jump in the rate of $^{14}\text{CO}_2$ evolution shortly after the breakage of the dialysis bag was visually ascertained. No abrupt change in the rate of $^{14}\text{CO}_2$ evolution was observed for any other case. Since the flask containing C. versicolor and Avicel was the only flask to exhibit both a complete breakdown of the dialysis bag and a sudden rise in $^{14}\text{CO}_2$ evolution rate, these two events might be related. The sudden change in $^{14}\text{CO}_2$ evolution rate, which is reminiscent of the change in $^{14}\text{CO}_2$ evolution rate encountered when supplemental cellobiose was added during the primary screening, may be due to the metabolism of cellulose acetate or events related to the breakage. It is worth mentioning that the rates and levels of

$^{14}\text{CO}_2$ evolution by C. versicolor in the presence of Avicel and PSC are comparable for the first 7-8 days of incubation as can be seen in Fig. 17. No out of the ordinary results were obtained when cellulose acetate, the material of dialysis bags, was used as the carbohydrate source (Fig. 38).

C. Effect of Carbohydrates on Lignin Metabolism

Kirk et al. (1976) demonstrated that there is an absolute requirement for a growth substrate during lignin fermentations by C. versicolor and P. chrysosporium. Carbohydrates can serve as growth substrates during lignin biodegradation. The above requirement may be common even to other lignin-degrading fungi and bacteria. The reason for the absolute requirement for a carbohydrate cosubstrate may be that the energy recovered from lignin metabolism is not sufficient to support growth. The conclusions of Kirk et al. (1976) are compatible with the natural situation where lignin resides in the form of lignocellulosics and probably the white-rot and other wood-decaying fungi have evolved to break down lignocellulosics as a whole. Hence, it is not surprising that lignin-degrading fungi cannot grow on lignin as the only carbon source. The obligatory requirement for a carbohydrate cosubstrate during lignin fermentation has considerable bearing on the economics of lignin bioprocessing.

As previously noted, both the addition of supplemental cellobiose and the breakdown of dialysis bags seemed to have an apparent effect on lignin metabolism. In the secondary screening, the type of

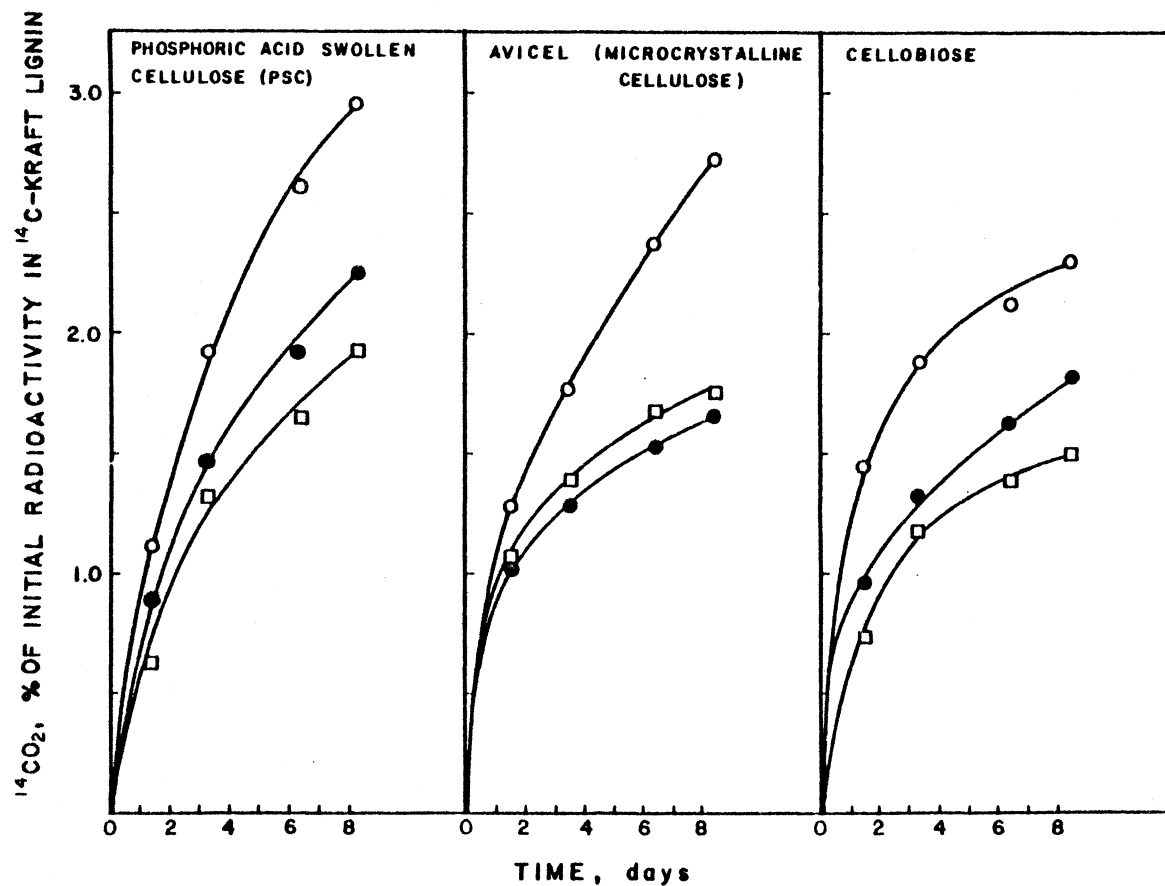


Figure 17. White-rot fermentations during secondary screening before the breakage of dialysis bags.

C. versicolor,

S. pulverulentum,

P. chrysosporium.

carbohydrate used significantly affected lignolytic activity. The importance of carbohydrates was further confirmed by studying the effect of six carbohydrates on lignin metabolism by C. versicolor.

Sigmacell, which is a microcrystalline cellulose powder like Avicel, was chosen as a positive control since crystalline carbohydrates seemed to support relatively high lignolytic activities. Soluble starch was included in the experiment since it is also a glucose polymer but has different types of linkages. It also is in between Avicel and cellobiose in terms of the ease of metabolism. Solka Floc, which is also a kind of cellulose but less crystalline than Avicel, was also included in the list of carbohydrates along with Avicel, PSC and cellobiose.

C. versicolor was grown on ^{14}C -labeled lignin and different carbohydrate cosubstrates, the initial concentration of each carbohydrate being 1% (w/v). Sigmacell supported the highest lignolytic activity and Avicel was a close second (Fig. 18). Solka Floc (not shown) supported lower lignolytic activity than Sigmacell or Avicel. Cellobiose supported the lowest lignolytic activity but there was no statistically significant difference between lignolytic activities in the presence of PSC and cellobiose. Soluble starch, which is in between Sigmacell and cellobiose in terms of digestibility, supported lignolytic activity which was about halfway between Sigmacell and Cellobiose. Though differences among lignolytic activities encountered in the presence of various carbohydrates are not overwhelming, a trend is apparent. The more recalcitrant the carbohydrate the higher is its

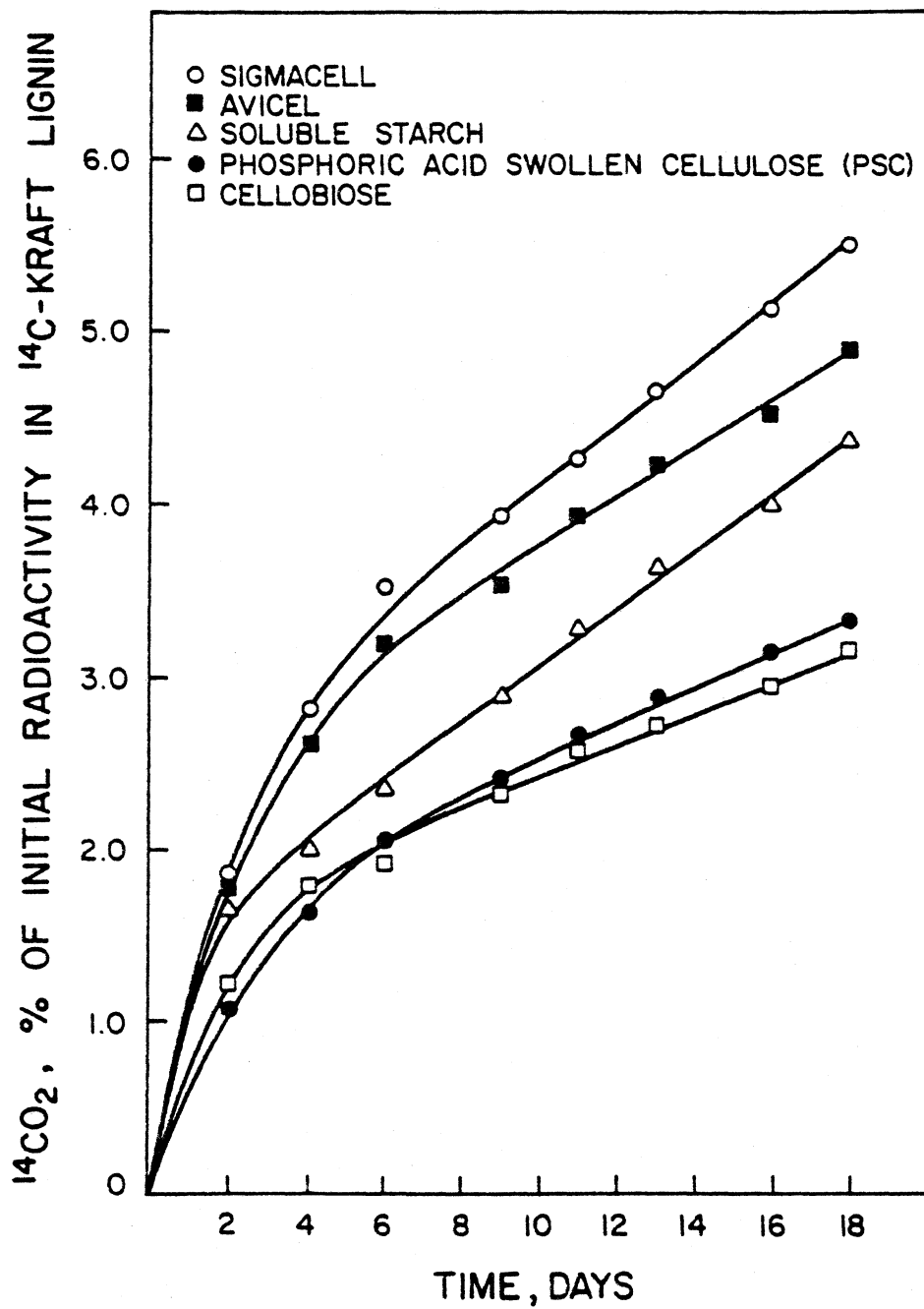


Figure 18. Effect of carbohydrates on lignin metabolism by *C. versicolor*. I.

ability to support oignolytic activity. The implication of catabolite repression is addressed later.

D. $^{14}\text{CO}_2$ vs. Time Curves

The shape of $^{14}\text{CO}_2$ vs. time curve is reminiscent of a first-order chemical reaction where the rate of product formation is initially high due to a high reactant concentration but drops to a much lower value as the reactant concentration, and hence the driving force, decreases. This explanation would be perfectly valid if lignin and CO_2 were the only substrate and product, respectively, and the lignin to CO_2 conversion was controlled by a rate-limiting reaction of the first order. One would intuitively expect to see a relatively high rate of $^{14}\text{CO}_2$ evolution during the latter part of the fermentation when the carbohydrate concentration has dropped to a low value. However, the observed shape of $^{14}\text{CO}_2$ vs. time curves shows a high initial $^{14}\text{CO}_2$ evolution rate and a low $^{14}\text{CO}_2$ evolution rate later during the fermentation. Kirk et al. (1976) also see a similar $^{14}\text{CO}_2$ vs. time curve but there is a lag in $^{14}\text{CO}_2$ evolution since they used a spore inoculum. My results do not show a lag in $^{14}\text{CO}_2$ evolution since I used vegetative inocula.

If lignolytic enzymes are inducible then $^{14}\text{CO}_2$ evolution rate would be expected to be low initially but high later in the fermentation as the system becomes fully induced. Recently Keyser et al. (1978) claimed that, at least for P. chrysosporium, the lignolytic activity appears in the absence of lignin or lignin derivatives and in response to nitrogen starvation, the rate and extent of $^{14}\text{CO}_2$

evolution from ^{14}C -labeled DHP not being altered by the addition of lignin. The above observation connotes that lignolytic enzymes are not inducible for the case of *P. chrysosporium*. This implication may apply to other white-rot fungi as well. Thus, the ever decreasing rate of $^{14}\text{CO}_2$ evolution is not incompatible with the notion that lignolytic enzymes are not inducible.

The possibility of aerosols from the flasks carrying radioactivity to the traps (Fig. 8) and giving erroneously high initial $^{14}\text{CO}_2$ evolution rates was investigated by flushing the flasks right after inoculation and measuring the resultant radioactivity. Flasks were shaken vigorously and flushed immediately. The radioactivity from such flushings was close to the background reading, thus proving that the high initial rate of $^{14}\text{CO}_2$ evolution was not an artifact of aerosols carrying radioactivity.

Monooxygenases are some of the first enzymes involved in the metabolism of simple phenolic compounds as they prepare the aromatic rings for cleavage by dioxygenases. Kirk (1975) has stated that the cleavage of aromatic rings in lignin would also involve an initial action by monooxygenases followed by the actual cleavage by dioxygenases. Monooxygenases and dioxygenases involved in lignin degradation are probably extracellular since, in wood, lignin is physically "fastened" by interpenetration to the polysaccharides (Kirk, 1975). Alternatively, these enzymes may not be extracellular but may be bound to the cell membrane in such a way as to allow a high enzyme concentration just outside the cell wall, the enzyme concentration

dropping exponentially as we go away from the cell wall (Fig. 19). A hypha-lignin contact will be essential in such a case to facilitate the action of aforementioned oxygenases.

If cell-lignin contact is imperative, as hypothesized above, then it is possible to explain the shape of $^{14}\text{CO}_2$ vs. time curves. In the early part of the fermentation, the carbohydrate concentration is high and the cell growth rate is also relatively high. Thus a large cell surface area facilitates better cell-lignin contact and faster lignin degradation. In other words, lignin metabolism is limited only by the "controlling chemical reaction". In the latter part of the fermentation, the carbohydrate concentration is low and the cell growth rate is substantially lower than that in the initial phase. Hence, the generation of new surface area is very slow and both the chemical reaction and the cell surface availability control lignin metabolism giving rise to a lower rate of lignin metabolism than that seen in the early part of the fermentation. The rate of lignin metabolism can be postulated to be a function of rates of cell growth and controlling chemical reaction:

Rate of lignin metabolism

$$= f(\text{cell growth rate}) \times g(\text{controlling reaction rate}).$$

E. Cell-Lignin Interactive System

If we look at a lignin fermentation from the vantage point of a chemical engineer, we visualize it to be a heterogeneous system

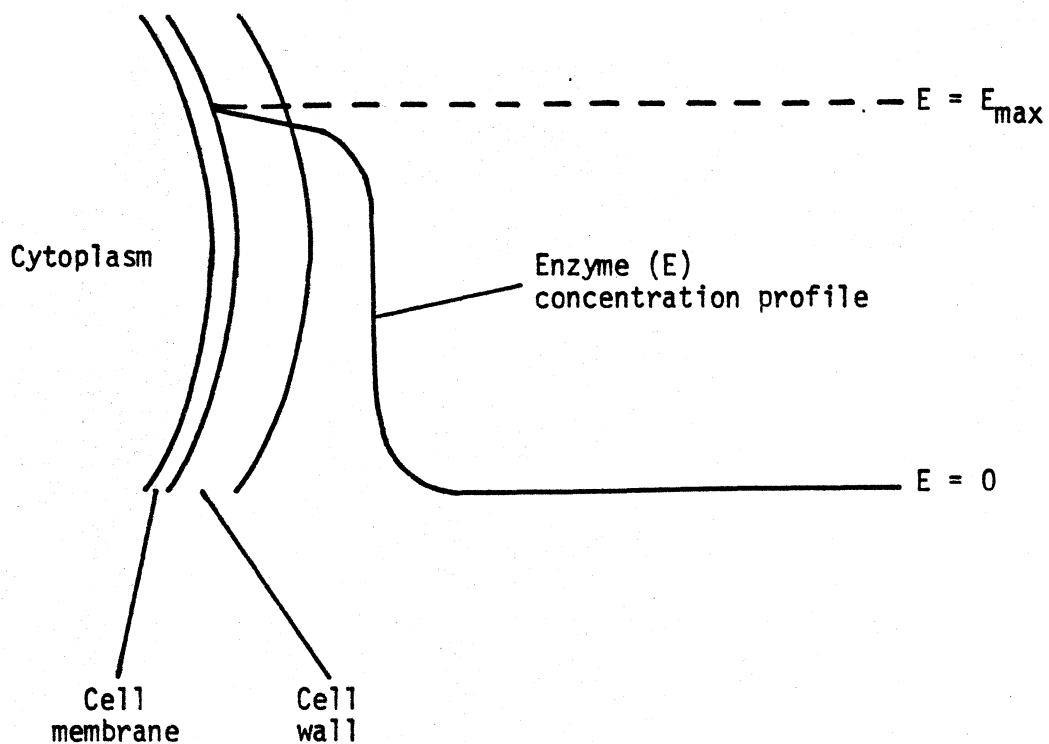


Figure 19. Hypothetical concentration profile of a membrane-bound enzyme involved in the rate-limiting step of lignin metabolism.

where both mass transfer and chemical reaction are key factors. If cell-lignin contact is vital and the controlling chemical reaction does take place on the cell surface, it is possible to speculate on the general kinetics of a typical lignin fermentation.

1. Chemical Reaction with Mass Transfer: Lignin molecules will have to diffuse through the bulk and the boundary layer around the cell in order to reach the site of action on the cell surface. The controlling reaction that takes place on the cell surface may be assumed to be, for the sake of simplicity, of a first order. Kirk (1975) has postulated that the rate limiting step in lignin metabolism may be the action of monooxygenase. Though there is no hard proof to claim that monooxygenases drive the controlling step in lignin, it can be conjectured so for the purpose of illustration.

Upon equating interphase mass transport to surface reaction we get,

$$k_g a (C - C_s) = R = k C_s$$

where a : surface area available for reaction

k_g : mass transfer coefficient

k : rate constant of the controlling reaction

R : effective reaction rate

C : bulk concentration of lignin or lignin derivative
involved in the controlling reaction

C_s : surface concentration of lignin or lignin derivative
involved in the controlling reaction

From the above equation we get,

$$C_s = \frac{C}{1 + \frac{k}{k_g a}} = \frac{C}{1 + Da}$$

Thus $R = kC_s = kC (1 + Da)$

where $Da = \text{Damoköhler number}$

$$= \frac{\text{chemical reaction rate}}{\text{mass transfer rate}}$$

When mass transfer is more rapid than chemical reaction, $Da \rightarrow 0$ and $R \approx kC$ and the system behaves as though it were homogeneous. Conversely, when the surface reaction is faster than the rate of reactant supply by diffusive mass transport, Da is large and $R \approx k_g aC$. In the latter case the effective rate is truly heterogeneous, depending as it does on the external surface area.

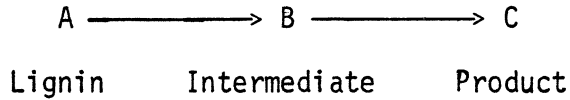
A typical fermentation with ^{14}C -labeled lignin can be divided into two phases, phase I which represents the early part of the fermentation with a high $^{14}\text{CO}_2$ evolution rate and phase II which represents the latter part of the fermentation with a relatively low $^{14}\text{CO}_2$ evolution rate. The rate of reactant transfer to the cell surface during phase I can be expected to be much higher than the controlling reaction rate. Hence, $R_I = kC_I$. The mass transfer rate during phase II will be much lower due to a decreased driving force and comparable to the chemical reaction rate. Thus,

$$R_{II} = kC_{II}/(1 + Da).$$

$$\text{Also } R_I/R_{II} = C_I(1 + Da)/C_{II} \\ > 1 \text{ since } Da > 0 \text{ and } C_I > C_{II}$$

Thus, the rate of reaction is higher during phase I than during phase II.

2. Yield of Intermediates: If lignin catalysis can be represented by following consecutive reactions, it is possible to predict the yield of intermediate products,



For catalysis over a cell surface we can equate the steady-state mass transport to surface reaction, assuming equality of liquid phase mass transfer coefficients for each of the transported species, i.e.,

$$(k_L a)_A = (k_L a)_B = (k_L a)_C$$

$$\text{For } A: k_L a (A - A_s) = k_1 A_s$$

$$\text{For } B: k_L a (B - B_s) = k_1 A_s - k_2 B_s$$

$$\text{For } C: k_L a (C_s - C) = k_2 B_s$$

The suffix s denotes surface concentrations of species and Da_1 , Da_2 and Da_3 are Damköhler numbers for A, B and C, respectively.

After some algebra we get,

$$A_s = A_0/(1 + Da)$$

$$\text{and } B_s = Da_1 A_0 / (1 + Da_1) (1 + Da_2) + B_0 / (1 + Da_2)$$

A_0 and B_0 in above equations represent the initial concentrations of A and B, respectively.

$$Da_1 = k_1/k_{\ell} a \text{ and } Da_2 = k_2/k_{\ell} a$$

The time constants of the system are as follows:

$$\tau_{R1} = 1/k_1, \tau_{R2} = 1/k_2 \quad \text{time constants for chemical events}$$

$$\tau_D = 1/k_{\ell} a \quad \text{time constant for diffusive events}$$

$$\text{Hence, } Da_1 = \tau_D / \tau_{R1} \text{ and } Da_2 = \tau_D / \tau_{R2}$$

Hence the yield of B is,

$$Y_B = - \frac{dB}{dA} = 1 - \frac{Da_1}{K(1 + Da_2)} - \frac{B(1 + Da_1)}{KA(1 + Da_2)}$$

where

$$K = k_1/k_2 = Da_1/Da_2$$

or

$$Y_B = \frac{1}{1 + Da_2} - \frac{B(1 + Da_1)}{KA(1 + Da_2)}$$

When the intermediate is not present at time = 0, $B_0 = 0$

and

$$- \frac{dB}{dA} = 1/(1 - Da_2) = Y_B$$

Thus,

$$Y_B \equiv \tau_{R2} / \tau_D$$

The yield of the intermediate then depends on the surface reaction time for its conversion to C, relative to the diffusive time for its escape from the catalytic surface. In diffusion-affected heterogeneous reactions, intermediate overexposure upon the catalytic surface due to an escape limitation invites overreaction to the ultimate product (Carberry, 1976, pp. 212-213). This may be one possible way of explaining the lack of accumulation of intermediates during lignin catalysis.

F. Kinetic Model for Lignin Catalysis

The premiere premise in developing the following kinetic model is that cell-lignin contact is mandatory for catalysis. The limiting assumptions necessary for modeling lignin catalysis are enumerated as follows:

- i) Lignin molecules adsorb to cell surface before reacting.
- ii) The controlling chemical reaction in lignin metabolism takes place on the cell surface.
- iii) Total metabolic effect

$$= \sum_{i=1}^n \left[\begin{array}{c} \text{metabolic effect of } i^{\text{th}} \text{ cell} \\ \text{per unit mass} \end{array} \right]_i \left[\begin{array}{c} \text{mass of} \\ i^{\text{th}} \text{ cell} \end{array} \right]_i$$

Where, n is the total number of cells at a given time. This assumption implies that cells are noninteractive which is, however, not true in practice.

- iv) The cell surface area is a linear function of cell mass.

1. Development of the Model: In such a model for heterogeneous catalysis, it is understood that rate phenomena of an exclusively chemical nature are under consideration, i.e., chemisorption, desorption or surface chemical reaction. Excluded by definition are all limiting physical-transport steps such as mass transfer of species from the bulk-fluid phase to the external surface of the catalyst. If monooxygenases are the enzymes responsible for the rate-limiting step in lignin catalysis and if these enzymes operate in a fashion akin to that depicted in Fig. 19, then the controlling reaction alluded to in assumption ii) may be the reaction between monooxygenases and lignin occurring on the cell surface.

S_0 , which is the concentration of total catalytic centers or sites, is a constant in the traditional gas-solid heterogeneous catalysis. However, in the present case, the concentration of total catalytic sites is variable since the cell mass and hence the total cell surface area changes during the fermentation. Where the total number of sites is changing with respect to time we have,

$$S_0 + \int_0^t \left(\frac{ds}{dt} \right) dt = S + S_a + S_b + S_c + \dots$$

where

S_0 = concentration of total sites at time zero

$\frac{ds}{dt}$ = rate of generation of new sites

S = concentration of unoccupied sites at time t

S_a, S_b, S_c = concentration of sites occupied by various adsorbed species

By the virtue of assumption iii),

$$\frac{ds}{dt} = m' \frac{dx}{dt}$$

where

X = cell concentration

$\frac{dx}{dt}$ = rate of cell growth

m' = proportionality constant

A typical growth curve can be mathematically represented as follows:

$$\begin{aligned} X &= X_0 & 0 \leq t \leq t_1 & \text{lag phase} \\ &= X_0 e^{\mu t} & t_1 < t \leq t_s & \text{log phase} \\ &= X e^{\mu t_s} & t \geq t_s & \text{stationary phase} \end{aligned}$$

where, μ is the specific growth rate, and t_1 and t_s are times denoting the beginning of log and stationary phases, respectively. X_0 is the initial cell concentration.

However, in fungal degradation of lignin I have repeatedly observed a linear rather than an exponential growth phase. The growth equations take the following form if the lag phase is absent and the growth phase is linear:

$$\begin{aligned} X &= X_0(1 + mt) & 0 \leq t \leq t_s & \text{linear growth phase} \\ &= X_0(1 + mt_s) & t \geq t_s & \text{stationary phase} \end{aligned}$$

where, m is a constant.

Hence, $\frac{dx}{dt} = m X_0$ growth phase

$= 0$ stationary phase

Thus, $\frac{ds}{dt} = m' m X_0$ growth phase

$$\int \left(\frac{ds}{dt} \right) dt = m m' X_0 \int dt = m S_0 t \quad 0 \leq t < t_s$$

$$= m S_0 t_s \quad t \geq t_s$$

It should be noted that $m' X_0 \equiv S_0$.

The controlling reaction is assumed to be represented by the following biomolecular reaction:



where, A: lignin or lignin derivative partaking
in the controlling step.

B: cofactor necessary to drive the controlling
reaction or supplier of energy such as a
carbohydrate which makes the reaction feasible.

C, D: products

The assumed reaction sequence is,

- i) $A + S = (AS)$ adsorption
- ii) $B + S = (BS)$ adsorption
- iii) $(AS) + (BS) = C + D + 2S$ surface reaction

If the surface reaction step is controlling and is irreversible then,

$$r = k_3(AS)(BS)$$

where, r is the rate of reaction and k_3 is the rate constant for step iii).

The other two steps are in equilibrium. Hence,

$$(AS) / AS = K_1 \quad \text{equilibrium constant for step i)}$$

$$(BS) / BS = K_2 \quad \text{equilibrium constant for step ii)}$$

Hence,

$$r = k_3 K_1 K_2 AB S^2$$

$$\begin{aligned} S_0 + S_0 mt &= S + (AS) + (BS) \\ &= S + SK_1 A + SK_2 B \end{aligned}$$

Thus,

$$S = \frac{S_0(1 + mt)}{1 + K_1 A + K_2 B}$$

and

$$r = S_0^2 k_3 K_1 K_2 \frac{AB(1 + mt)^2}{(1 + K_1 A + K_2 B)^2}$$

or

$$r = \frac{KAB(1 + mt)^2}{(1 + K_1 A + K_2 B)^2} \quad 0 \leq t \leq t_s$$

$$= \frac{KAB(1 + mt_s)^2}{(1 + K_1 A + K_2 B)^2} \quad t \geq t_s$$

where

$$K \equiv S_0^2 k_3 K_1 K_2$$

If K_1 and K_2 are assumed to be very small then K_1A and K_2B can be neglected with respect to 1 and we get,

$$r \approx KAB(1 + mt)^2$$

2. Consequences of the Model: If B represents the energy source such as a carbohydrate cosubstrate, it is evident that the initial reaction rate is going to be high since A and B are initially at their highest values. Later on during the fermentation, the carbohydrate concentration is going to drop exponentially thus more than nullifying the effect of the term, $(1 + mt)^2$. Lignin concentration is also expected to drop, if not drastically. Thus, the latter reaction rate is going to be slower than the initial rate.

Does this model predict a maximum rate of reaction during the fermentation? Let us assume, for the sake of mathematical simplicity, that dA/dt is very small compared to dB/dt and B decreases according to the equation, $B = B_0 e^{-bt}$, where B_0 is the initial concentration of B and b is a positive constant. Then, r will be at its maximum or minimum at

$$t = (2m-b)/bm, \quad 2m > b$$

The second derivative of r, d^2r/dt^2 , is negative at the above value of t since $d^2r/dt^2 = -2m^2$ when $t = (2m-b)/bm$ and m^2 is a positive number. Then r is maximum at $t = (2m-b)/bm$. It can be demonstrated that the maximum reaction rate will be observed during the early

phase of the fermentation, the exact time of occurrence depending on the relative values of m and b .

Since the model is highly speculative and most of the parameters are unknown or not easily estimated, it is difficult to apply it to the real situation. Nevertheless, it qualitatively represents the system and as more information about lignin degradation pathways becomes available, it may serve as a stepping stone.

G. Aspergillus fumigatus

During a C. versicolor fermentation a sudden clarification of the lignin medium was observed. The medium originally contained particulate lignin and was brown in color. The growth was in the form of a big clump and lignin seemed to have been trapped inside, as was confirmed after homogenizing the clump. Some flasks also showed green sporulation although the vegetative growth was white in color. Upon isolation and taxonomic evaluation*, the contaminant was found to be an Aspergillus fumigatus strain. A. fumigatus is a facultatively thermophilic fungus which is known to cause soft rot in wood and is also very common at high temperature in compost. A. fumigatus belongs to the Ascomycetes family.

* Identification was done by Dr. O. M. Miller of the Mycology Department, VPI & SU.

1. Comparison with *C. versicolor*: Since *A. fumigatus* clarified the lignin-containing medium in a day or two, it was compared with *C. versicolor* which was then the most efficient lignin degrader in my culture collection.

When grown on soluble starch and ^{14}C -labeled kraft lignin, *A. fumigatus* liberated about 38% of the initial radioactivity as $^{14}\text{CO}_2$ whereas *C. versicolor* liberated about 6% (Fig. 20). For the cases where ^{14}C -labeled natural lignin was used as a substrate, none of the organisms reported in the literature evolved more $^{14}\text{CO}_2$ than *A. fumigatus*, the comparisons being made for the same period of incubation. The relatively low $^{14}\text{CO}_2$ evolution rate during the early part of the *A. fumigatus* fermentation may be due to a relatively high proportion of spores in the inoculum. Such a relatively low $^{14}\text{CO}_2$ evolution rate was not seen in the initial stages of any other *A. fumigatus* fermentations.

2. Lignin Derived CO_2 /Total CO_2 : Since there are two carbon sources in a lignin fermentation it is imperative to distinguish between their metabolism. The weight of CO_2 evolved (in mg) from lignin can be estimated by following $^{14}\text{CO}_2$ evolved (in dpm) from ^{14}C -labeled lignin and assuming a constant specific activity of lignin (dpm/mg) throughout the fermentation. Total CO_2 , CO_2 from both lignin and carbohydrate, can be determined by a titrimetric method. Thus, CO_2 from carbohydrate is calculated by difference. The ratio

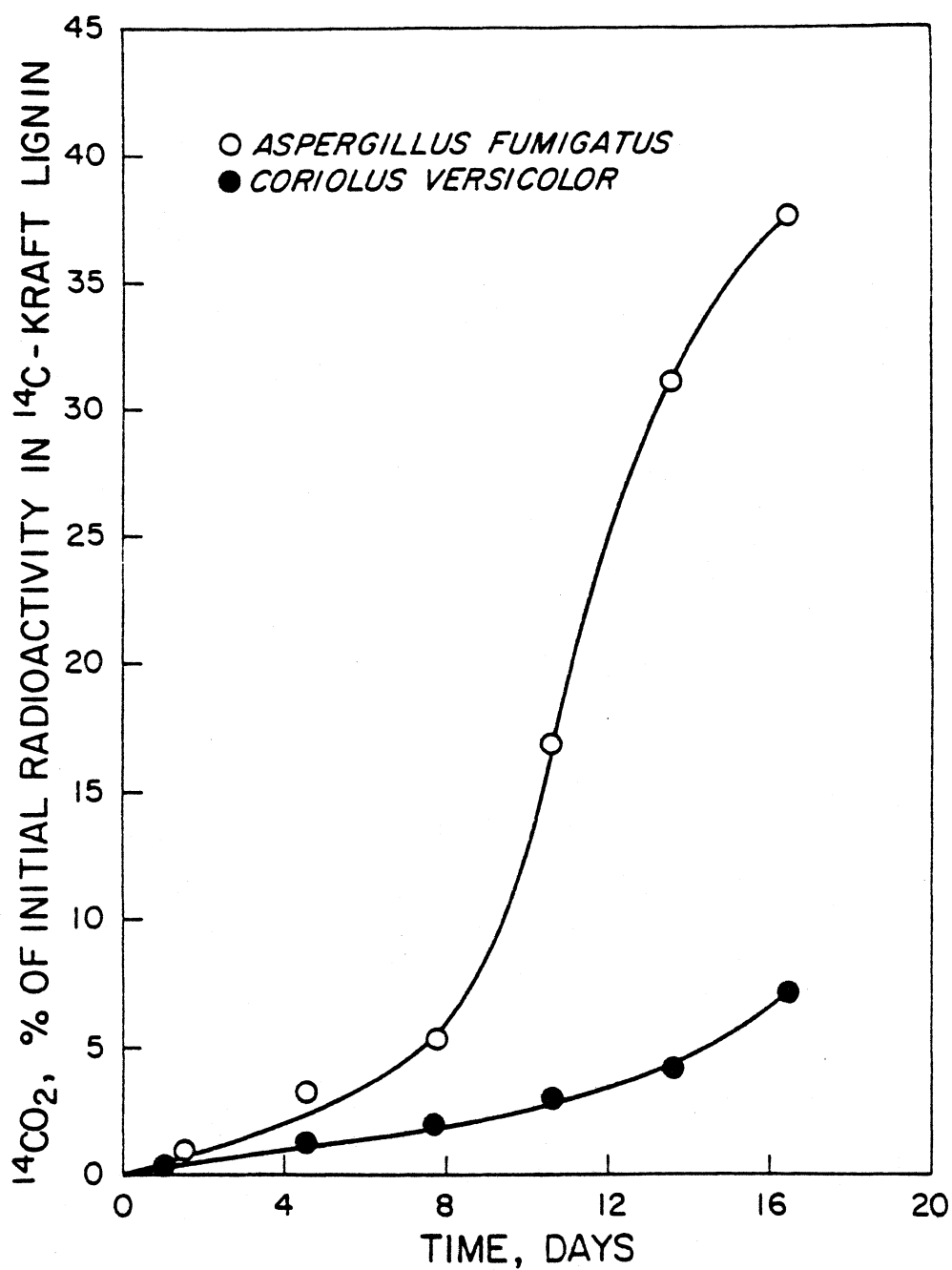


Figure 20. Lignolytic activities of *A. fumigatus* and *C. versicolor*.

of lignin derived CO_2 /total CO_2 represents the efficiency at which the organism metabolizes lignin, higher the ratio more efficient is the organism.

A. fumigatus exhibited higher ratios of lignin derived CO_2 /total CO_2 than did C. versicolor (Fig. 21). For both cultures the ratio was variable during the fermentation. This may mean that there is no stoichiometric link between lignin metabolism and carbohydrate metabolism. A. fumigatus also showed a maximum for the lignin derived CO_2 /total CO_2 vs. time curve. There is a good correlation between the rate of $^{14}\text{CO}_2$ evolution and lignin derived CO_2 /total CO_2 . Thus, as the rate of $^{14}\text{CO}_2$ evolution drops to a relatively low value so does the ratio of lignin derived CO_2 /total CO_2 . The maximum instantaneous rate of $^{14}\text{CO}_2$ evolution is seen at about 10 days into the fermentation whereas the maximum lignin derived CO_2 /total CO_2 occurs at about 12 days. This apparent discrepancy is small and can be attributed to experimental errors and the nature of the two pieces of data. $^{14}\text{CO}_2$ vs. time is a cumulative or integral plot as against lignin derived CO_2 /total CO_2 vs. time, which is a differential plot. Thus, within limitations, the rate of $^{14}\text{CO}_2$ evolution and lignin derived CO_2 /total CO_2 agree with each other well.

Both the rate of $^{14}\text{CO}_2$ evolution and lignin derived CO_2 /total CO_2 increase during the early part of the fermentation. This is, as discussed earlier, possibly due to the relatively high cell growth rate and better cell-lignin contact. In the latter part of the fermentation the ratio of lignin derived CO_2 /total CO_2 decreases

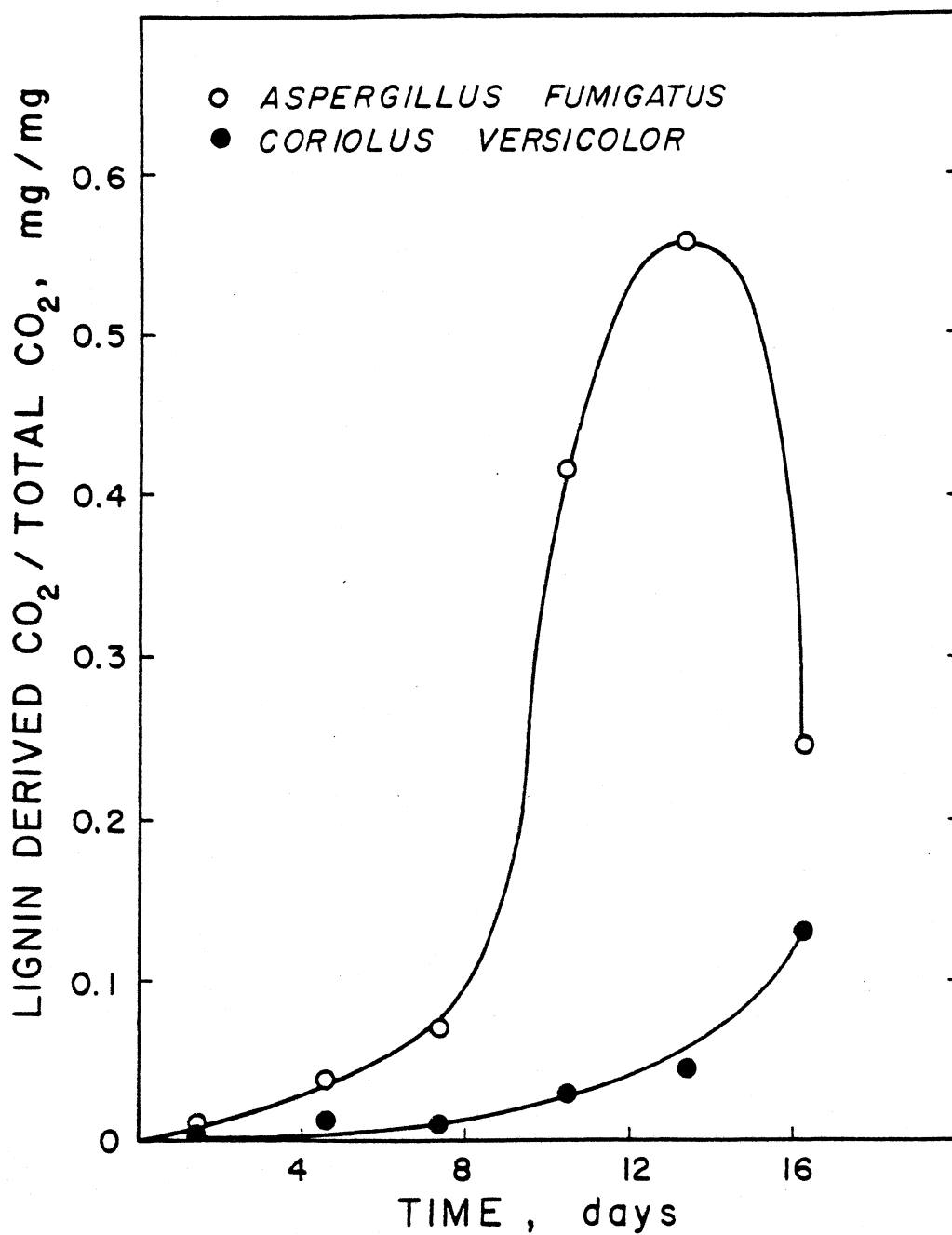


Figure 21. Lignin derived CO₂/total CO₂ ratios during A. fumigatus and C. versicolor fermentations.

since carbohydrate concentration drops to a low value, cell growth rate is low and both these factors affect lignin metabolism. The decrease in this ratio is probably accentuated by the fact that even though the rate of carbohydrate metabolism is also affected, it is not affected as much as the rate of lignin metabolism. Thus, the ratio of lignin derived CO_2 /total CO_2 goes through a maximum. This has some important process implications. Firstly, if the effectiveness of lignin metabolism and the production of lignolytic enzymes are assumed to be related to lignin derived CO_2 /total CO_2 then the batch fermentation should be harvested when this ratio is maximum. In a standardized batch fermentation, it will then be possible to predict an optimal harvest time. Secondly, it may be possible to devise a continuous or semicontinuous process where the conditions are so stipulated as to yield maximum lignin metabolism. In continuous or semicontinuous culture studies designed to arrive at such optimal conditions, the ratio of lignin derived CO_2 /total CO_2 can be used as an index of the efficiency of lignin metabolism. It might be added that the plot of lignin derived CO_2 /carbohydrate derived CO_2 vs. time should look similar to that of lignin derived CO_2 /total CO_2 since lignin derived CO_2 /carbohydrate derived $\text{CO}_2 = x/(1-x)$, where x is the ratio of lignin derived CO_2 /total CO_2 .

Figure 22 illustrates the parameters of lignin metabolism by A. fumigatus with respect to starch depletion. Starch was chosen as the carbohydrate cosubstrate since it is almost as effective as cellulose in supporting lignolytic activity and it is nearly impossible

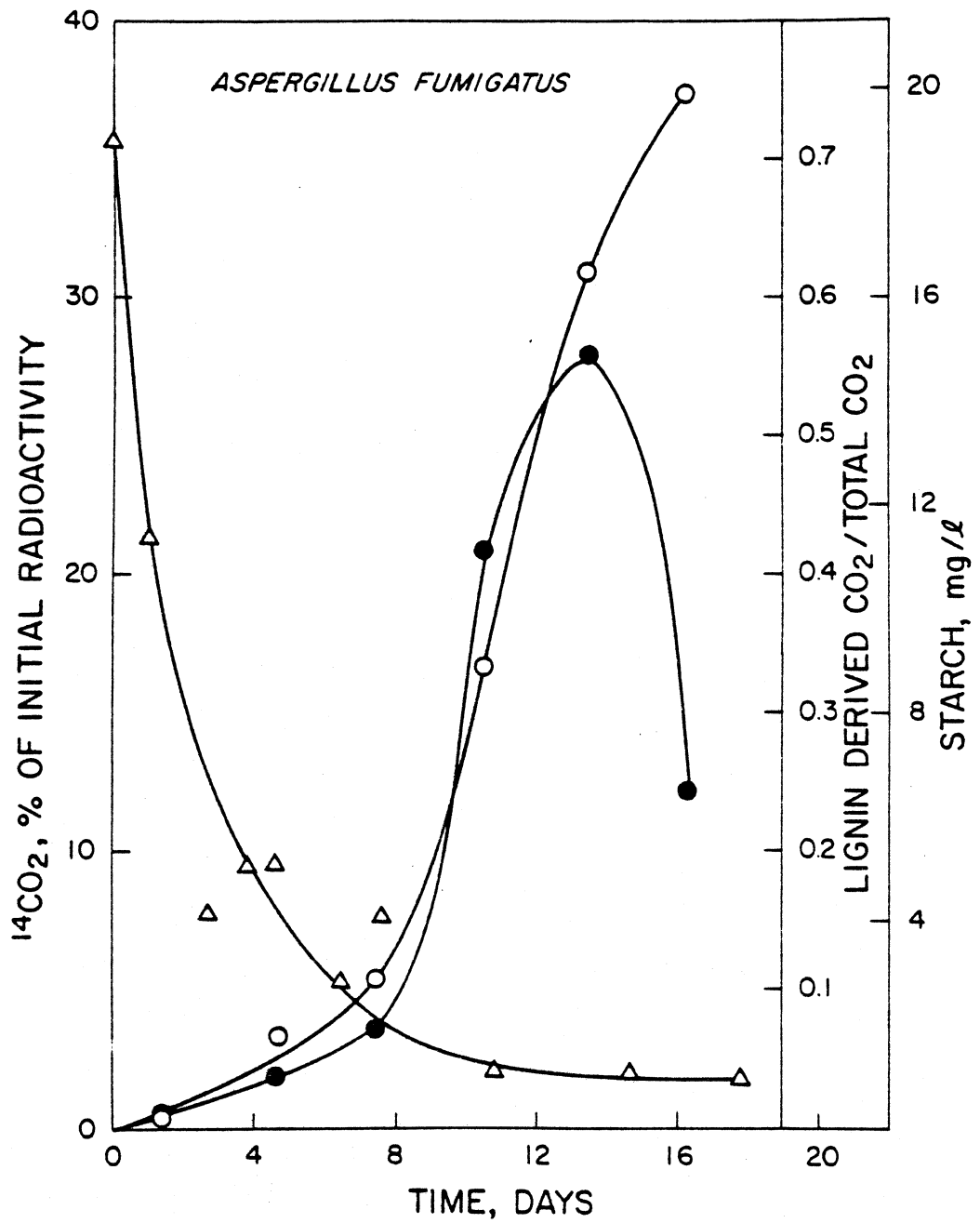


Figure 22. Lignin metabolism in relation to carbohydrate metabolism during an *A. fumigatus* fermentation.

○—○ $^{14}\text{CO}_2$, ●—● lignin derived CO_2 /total CO_2 ,
 ▲—▲ starch.

to assay cellulose. However, the iodine assay for starch posed a problem too. When assayed, the time zero sample containing a known amount of starch showed a starch concentration two orders of magnitudes lower than it should have. The possibility of losing starch either by adsorption to or retention on the filter paper was eliminated by running proper controls. Since the controls containing no cells or lignin did not show any anomaly when assayed for starch, the decrease in starch contents of the initial fermentation sample may be due to the adsorption of starch to lignin and/or cells. Thus the starch concentration vs. time plot, not being replete with absolute values, represents only a trend in starch availability during the fermentation. The apparent starch concentration drops exponentially during the first 5-6 days of incubation and levels off to a low value in the latter part of the fermentation.

It appears that both the rate of $^{14}\text{CO}_2$ evolution and the ratio of lignin derived CO_2 /total CO_2 decrease as the starch concentration drops to a low level. The reason that the highest rate of $^{14}\text{CO}_2$ evolution and lignin derived CO_2 /total CO_2 are seen at relatively low starch concentration may be partly due to the uncertainty in the iodine-starch assay. Furthermore, the starch data are instantaneous in nature whereas the $^{14}\text{CO}_2$ data and the lignin derived CO_2 /total CO_2 data are integral and differential, respectively.

H. General Process Considerations

Decisions regarding the mode of operation and the type of catalysis are crucial to the success of a future process for lignin utilization. These decisions are dictated by the kinetics of the system and nature of lignolytic enzymes.

1. Mode of Operation: A future lignin biotransformation process can be a batch, a semibatch or a continuous process. Since the kinetics of lignin fermentations is rather slow, a continuous process is unattractive. A continuous process will have to be operated at low dilution rates, especially for fungal fermentations since fungal specific growth rates are relatively small in magnitude. Thus, due to a very high residence time, such a process will approach a batch process in the limit. Hence, a batch or a semibatch, also referred to as fed-batch, mode of operation is better fitted for large-scale lignin fermentations. A semibatch fermentation is especially suited where the operating conditions call for maintaining a specific medium component at a desired level.

2. Type of Catalysis: Lignin bioprocessing can be achieved either via fermentations with viable cells or an enzymatic treatment in the absence of cells. The latter course is not a practical one since not all the lignolytic enzymes are extracellular. Drew et al. (1978) found that a concentrated preparation of extracellular enzymes from a C. versicolor lignin fermentation had almost no

capacity to degrade or transform lignin. Even when it becomes feasible to extract membrane-bound and intracellular enzymes, the in vitro lignin treatment may not be economical if costly cofactors are needed for the activity of enzymes. Thus, a traditional lignin fermentation is the preferred type of catalysis.

I. Multi-Stage Lignin Fermentations

Cell-lignin contact is apparently essential for effective lignin degradation. Drew et al. (1978) found that the cell-associated lignin fraction had undergone substantial structural modification compared to other fractions. A large amount of inoculum consisting of very small individual cells should enhance lignin degradation by providing a substantial cell surface area and fostering effective cell-lignin contact. Thus, the first stage in the multi-stage fermentation was conceived to be a growth stage. Glucose was used as the sole carbon source to yield a high-density biomass during the growth stage. A high level of agitation, 600-700 rpm, was employed to keep the mycelial size minimal without causing any shear damage. The mycelia, when examined under the microscope, showed no apparent cellular damage.

At the time of the conception of multi-stage fermentations, lignolytic enzymes were thought to be inducible. This was a perfectly logical assumption since it is discordant with the economics of cellular operation to constitutively produce an array of enzymes

that are needed to degrade a refractory material like lignin. Cellulose, which is more abundant and less intractable than lignin, is broken down by cellulases which are predominantly inducible and not constitutive. Thus, the second stage of the multi-stage fermentation was an induction stage where the biomass from the growth stage was exposed to possible inducers of lignolytic enzymes. However, Keyser et al. (1978) have recently claimed that, at least for P. chrysosporium, lignolytic activity appears in the absence of lignin and lignin-related compounds and in response to nitrogen starvation. They also reported that addition of lignin did not cause an increase in lignolytic activity. This means that lignolytic activity by P. chrysosporium is not inducible but rather is de-repressed by nitrogen starvation. If this claim is confirmed and applies to A. fumigatus as well, the inclusion of an induction stage was, in retrospect, unnecessary.

The third and final stage of the fermentation was a biotransformation stage where the supposedly induced biomass from the induction stage was contacted with kraft lignin.

The entire multi-stage fermentation was carried out in a 14 l New Brunswick fermenter. The three Aspergillus fermentations reported in the following are designated as AspI, AspII and AspIII.

1. AspI: Details of the fermentation are as follows:

Growth Stage:

C Source: glucose, 5% (w/v)

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other components: various salts

Duration: 6 days

Cells were allowed to settle at the end of this stage and the supernatant was drained off.

Induction Stage:

C Source: soluble starch, 1% (w/v)

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other components: various salts

Inducers: vanillin and vanillyl alcohol

Duration: 2.5 days

At the end of this stage cells were allowed to settle and the supernatant was drained off.

Biotransformation Stage:

C Source: Lignin, 0.1% (w/v) and soluble starch,
1% (w/v)

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other components: various salts

Duration: 5 days

The following conditions were maintained throughout the three stages:

operating volume = 8 l

temperature = 28 C

and

pH = 5.0.

Due to the very high biomass present during the fermentation, no representative samples could be collected and no kinetic data could be gathered. A vanilla-like aroma could be sensed during the early part of the induction stage, which was probably due to the metabolism of vanillin and vanillyl alcohol. The fermentation medium lost its brown color within the first two days of the biotransformation stage. A rather heavy sporulation was observed during the last two days of the biotransformation stage. This might have been due to carbohydrate limitation though actual starch concentrations could not be ascertained due to sampling difficulties.

The fermentation was harvested as described in Materials and Methods. The enzyme concentrate contained 174.5 mg protein and showed a weak, phenoloxidase activity (specific phenol oxidase activity = 0.003) and no cellobiose dehydrogenase activity.* The analysis of degraded lignin, which appears later in Table 5, showed that A. fumigatus brought about considerable demethoxylation.

2. AspII: In order to gather representative kinetic data, another multi-stage fermentation was carried out. Details of the fermentation are as follows:

Growth Stage:

C Source: glucose, 5% (w/v)

*This information was obtained from Dr. P. L. Hall of the Chemistry Dept., VPI & SU.

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other components: various salts

Duration: 5.5 days

The biomass obtained was much less profuse than that in Aspl. This might have been due to an inadvertent omission of a medium component. At the end of this stage, cells were allowed to settle and the supernatant was drained off.

Induction stage:

C Source: soluble starch, 0.2% (w/v)

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other components: various salts

Inducers: vanillin and a low molecular weight
lignin fraction prepared by dialysis

Duration: 2 days

The biomass didn't increase appreciably during this stage, possibly due to a carbohydrate limitation. Cells were allowed to settle at the end of this stage and the supernatant was drained off.

Biotransformation stage:

C Source: lignin, 0.1% (w/v) and soluble starch,
1% (w/v)

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other components: various salts

Duration: 5 days

Samples were withdrawn during this stage to determine characteristics of the fermentation.

The same conditions mentioned in AspI were maintained throughout all the three stages.

Fig. 23 describes the relations among biomass, dissolved oxygen (DO) demand and starch consumption during the time course of the biotransformation stage. The biomass increased during the first two days but declined later mostly due to carbohydrate limitation. Such a rapid increase in cell mass at the expense of carbohydrate followed by an abrupt decline when the carbohydrate becomes limiting seems to be characteristic of fungal lignin-fermentations when carried out in a deep-tank fermenter. S. pulverulentum showed similar behavior when grown in a deep-tank fermenter containing lignin and cellobiose (Fig. 24).

As seen earlier in a shake-flask fermentation, the starch concentration dropped exponentially during the first two days of the biotransformation stage and leveled off to a low value. The same problem mentioned earlier was encountered in assaying starch. Hence, the starch concentration curve is only indicative of relative carbohydrate availability and does not represent absolute starch levels. The dissolved oxygen demand corresponded well with the dry cell weight. The dissolved oxygen demand could not be determined for the early part of the fermentation due to probe fouling. Decolorization of the lignin medium was seen as usual. An attempt

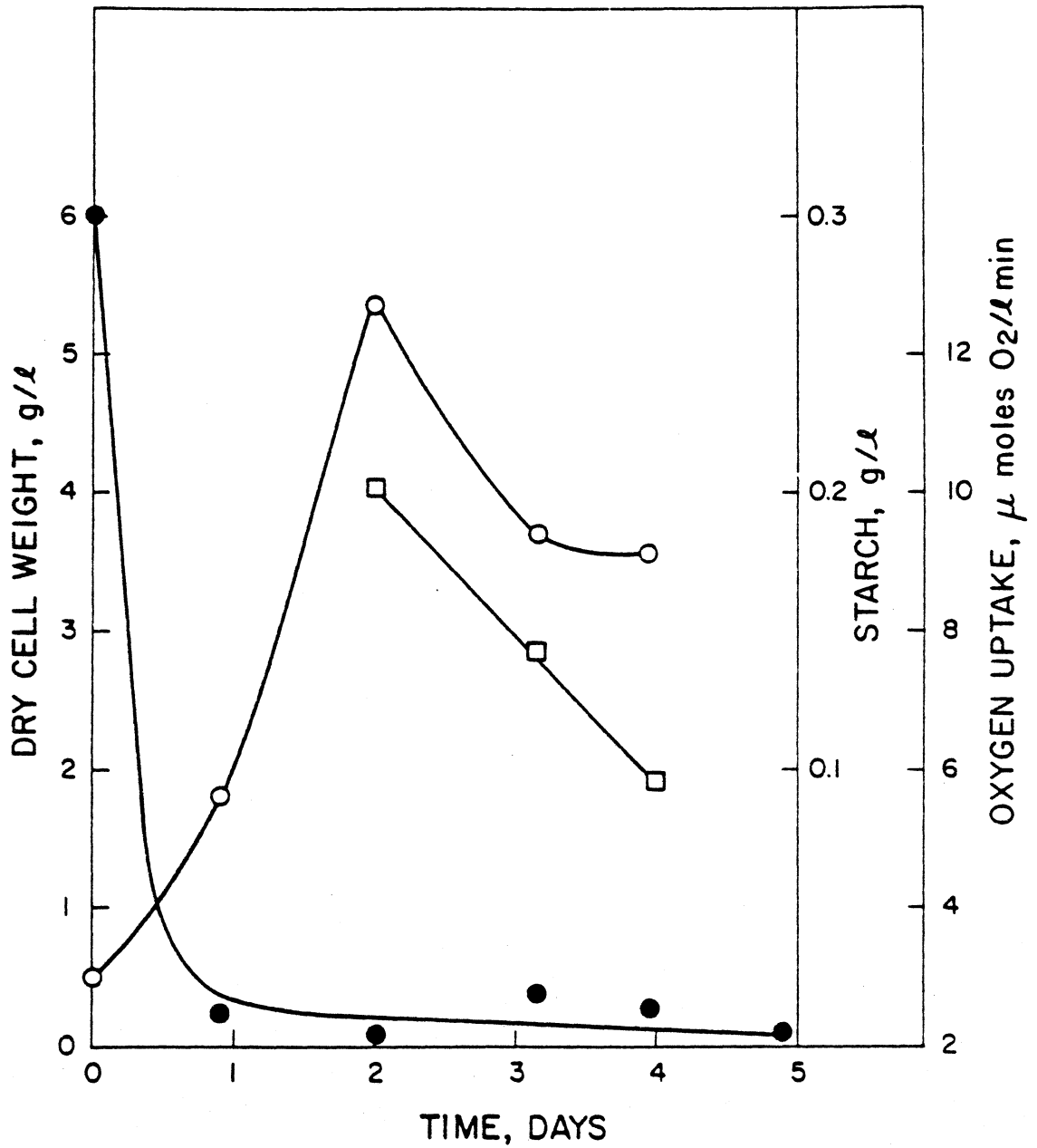


Figure 23. Multi-stage lignin fermentation by *A. fumigatus*: Biotransformation stage of AspII.

○—○ dry cell weight, ●—● starch,
 □—□ oxygen uptake.

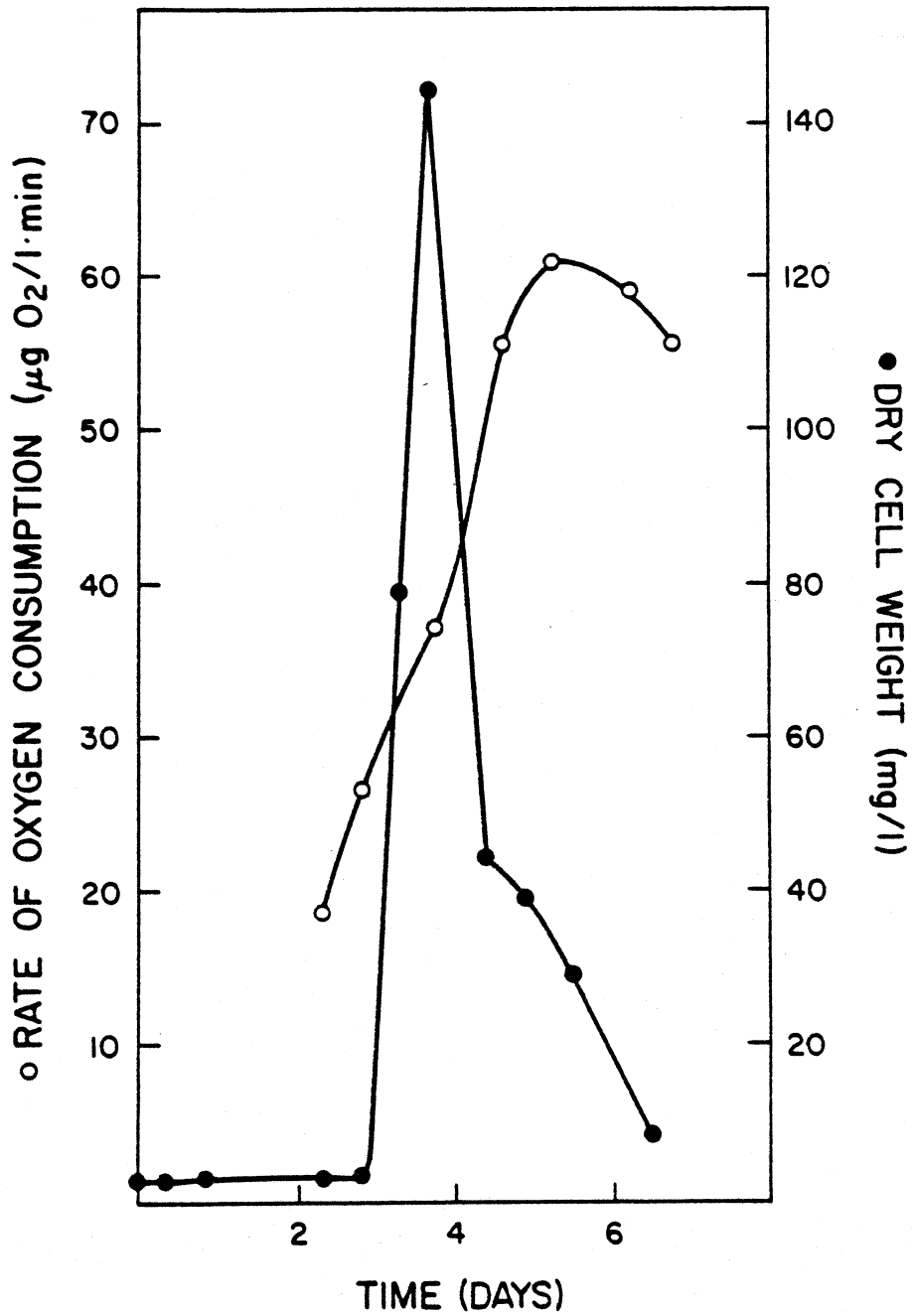


Figure 24. Lignin fermentation by *S. pulverulentum* in a deep-tank fermenter.

- rate of oxygen consumption,
- dry cell weight.

was made to follow lignin concentration by the chlorine number method, but the data obtained was inconsistent due to the interference caused by cells. Hence, lignin consumption data do not appear in Fig. 23. The analysis of degraded lignin appears in Table 5.

3. Lignolytic Efficiency: Since the chlorine number method was found unsuitable for following lignin consumption, two new parameters were defined to study a large-scale lignin-fermentation. The parameters were lignolytic efficiency and normalized lignolytic efficiency.

In order to determine these parameters, samples are withdrawn from the fermenter during the biotransformation stage and the cells from these samples are exposed to ^{14}C -labeled kraft lignin. The latter sub-fermentation is carried out in special flasks described in Fig. 8, and $^{14}\text{CO}_2$ and lignin derived CO_2 /total CO_2 are determined as a function of time for each sample. The schematic diagram of the setup for determining lignolytic efficiency is shown in Fig. 25. Definitions of lignolytic and normalized lignolytic efficiencies appear in Fig. 26.

If a sample is withdrawn from the deep-tank fermenter at x days after commencing the biotransformation stage, the performance of the fermentation at x days can be judged by determining lignolytic efficiencies. Cells from the sample taken at x days are cultivated in a special flask in the presence of ^{14}C -labeled lignin, and $^{14}\text{CO}_2$ and

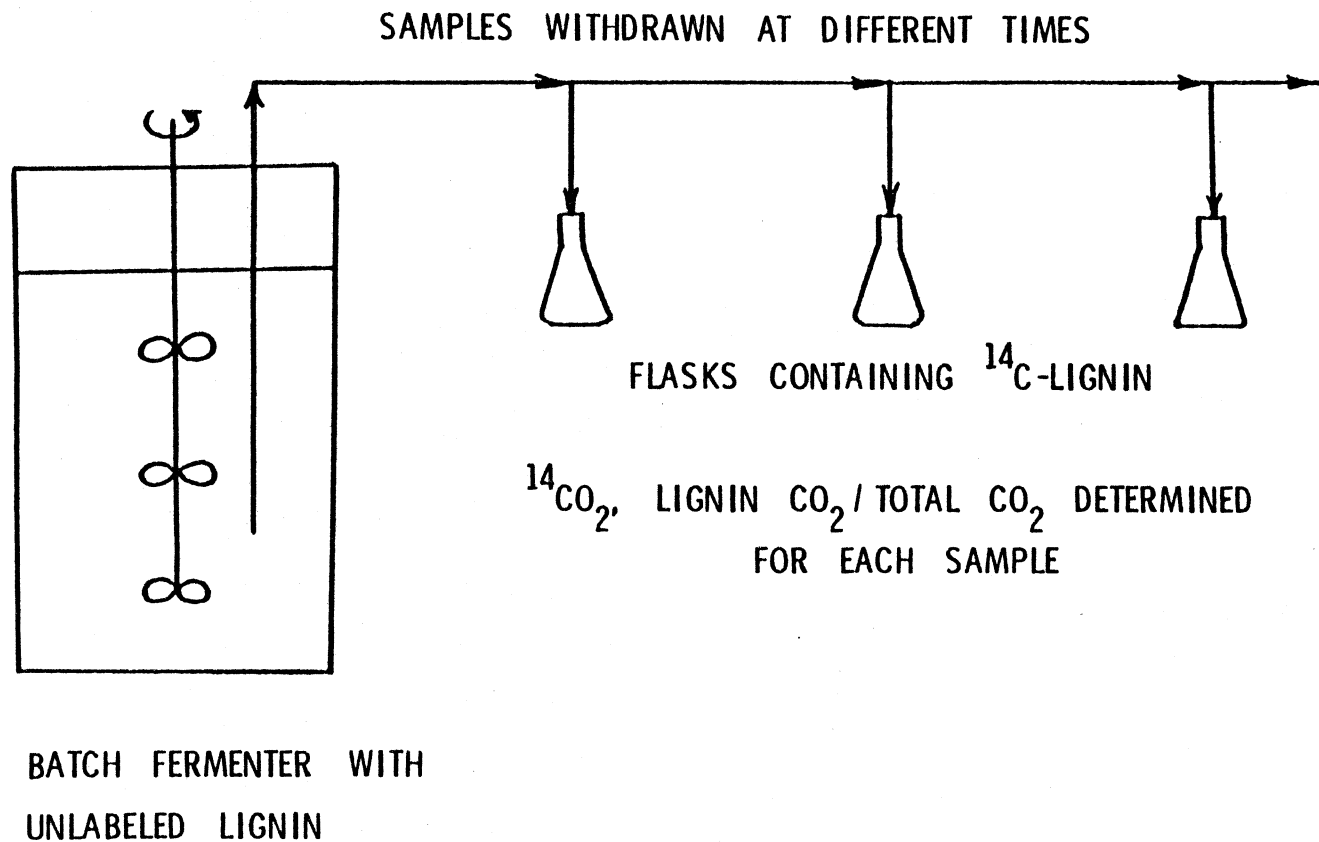
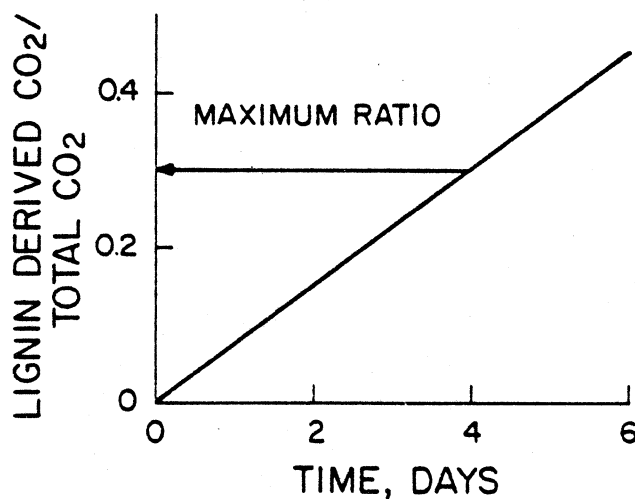
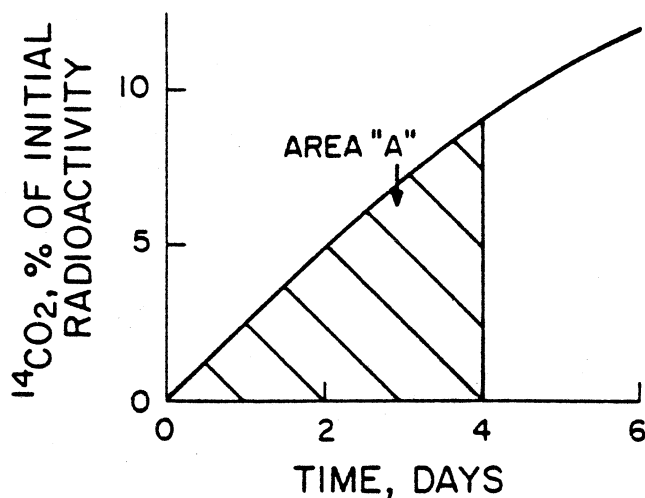


Figure 25. Schematic diagram of the setup for determining lignolytic efficiencies.



LIGNOLYTIC EFFICIENCY

$$= \left(\frac{\text{AREA "A"}}{4} \right) \times \text{MAXIMUM RATIO OF } \left(\frac{\text{CO}_2 \text{ FROM LIGNIN}}{\text{TOTAL CO}_2} \right) \times 1000$$

NORMALIZED LIGNOLYTIC EFFICIENCY

$$= \text{LIGNOLYTIC EFFICIENCY} \div \text{DCW OF INOCULUM IN g/l}$$

Figure 26. Definitions of lignolytic and normalized lignolytic efficiencies.

lignin derived CO_2 /total CO_2 are followed as a function of time. The time course of the sub-fermentation is from x days to $x + 4$ days. However, for simplicity, the time course of a typical sub-fermentation in Fig. 26 is shown to be from 0 to 4 days. At the end of $x + 4$ days, it is then possible to calculate both lignolytic and normalized lignolytic efficiencies for the sample withdrawn at x days. Lignolytic and normalized lignolytic efficiencies thus determined are characteristics of the deep-tank fermentation at x days. Since shake-flask fermentations are a little sluggish compared to deep-tank fermentations, the sub-fermentation was allowed to proceed for 4 days. Though the culture can change in 4 days, the $^{14}\text{CO}_2$ data obtained for this period of time is statistically more reliable than those obtained for a shorter period of time. Furthermore, the $^{14}\text{CO}_2$ evolution is mostly linear for the first 3-4 days and hence, the duration of sub-fermentations should not adversely affect the calculation of lignolytic efficiencies. Though the plot of lignin derived CO_2 /total CO_2 vs. time shown in Fig. 26 is a straight line, it does not necessarily have to be so. It is evident that separate sets of $^{14}\text{CO}_2$ vs. time and lignin derived CO_2 /total CO_2 vs. time plots have to be established for each sample.

Lignolytic efficiency is an index of the ability of an organism to metabolize lignin and embodies the effectiveness with which the organism carries out lignin metabolism in relation to carbohydrate metabolism. Normalized lignolytic efficiency is a specific

efficiency which takes into consideration the effect of inocula levels on the determination of lignolytic efficiency. Thus normalized lignolytic efficiency allows us to study the intrinsic lignolytic-capacity of an organism during a large-scale fermentation.

The units of lignolytic efficiency and normalized lignolytic efficiency are % of initial radioactivity and (% of initial radioactivity) 1/g, respectively.

4. AspIII: Details of the fermentation are as follows:

Growth stage:

C Source: glucose, 1% (w/v)

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other Components: various salts

Duration: 1.2 days

The yield of biomass was substantial. At the end of this stage, cells were allowed to settle and the supernatant was drained off.

Induction stage:

C Source: soluble starch, 0.2% (w/v)

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other Components: various salts

Inducers: a low molecular weight lignin fraction

Duration: 1.2 days

The biomass decreased during this stage. Cells were allowed to settle at the end of this stage and the supernatant was drained off.

Biotransformation stage:

C Source: Lignin, 0.1% (w/v) and soluble starch,
1% (w/v)

N Source: $\text{NH}_4\text{H}_2\text{PO}_4$

Other Components: various salts

Duration: 5 days

Samples were withdrawn during this stage for the determination of lignolytic efficiencies.

The following conditions were maintained throughout all the three stages:

operating volume = 8 l

temperature = 37 C

and

pH = 5.0.

Fig. 27 describes the relation between dry cell weight and lignolytic efficiencies during the biotransformation stage. As seen during earlier fermentations, the biomass increased sharply during the first 1-2 days but declined later as starch became limiting.

As expected, there was a good correlation between biomass and lignolytic efficiency. Lignolytic efficiency at 3.5 and 4.5 days is much lower than at 2.5 days though the biomass remains fairly constant between 2.5 and 4.5 days. This means that the cells older than 2.5 days are less efficient in lignin metabolism than cells with the age of or younger than 2.5 days. The cell age referred to

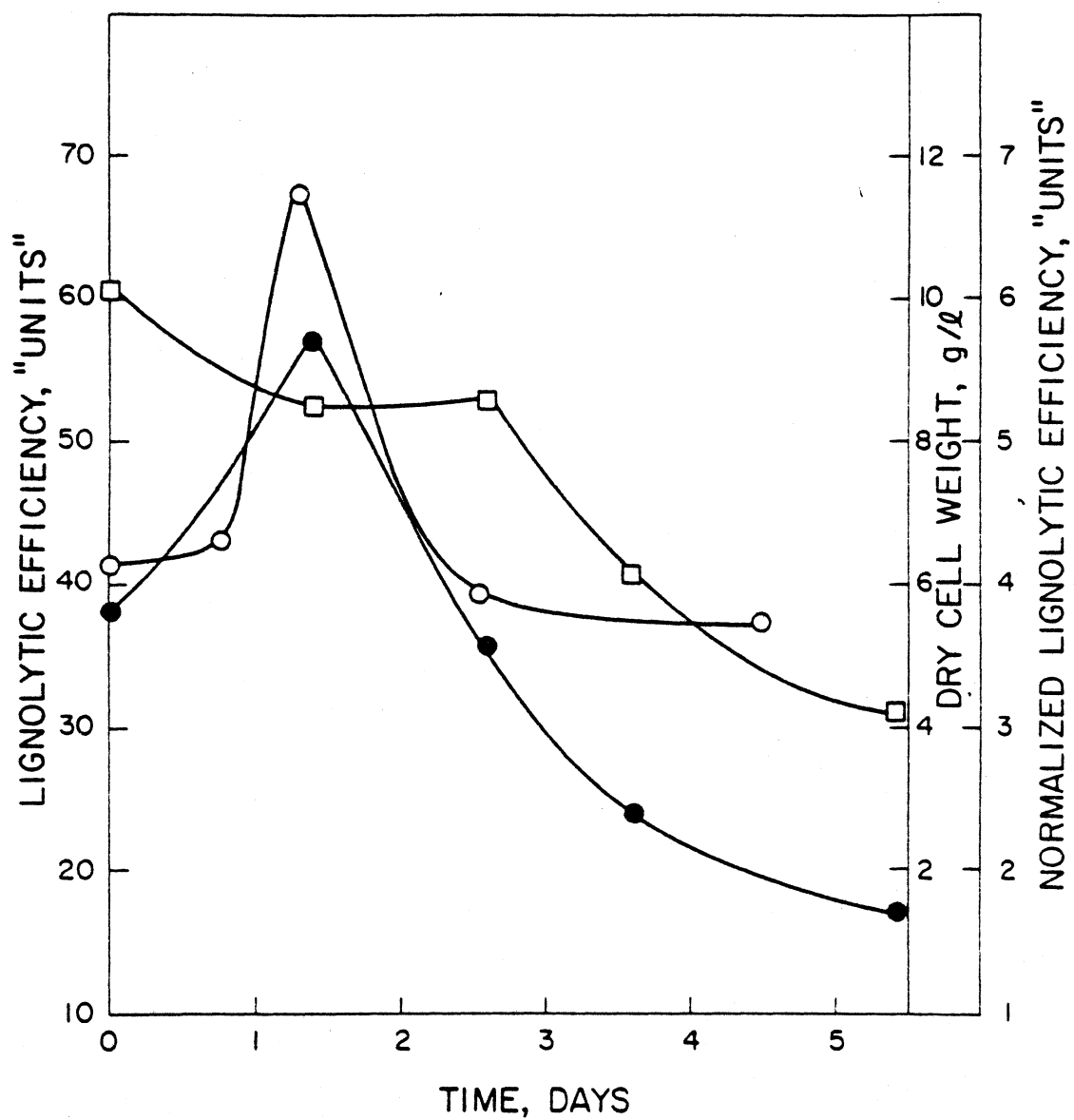


Figure 27. Lignolytic efficiencies during the biotransformation stage of AspIII.

○—○ dry cell weight, ●—● lignolytic efficiency,
 □—□ normalized lignolytic efficiency.

is measured within the time frame of the biotransformation stage. As mentioned earlier, the biomass decreases during the latter part through lysis caused by carbohydrate limitation. Thus, the cell population older than 2.5 days may be different in characteristic, having survived lysis and being exposed to an environment limited in energy supply. The older cells may have lost some of their metabolic efficiency.

Unlike lignolytic efficiency, normalized lignolytic efficiency decreased continually from time zero. Lignolytic efficiency and biomass are both higher at 1.5 days than at time zero, whereas normalized lignolytic efficiency is lower at 1.5 days than at time zero. This may be due to the relatively high inoculum (12 g dcw/l) in the flask used to determine lignolytic efficiency at 1.5 days. Such high inocula may entail a suboptimal cell-lignin contact. Thus, even though lignolytic efficiency is higher at 1.5 days than at time zero, it did not increase in proportion with the increase in biomass. This resulted in a lower normalized lignolytic activity at 1.5 days than at time zero since normalized lignolytic efficiency is directly proportional to lignolytic efficiency but inversely proportional to the dry cell weight of inocula. This continual decrease in normalized lignolytic efficiency is reminiscent of the continual decrease in the $^{14}\text{CO}_2$ evolution rate seen in shake-flask fermentations.

One of the purposes behind monitoring lignolytic efficiencies during lignin fermentations was to determine an optimal harvest time for a typical batch fermentation. When lignin fermentations operated

in the batch mode are standardized, it is possible to predict, within experimental limitations, an optimal harvest time if such a parameter exists for the system at hand. If lignolytic efficiencies, normalized or otherwise, are assumed to reflect lignolytic enzyme production or lignin modification, then it is possible to ascertain an optimal harvest time through studies employing above parameters of metabolic efficiency. Lignolytic efficiency vs. time curve showed a maximum which corresponded with the maximum seen in the plot of dry cell weight vs. time. No maximum was observed in the plot of normalized lignolytic efficiency vs. time. Thus an absolute harvest time is hard to assign to this particular fermentation.

The analysis of degraded lignin appears in Table 5.

J. Analyses of Degraded Lignins*

Contents of a deep-tank fermentation were filtered to obtain a retentate containing both cells and modified lignin. The retentate was then extracted with alkali and the acid insoluble fraction of the alkaline solution was recovered by freeze-drying. Lignin thus obtained was designated as "degraded lignin" and structurally analyzed to gain insights into lignin fermentations.

* Data presented in this section were obtained from Dr. W. G. Glasser of the Dept. of Forest Products, VPI & SU.

1. Elemental and Functional Composition: The elemental and functional analyses of lignins from A. fumigatus and C. versicolor fermentations appear in Table 5. The most important information in these analyses is the extent of loss of functional groups. The relative loss of OCH_3 groups is a quantitative measure of lignin degradation. The three Aspergillus degraded lignins showed the following degree of demethoxylation:

AspI = 62.48%

AspII = 29.93%

AspIII = 54.31%.

Thus, AspI and AspIII lignins showed comparable degrees of demethoxylation whereas AspII lignin suffered demethoxylation half that of AspI and AspIII. AspI and AspIII fermentations were quite similar in terms of relatively high yields of biomass during growth stages and overall behavior. AspII fermentation, however, showed a poor yield of biomass during its growth stage. As a rough estimate, the biomass yield from the growth stage of AspII was 3-4 fold lower than that from growth stages of AspI or AspIII. This fact could have led to lower overall lignin metabolism during the biotransformation stage of the AspII fermentation and hence to substantially low demethoxylation in AspII lignin. AspI and AspIII lignins are hence regarded as representative Aspergillus degraded lignins.

In comparison to Aspergillus lignins a typical Coriolus lignin showed very meagre amount of demethoxylation, i.e., 11.17%.

Table 5
Analyses of Degraded Lignins^a

Elemental and Functional Group Composition, %	Untreated Kraft Lignin	AspI Lignin	AspII Lignin	AspIII Lignin	Typical Coriolus Degraded Lignin
C	64.33 ^b	65.41 ^c	60.88 ^c	58.86 ^c	56.89 ^c
H	5.88 ^b	7.18 ^c	5.81 ^c	6.47 ^c	5.34 ^c
N (protein)	-	2.05	1.43	1.08	-
S	2.05	2.69	6.04	4.40	2.21
OCH ₃	13.70	5.14	9.60	6.26	12.17
Total OH	11.86	5.14	9.04	8.20	10.37
Ash	0.66	1.73	1.19	2.50	9.32

^aData obtained from Dr. W. G. Glasser of the Dept. of Forest Products, VPI & SU.

^bCorrected for impurities.

^cNot corrected for impurities.

Thus A. fumigatus brings about substantial lignin degradation compared to C. versicolor. If we compare relative degrees of demethoxylation and extents of $^{14}\text{CO}_2$ evolution from ^{14}C -labeled lignin by A. fumigatus and C. versicolor, we find a striking agreement between the two.

$$\frac{\text{OCH}_3 \text{ loss by } \underline{\text{A. fumigatus}} \text{ (AspI)}}{\text{OCH}_3 \text{ loss by } \underline{\text{C. versicolor}}} = \frac{62.5\%}{11.17\%} = 5.59$$

$$\frac{^{14}\text{CO}_2 \text{ evolution by } \underline{\text{A. fumigatus}}}{^{14}\text{CO}_2 \text{ evolution by } \underline{\text{C. versicolor}}} = \frac{37.5\%}{7.1\%} = 5.28 \quad (\text{see Fig. 21})$$

The above ratios show that the effectiveness of lignin metabolism by A. fumigatus relative to that by C. versicolor is about the same whether demethoxylation or $^{14}\text{CO}_2$ evolution is used as the criterion for comparison. The difference between the above quotients can be attributed to the fact that demethoxylation data are based on deep-tank experiments whereas $^{14}\text{CO}_2$ data are based on shake-flask experiments. The mixing characteristics in the two situations can be widely different. Furthermore, the loss of OCH_3 groups represents a specific change in lignin vis-a-vis $^{14}\text{CO}_2$ evolution which is indicative of gross metabolic changes brought about in lignin. Thus demethoxylation data and $^{14}\text{CO}_2$ data are compatible with each other.

As can be seen from Table 5, the lignin sample with the most demethoxylation, AspI lignin, shows the most decrease in total hydroxyl groups and the lignin sample with the least demethoxylation, Coriolus lignin, shows the least decrease in total hydroxyl groups.

Before the ring cleavage by fungal dioxygenases can take place, the aromatic ring has to be converted to an o-diphenol structure (Fig. 5). At least part of the o-diphenol structures are formed via demethylation which would result in the loss of methoxyl groups. A corresponding increase in hydroxyl groups would result if the ring were not further metabolized. Thus, the fact that a concomitant decrease in hydroxyl groups is seen with demethylation supports the notion that aromatic rings in lignin are cleaved. It is interesting to note that the ratio of OH loss by A. fumigatus to that by C. versicolor is in the same vicinity as the ratio of OCH₃ losses.

$$\frac{\text{total OH loss by } \underline{\text{A. fumigatus}} \text{ (AspI)}}{\text{total OH loss by } \underline{\text{C. versicolor}}} = \frac{56.83\%}{12.56\%} = 4.52$$

2. C₆-C₃ Formulae: Since the basic phenylpropane unit contains a total of 9 carbons, lignin analysis data are frequently reported on the basis of a C₆-C₃ unit (see Table 2). Table 6 gives such C₉ formulae for AspI, AspII and AspIII lignins. The OCH₃ losses based on C₉ empirical formulae are as follows:

AspI	=	60.49%
AspII	=	19.75%
AspIII	=	46.91%

These figures are slightly different than the ones calculated from Table 5. It might be added that the average unit of a typical degraded

Table 6
Analyses of Aspergillus Lignins Based on C₆-C₃ Units^a

	Untreated Kraft Lignin	AspI Lignin	AspII Lignin	AspIII Lignin
Chemical purity, %	98.6	83	88	84
Carbohydrate impurities, %	0.723	2.59	2.26	6.99
Hexosans/Pentosans	0.51/0.22	2.14/0.45	1.81/0.45	4.81/2.18
Protein, %	---	12.55	8.76	6.6
C ₉ -Empirical Formula				
H - Aromatic	2.52	3.06	2.43	2.85
- Sidechain	4.23	6.36	4.54	6.53
- OH by NMR ^b	1.44	1.97	1.30	1.23
- OH by chem. anal.	1.28	0.51	1.08	0.78
Total H	8.10	10.66	8.16	10.39
O - Aromatic OH (by NMR)	0.56	0.49	0.42	0.47
- Aliphatic OH	0.80	0.75	0.77	0.54
Total O	2.26	1.72	2.17	2.53
OCH ₃	0.81	0.32	0.65	0.43
SH	0.14	0.15	0.38	0.29
CO	---	0.13	---	---

^aData obtained from Dr. W. G. Glasser of the Dept. of Forest Products, VPI & SU.

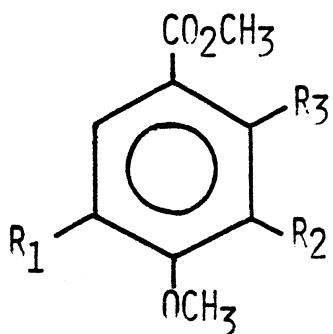
^bNeutron magnetic resonance.

lignin may, in all likelihood, be closer to C_8 than to C_9 , the actual number of carbons per unit depending on the type of degradation.

Thus the empirical formulae based on the C_9 units are only a convenient way for comparisons among lignin samples.

3. Permanganate Oxidation: Lignin degradation by permanganate oxidation and subsequent analyses of oxidation products is by far the most sensitive "fingerprinting" technique in lignin chemistry. The results of permanganate oxidation of Aspergillus lignins appear in Table 7.

Kraft lignin samples yield, on the average, 20-25 mg of monomeric and dimeric products per 100 mg lignin oxidized. About two-thirds of the total oxidation products are accounted for by veratric acid (compound II, Fig. 28). Aspergillus lignins, on the other hand, yielded only about 10-15 mg of monomeric and dimeric products per 100 mg lignin oxidized. Veratric acid constituted a much smaller portion of the total oxidation products. AspII lignin, which showed relatively low demethoxylation, was more akin to untreated lignin in terms of the yield of oxidation products than AspI and AspIII lignins which showed considerable demethoxylation. Coriolus lignin, which exhibited little demethoxylation, proved to be almost indistinguishable from untreated lignin. Coriolus lignin yielded about 20 mg of monomers and dimers per 100 mg lignin oxidized, about two-thirds of the total oxidation products being veratric acid. Thus, it is confirmed that A. fumigatus brings about substantial changes in the lignin structure whereas C. versicolor is responsible



- I: $\text{R}_1=\text{R}_2=\text{R}_3=\text{H}$
 II: $\text{R}_1=\text{OCH}_3$, $\text{R}_2=\text{R}_3=\text{H}$
 III: $\text{R}_1=\text{R}_2=\text{OCH}_3$, $\text{R}_3=\text{H}$
 IV: $\text{R}_1=\text{CO}_2\text{CH}_3$, $\text{R}_2=\text{R}_3=\text{H}$
 V: $\text{R}_1=\text{OCH}_3$, $\text{R}_2=\text{CO}_2\text{CH}_3$, $\text{R}_3=\text{H}$
 VI: $\text{R}_1=\text{OCH}_3$, $\text{R}_2=\text{H}$, $\text{R}_3=\text{CO}_2\text{CH}_3$
 VII: $\text{R}_1=\text{R}_2=\text{OCH}_3$, $\text{R}_3=\text{CO}_2\text{CH}_3$
 VIII: $\text{R}_1=\text{R}_2=\text{CO}_2\text{CH}_3$, $\text{R}_3=\text{H}$

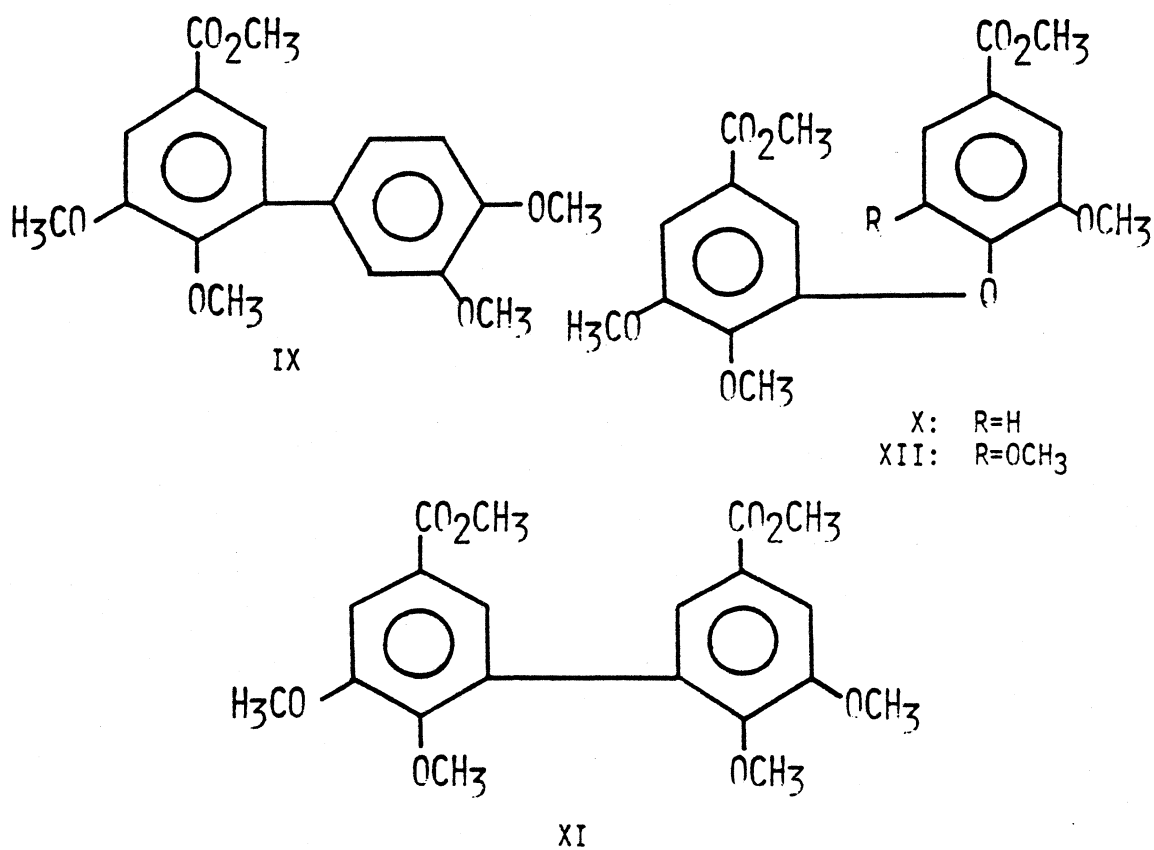


Figure 28. Monomeric and dimeric products from permanganate oxidation of lignin.

Table 7

Permanganate Oxidation of Aspergillus Lignins^a

	Untreated Kraft Lignin	AspI Lignin	AspII Lignin	AspIII Lignin
Permanganate oxidation products by GC, ^b mg ester/ 100 mg lignin				
IC	1.83/ 2.15 ^d	0.67/ 2.66	1.32/ 1.65	1.04/ 0.96
II	14.32/17.37	1.32/ 3.36	6.12/10.82	3.81/ 6.14
III	0.07/ 0.26	0.62/ 5.03	0.75/ 1.12	0.38/ 0.38
IV	0.01/ 0.01	0.02/ 2.44	1.25/ 0.74	0.46/ 0.60
V	2.25/ 1.39	3.37/ 1.09	1.77/ 1.23	0.72/ 0.72
VI	0.43/ 0.27	2.47/ 0.67	0.51/ 0.36	0.41/ 0.19
VII	0.38/ ---	---	---	---
VIII	--- / ---	--- / 0.37	0.09/ ---	0.53/ 0.03
IX	0.11/ 0.17	0.12/ 0.53	0.71/ 0.37	0.49/ 0.24
X	0.57/ 0.58	0.29/ 0.06	0.38/ 0.29	0.29/ 0.25
XI	1.68/ 2.05	0.66/ 0.42	1.53/ 1.26	0.56/ 0.91
XII	---	0.0 / 0.0	---	---
Total	21.66/24.25	9.53/16.62	14.43/17.84	8.69/10.42
Permanganate oxidation products by GPC, ^e %				
1- and 2-mers	43.27/40.78	41.29/40.75	42.18/52.14	39.40/55.17
3-mers	20.29/15.70	19.02/17.92	6.66/16.94	19.10/18.80
4-mers	13.63/13.07	14.18/14.34	5.60/13.70	14.22/11.92
5-mers	11.60/13.56	12.83/11.96	4.87/11.99	13.11/ 8.61
6- and higher oligomers	11.21/16.89	12.69/15.04	6.67/ 7.47	14.18/ 5.50

Table 7 (Continued)

^aData obtained from Dr. W. G. Glasser of the Dept. of Forest Products, VPI & SU.

^bGas chromatography.

^cSee Fig. 28 for structures of compounds I, II, . . . , XII.

^d(data for unhydrolyzed samples)/(data for hydrolyzed samples).

^egel permeation chromatography.

for only marginal changes in lignin. It is also fair to conclude that various indices of lignin modification such as $^{14}\text{CO}_2$ evolution, OCH_3 loss, yields of monomers and dimers and the specific yield of veratric acid after permanganate oxidation are all congruent with each other within experimental limitations.

The GPC (gel permeation chromatography) analysis of oxidation products of Aspergillus lignins (Table 7) reveals that little change in the molecular weight distribution of such products occurs when compared to untreated lignin even though greatly reduced yields of veratric acid and monomeric and dimeric products are encountered. This is due to the nature of mixtures of permanganate oxidation products which contain significant concentrations of monomers and/or dimers of unidentified structures. Where it is possible to positively identify approximately 90% of all permanganate oxidation products from a wood or pulp lignin, only 50% of such products from Aspergillus lignins can be so identified (Glasser, 1978). This means that Aspergillus lignins contain a large amount of structures not native to untreated lignin. This corollary lends credence to the contention that A. fumigatus severely changes the lignin structure.

4. Carboxylation: Dearomatization of rings in the lignin macromolecule should bring about an increase in carboxyl groups (see Fig. 5). Modified lignins enriched in COOH groups can be utilized in copolymerization since the above functionality confers attractive properties on an otherwise chemically intractable biopolymer. Hsu and Glasser (1975) have demonstrated the feasibility of producing polyurethane foams from carboxylated lignins.

K. Effect of Fermentation Parameters

Environmental parameters such as temperature, pH, dissolved oxygen concentration, mixing characteristics, medium composition etc. can have significant bearing on the performance of any given fermentation. The effects of carbohydrate, a crucial medium component, and temperature have already been discussed in connection with white-rot fungi. Regulatory phenomena such as catabolite repression, induction and cell-differentiation can also very subtly control a fermentation system. However, any adverse effect of regulatory phenomena can be alleviated by the proper control of environmental parameters. For example, catabolite repression can be surmounted by sufficiently lowering the concentration of the substance causing repression of lignolytic enzymes.

1. Temperature: A. fumigatus, a soft-rot fungus belonging to Ascomycetes, grows optimally at 37 C. When cultivated on ^{14}C -labeled lignin and starch, A. fumigatus exhibited slightly higher lignolytic activity at 37 C than at 28 C (Fig. 29). Conversely, S. pulverulentum and P. chrysosporium, which are white-rot fungi belonging to Hymenomycetes, showed much higher lignolytic activity at 28 C than at their optimal temperature for growth, 38 C (Fig. 13).

2. Nitrogen Levels: Before ^{14}C -labeled lignin became available, growth of lignin-degrading fungi in the presence of lignin was used as the criterion to study lignin degradation. S. pulverulentum was

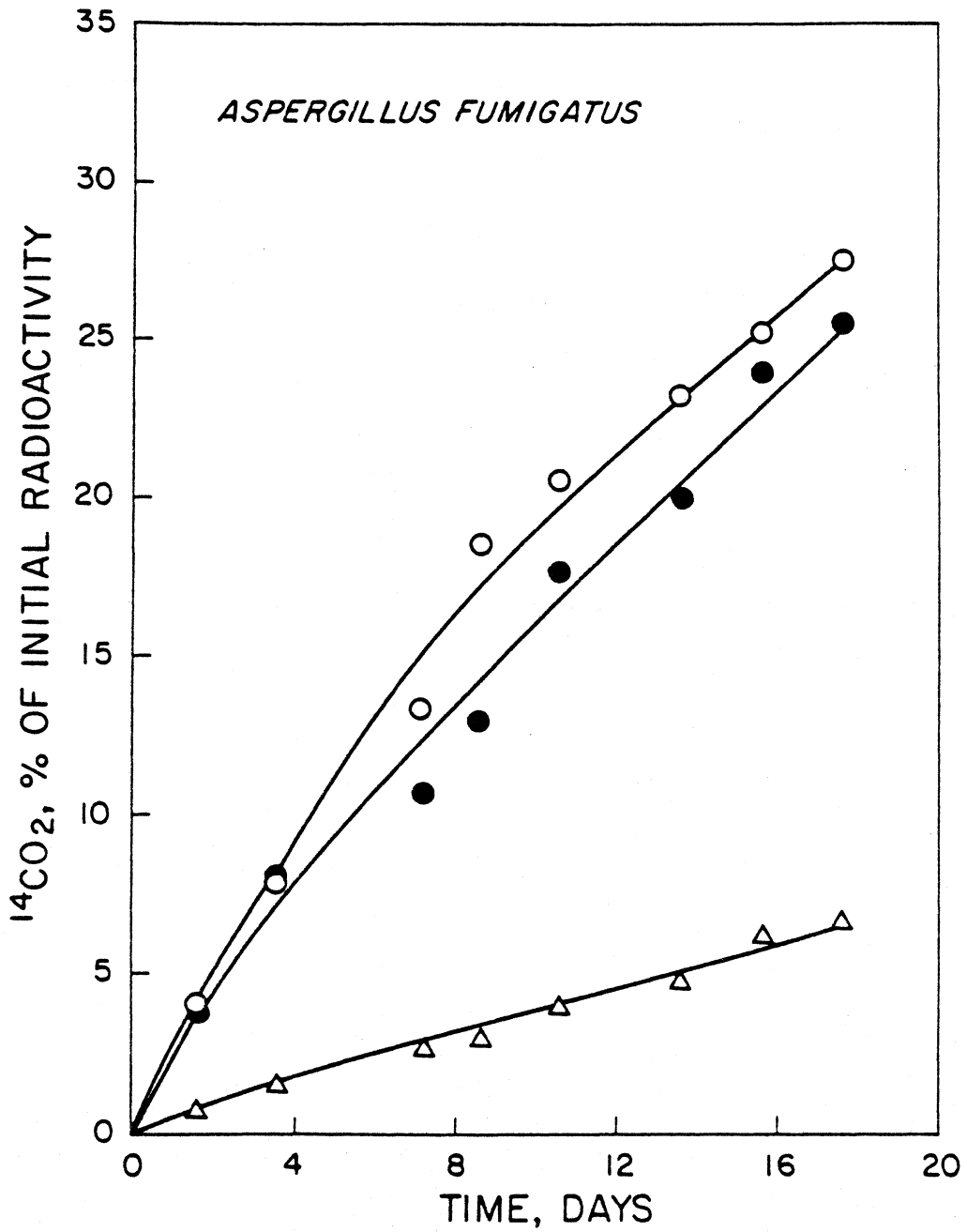


Figure 29. Effect of nutrient nitrogen and temperature on lignin metabolism by *A. fumigatus*: Starch as a cosubstrate.

○—○ 17.4 mM nitrogen, 37 C; ●—● 17.4 mM nitrogen, 28 C; ▲—▲ 2.6 mM nitrogen, 28 C.

grown on media containing lignin and cellobiose with variable nutrient nitrogen levels. $(\text{NH}_4)_2\text{HPO}_4$ and urea were used separately as nitrogen sources, the concentration of each being varied from 0.05 g/l to 3 g/l. The experiment was carried out in shake-flasks and dry cell weights were determined after 7.5 days of incubation. Higher levels of growth were observed where low initial concentrations of nitrogen sources were used (Fig. 30). Maximum growth resulted in flasks containing initial concentrations of 0.25 g $(\text{NH}_4)_2\text{HPO}_4$ /l and 0.05 g urea/l. This was a rather unexpected result since under most circumstances higher nitrogen concentrations lead to better growth due to better protein synthesis. White-rot fungi inhabiting wood are exposed to nutrient nitrogen limitation and hence may have evolved to function better under such a limitation. In spite of the above observations, low nitrogen levels were not used in subsequent studies until Kirk et al. (1978) published similar conclusions with P. chrysosporium using ^{14}C -labeled DHP.

A. fumigatus, when grown on soluble starch and ^{14}C -labeled lignin, supported higher lignolytic activity at 17.4 mM nitrogen, or 2 g $\text{NH}_4\text{H}_2\text{PO}_4$ /l, than at 2.6 mM nitrogen, or 0.3 g $\text{NH}_4\text{H}_2\text{PO}_4$ /l, (Fig. 29). However, A. fumigatus demonstrated higher lignolytic activity at 2.6 mM nitrogen than at 17.4 mM nitrogen when glucose was used as a cosubstrate (Fig. 31). Thus the former was in contrast with and the latter was in agreement with the results of Kirk et al. (1978). Regardless of the type of carbohydrate used, Kirk et al.

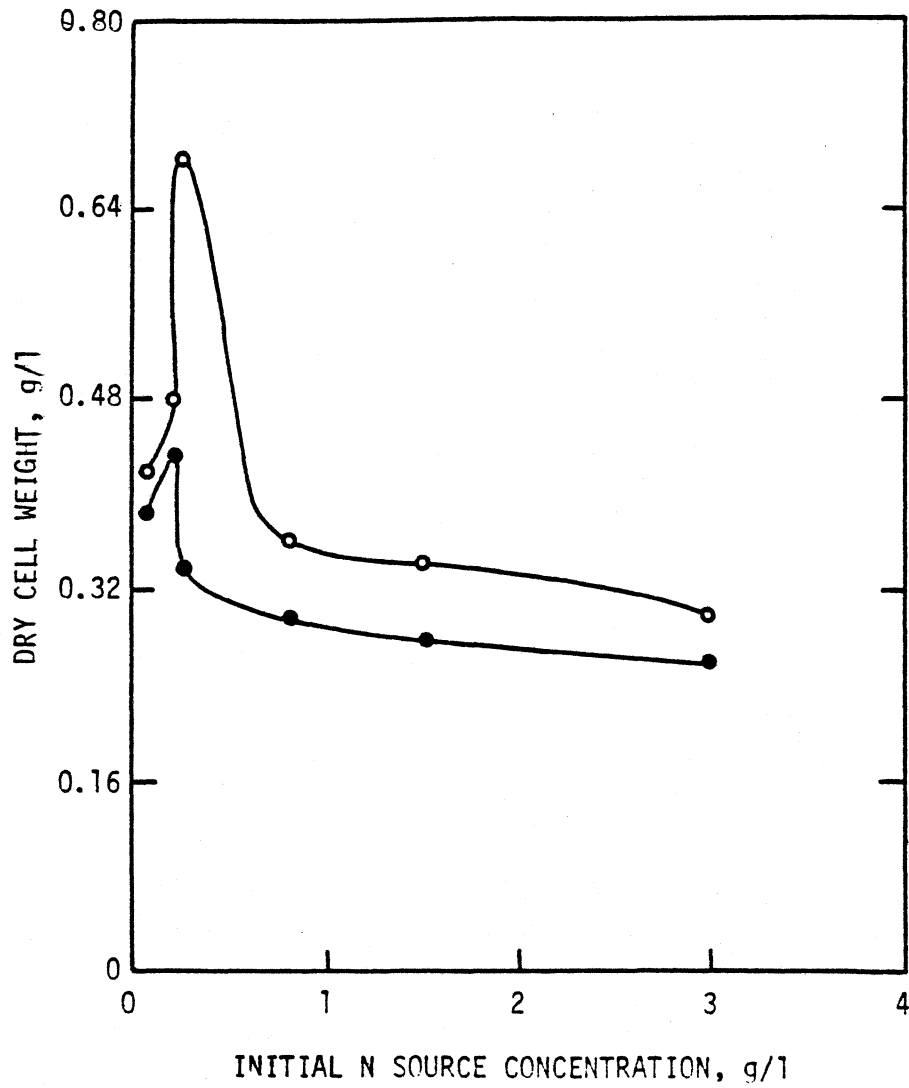


Figure 30. Effect of nutrient nitrogen on growth of *S. pulverulentum*.

○—○ $(\text{NH}_4)_2\text{HPO}_4$, ●—● urea.

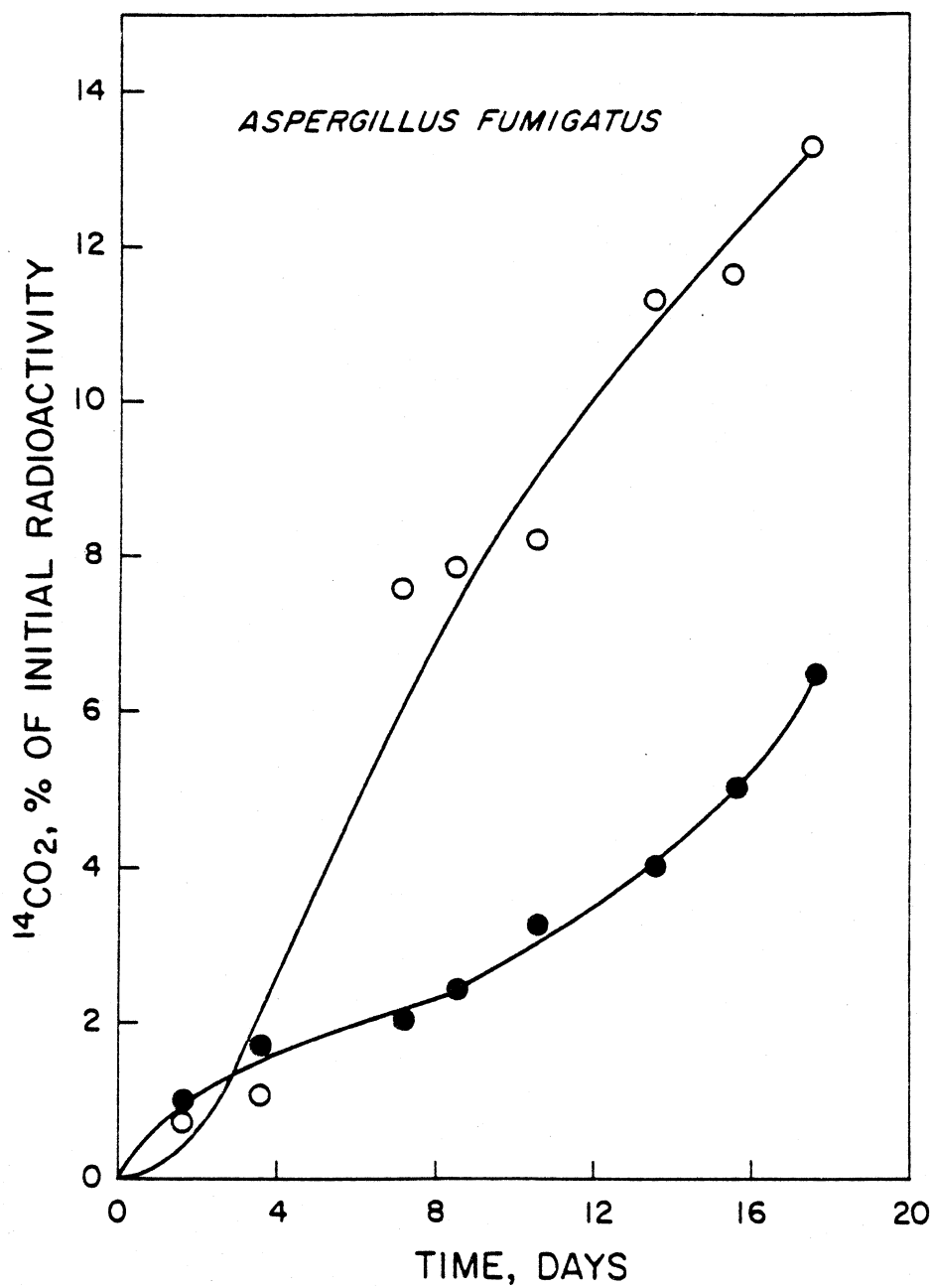


Figure 31. Effect of nutrient nitrogen on lignin metabolism by *A. fumigatus*: Glucose as a cosubstrate.

○—○ 2.6 mM nitrogen, 28 C

●—● 17.4 mM nitrogen, 28 C

(1978) saw higher lignolytic activity at low nitrogen levels than at high nitrogen levels.

This means that for A. fumigatus, the nitrogen effect depends on the type of cosubstrate used. Thus, unlike in the case of P. chrysosporium, the type of carbohydrate and nitrogen concentration together may control lignolytic activity of A. fumigatus than nitrogen concentration alone. Keyser et al. (1978) very recently claimed that lignolytic activity by P. chrysosporium was seen solely in response to nitrogen starvation. In light of the differences between A. fumigatus, a soft-rot fungus, and P. chrysosporium, a white-rot fungus, in terms of nitrogen effects and temperature effects, it is possible that these two fungi exhibit different types of regulation in their mode of lignin metabolism.

A. fumigatus exhibited about the same lignolytic activity at 2.6 mM nitrogen with starch as the cosubstrate as at 17.4 mM nitrogen with glucose as the cosubstrate (Table 8). This led me to believe that the carbon/nitrogen (C/N) ratio may play an important role in lignin metabolism by A. fumigatus.

3. Glucose Consumption and Nitrogen Concentration: Corresponding to each flask containing ^{14}C -labeled lignin and unlabeled glucose, the experiment discussed above also included another flask containing unlabeled lignin and ^{14}C -labeled glucose. The concentrations of all species in corresponding flasks were exactly the same. Lignolytic activity of A. fumigatus was determined, as usual, from

Table 8
Effect of Nitrogen Levels on Lignin Metabolism
by A. fumigatus

Carbohydrate	Nitrogen Concentration, mM	Lignolytic Activity after 17.6 days
Starch	17.4	25.5
Starch	2.6	6.60
Glucose	17.4	6.55
Glucose	2.6	13.30

$^{14}\text{CO}_2$ evolution from flasks containing ^{14}C -labeled lignin. Glucose consumption was estimated by following $^{14}\text{CO}_2$ from flasks containing ^{14}C -labeled glucose and assuming a constant yield of CO_2 from glucose. If we assume that $Y_{\text{CO}_2/\text{glucose}} = 0.5$ (mg CO_2 /mg glucose) then the concentration of glucose remaining in the flask is,

$$\begin{aligned} &\text{glucose concentration} \\ &= (\text{Initial glucose concentration}) \left[1 - \frac{\begin{array}{c} ^{14}\text{CO}_2 \text{ from } ^{14}\text{C-glucose,} \\ \text{fraction of initial} \\ \text{radioactivity} \end{array}}{Y_{\text{CO}_2/\text{glucose}}} \right] \\ &= (\text{Initial glucose concentration}) [1 - 2(\text{ } ^{14}\text{CO}_2 \text{ from } ^{14}\text{C-glucose} \\ &\quad \text{of initial radioactivity})]. \end{aligned}$$

Hence, glucose concentration could be calculated as a function of time. The rate of glucose consumption and the total amount of glucose consumed by A. fumigatus were about the same at 2.6 and 17.4 mM nitrogen concentrations (Figs. 32 and 33). Thus, nitrogen concentration, while significantly affecting lignolytic activity by A. fumigatus, does not affect overall glucose metabolism.

4. Loss of Activity of A. fumigatus: The idea of C/N ratio being important in lignin metabolism could not be pursued since the A. fumigatus culture somehow lost its lignolytic activity by an order of magnitude. The experiment involving different carbohydrate levels and different nitrogen levels was repeated three times with A. fumigatus and each time dismally low lignolytic activities were

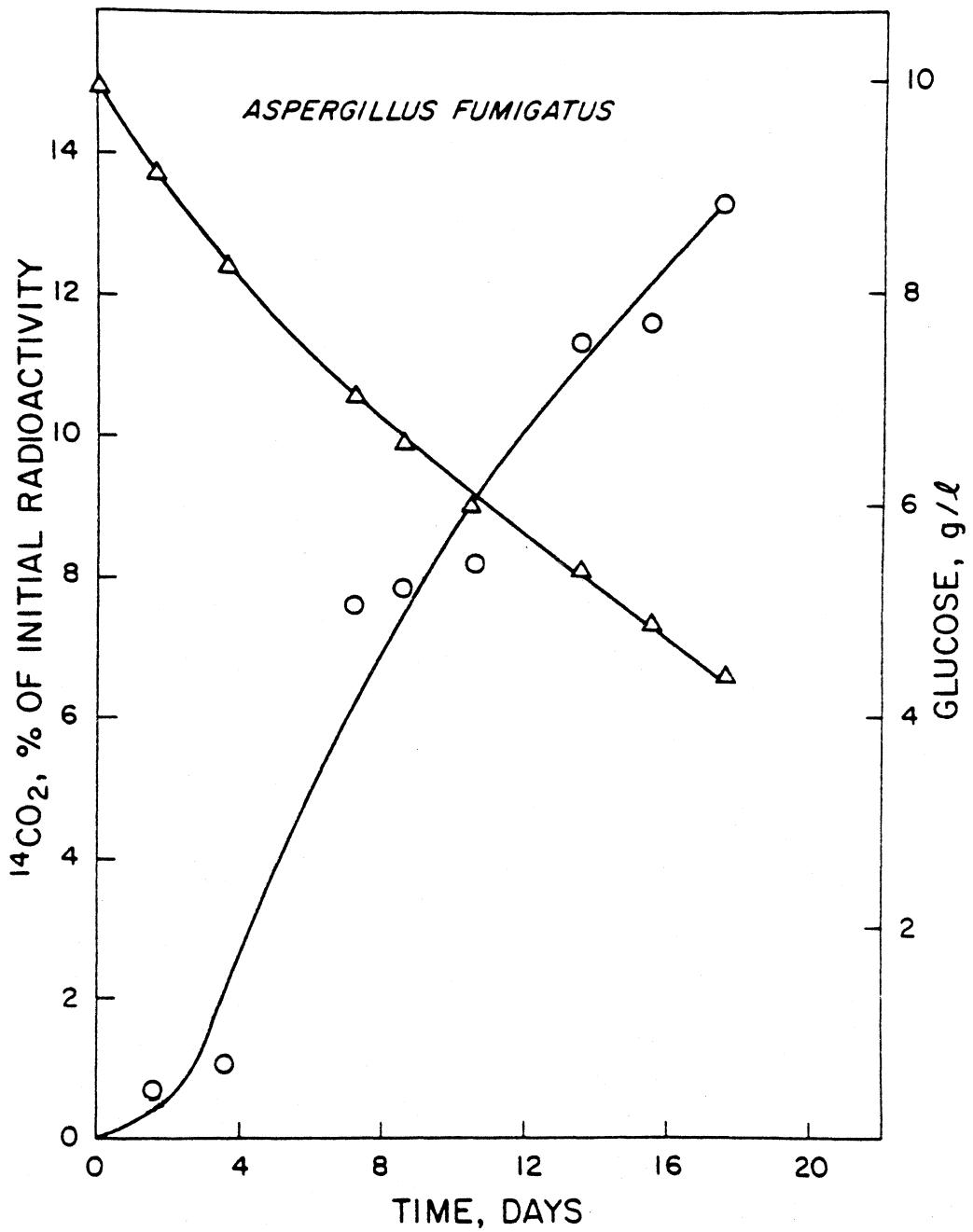


Figure 32. Lignolytic activity and glucose consumption during a lignin fermentation by *A. fumigatus*: 2.6 mM nitrogen.

○—○ $^{14}\text{CO}_2$, △—△ glucose.

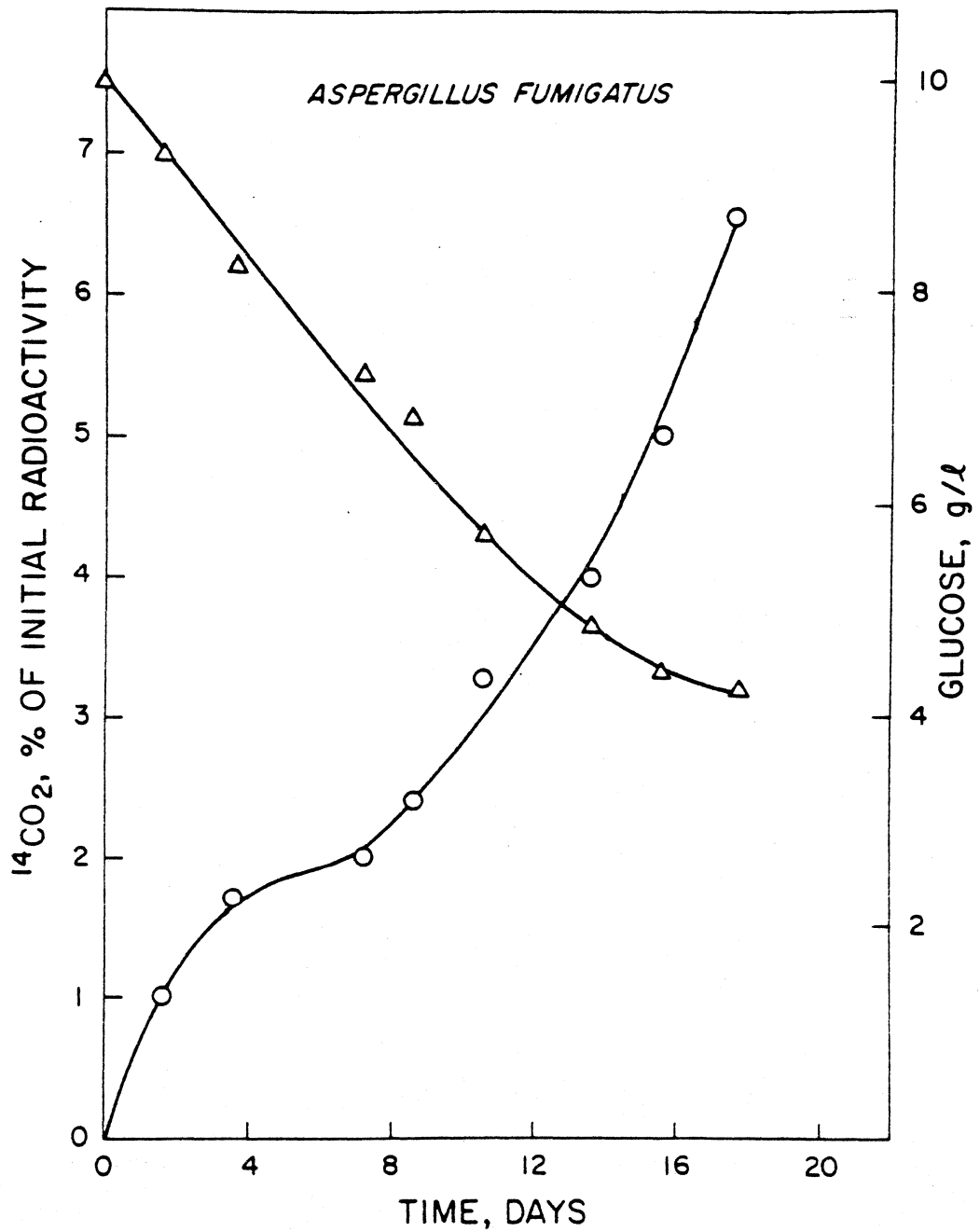


Figure 33. Lignolytic activity and glucose consumption during a lignin fermentation by *A. fumigatus*: 17.4 mM nitrogen.

○—○ $^{14}\text{CO}_2$, ▲—▲ glucose.

observed. All factors affecting various facets of the experiment were carefully examined and nothing abnormal or artifactual could be discerned. Cultures from three old stock slants were checked out but they yielded results only marginally better than the low-activity culture (Table 9). It might be added that there was a three month gap between the last successful and the first unsuccessful experiment.

Though the culture from the Mycology Dept. stock was better than the low-activity culture at hand, lignolytic activities seen were much too low compared to those in previous successful runs. It is quite possible that A. fumigatus underwent strain degeneration and reverted to the wild type culture. A modified dialysis fermentation was carried out in order to eliminate the possibility that the A. fumigatus strain could accumulate lignin-derived products even though it had lost the ability to evolve large amounts of CO₂ from lignin.

The dialysis fermentation contained soluble starch and ¹⁴C-labeled lignin as substrates. A dialysis bag (3500 MW cutoff) tied at both ends was included in each flask to detect any accumulation of lignin-derived products. Dialysis bags were periodically removed from both inoculated and uninoculated flasks and replaced with fresh dialysis bags. In addition to following ¹⁴CO₂ evolution, the radioactivity of the contents of dialysis bags was also measured. The rate of accumulation of ¹⁴C-labeled material of low molecular weight was always lower in the inoculated case than in the uninoculated control

Table 9
Loss of Lignolytic Activity of A. fumigatus

Source of <u>A. fumigatus</u> (VPI 887)	Lignolytic Activity			
	0.82 days	1.82 days	3.23 days	5.2 days
January '78 slant	0.63	0.85	1.16	1.25
February '78 slant	0.73	1.09	1.83	1.95
Slant from Mycology Dept. stock	1.31	1.74	2.49	2.71

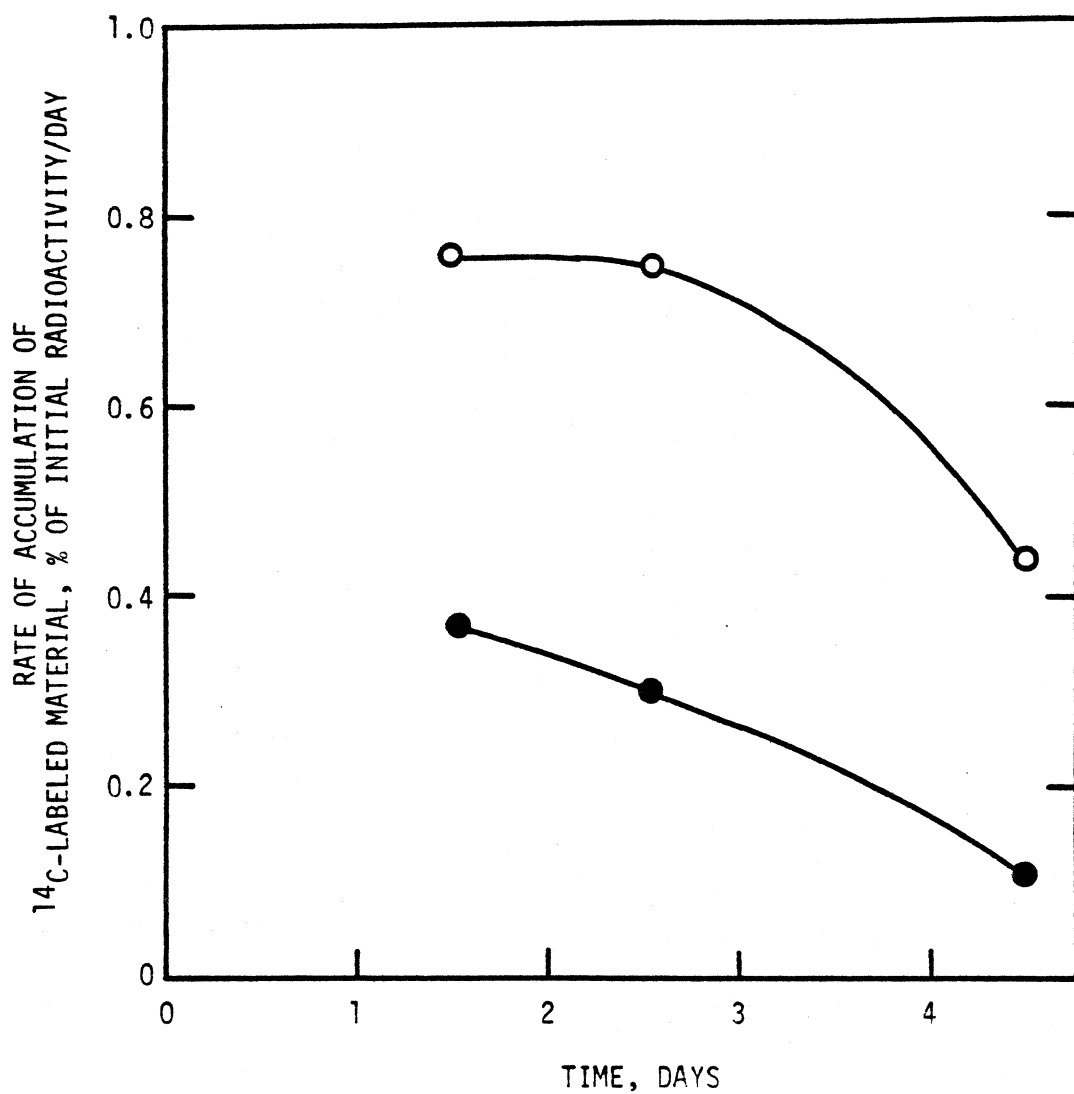


Figure 34. Lack of accumulation of lignin-derived low molecular weight products during a dialysis fermentation by *A. fumigatus*.

○—○ uninoculated control, ●—● *A. fumigatus*

(Fig. 34). Thus, A. fumigatus, besides evolving much lower amount of $^{14}\text{CO}_2$ than before, did not accumulate any lignin-derived low molecular weight compound either.

Due to the possible genetic instability of A. fumigatus, following studies were conducted with C. versicolor which, while not being very potent in lignin metabolism, had earlier given consistent results.

5. C/N Ratio: Since the combination of the type of carbohydrate and nitrogen concentration seemed important, the effect of such combinations on lignin metabolism by C. versicolor was studied. Avicel, soluble starch and glucose were the types of carbohydrates used and the nitrogen concentrations employed were 2.6, 17.4 and 34.8 mM nitrogen. Regardless of the carbohydrate type used, C. versicolor demonstrated very little differences among lignolytic activities seen at the three nitrogen concentrations (Figs. 35, 36 and 37). The differences among lignolytic activities at various nitrogen concentrations were statistically insignificant when starch and Avicel were used as cosubstrates. When glucose was used as the cosubstrate, the lignolytic activity at 2.6 mM nitrogen concentration was only slightly higher than at 17.4 or 34.8 mM nitrogen. The overall levels of lignolytic activity by C. versicolor were about the same as those previously seen under similar conditions but were an order of magnitude lower than those of successful A. fumigatus experiments.

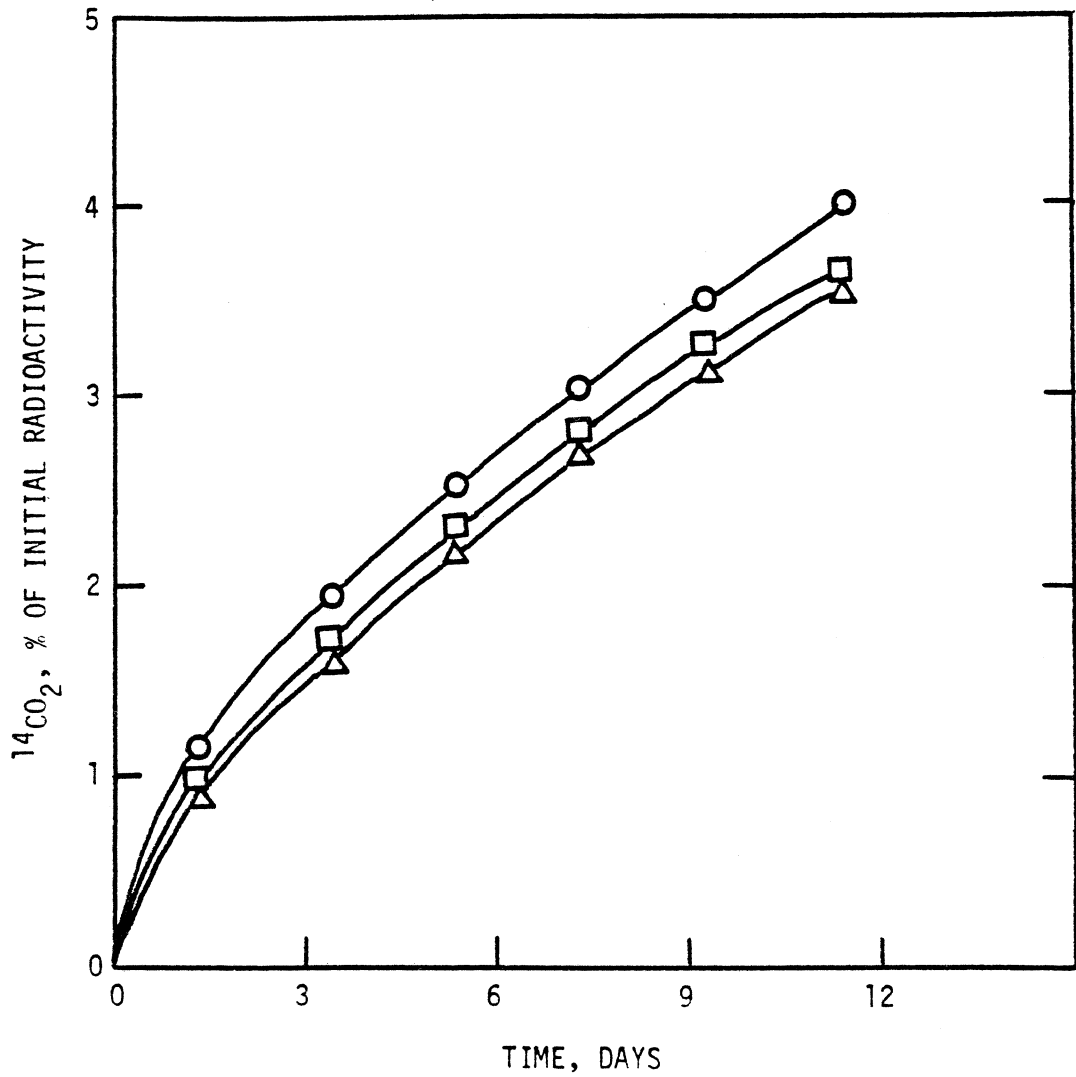


Figure 35. Effect of nitrogen concentration on lignin metabolism by *C. versicolor*: Avicel as a cosubstrate.

○—○ 2.6 mM nitrogen, □—□ 17.4 mM nitrogen,
△—△ 34.8 mM nitrogen.

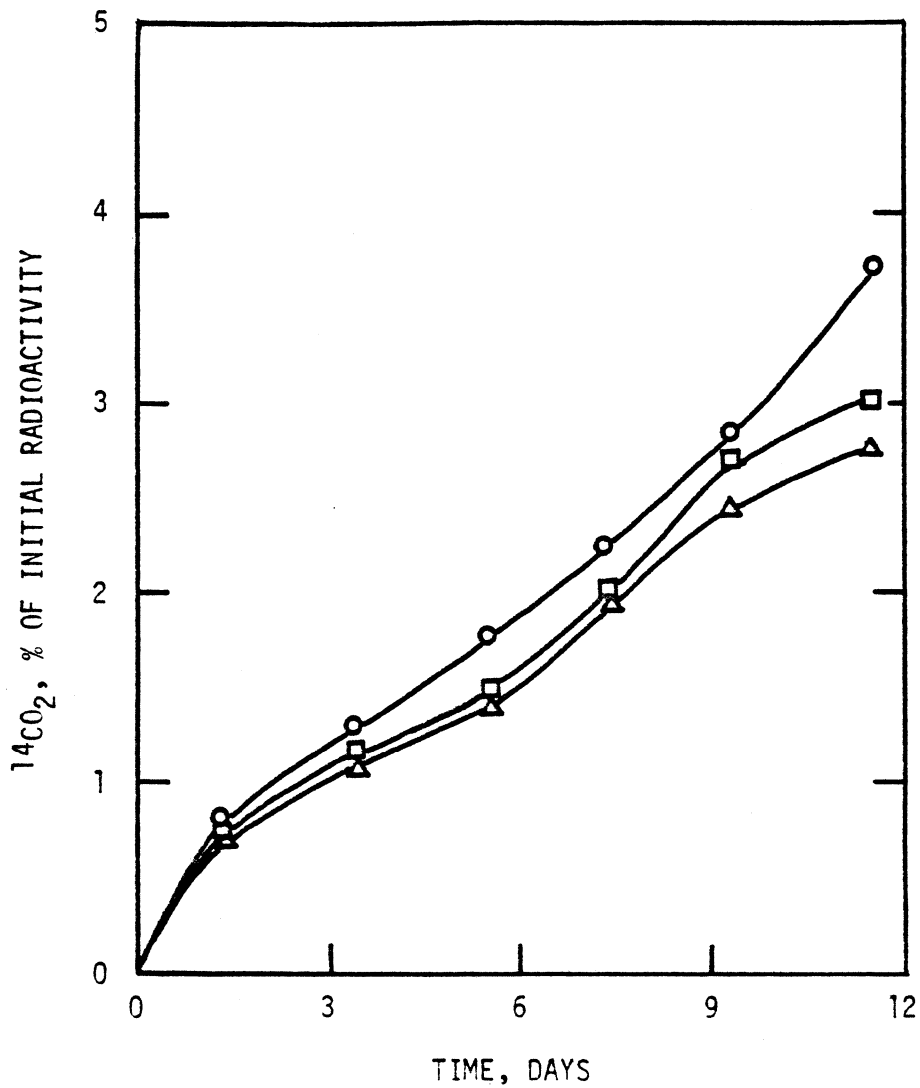


Figure 36. Effect of nitrogen concentration on lignin metabolism by *C. versicolor*: Soluble starch as a cosubstrate.

○—○ 2.6 mM nitrogen, □—□ 17.4 mM nitrogen,
▲—▲ 34.8 mM nitrogen.

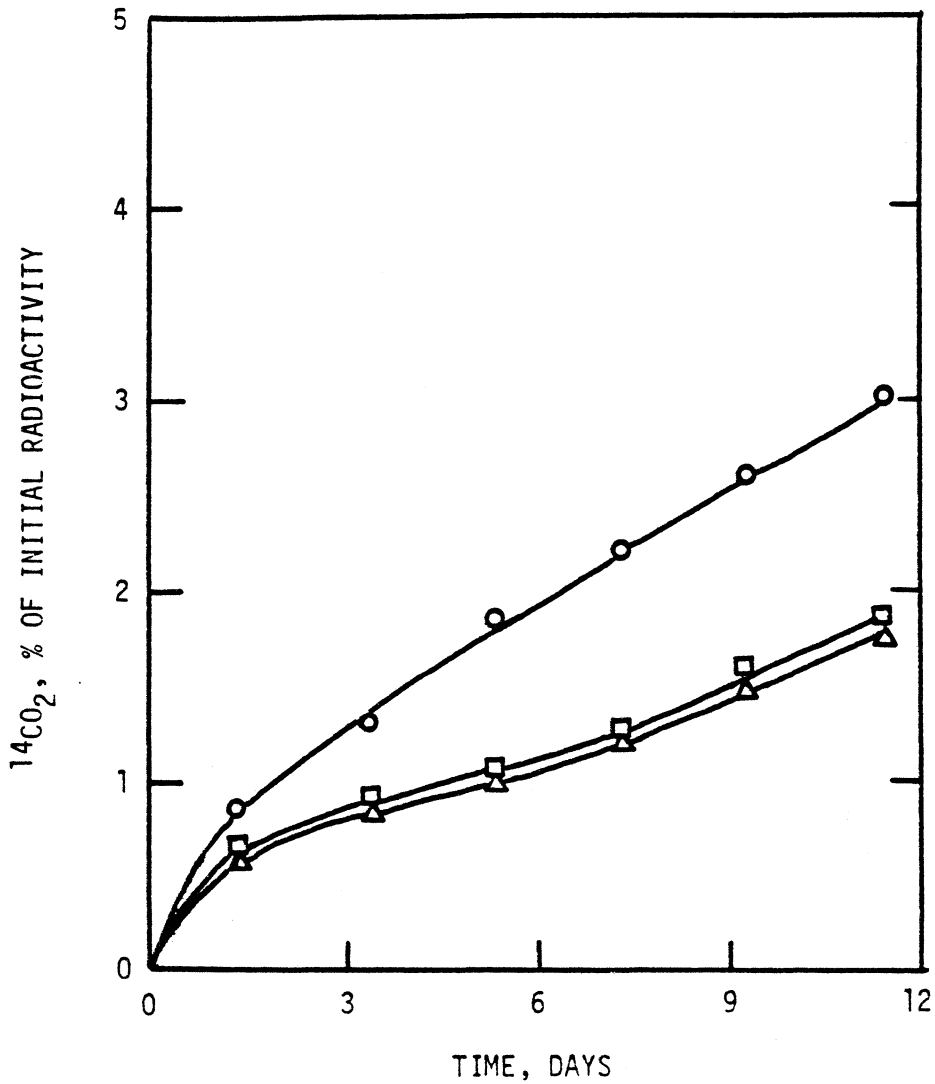


Figure 37. Effect of nitrogen concentration on lignin metabolism by *C. versicolor*: Glucose as a cosubstrate.

○—○ 2.6 mM nitrogen, □—□ 17.4 mM nitrogen,
▲—▲ 34.8 mM nitrogen.

C. versicolor is not as sensitive to nitrogen concentration as the active strain of A. fumigatus.

At 17.4 mM nitrogen concentration, cellulose acetate and Avicel supported higher lignolytic activity than did starch or glucose, though the differences among various conditions were not overwhelming (Fig. 38).

Though only a marginal effect of nitrogen on lignin metabolism by C. versicolor was seen, the hypothesis of the importance of C/N ratio in lignin metabolism was tested again. Initial glucose concentrations of 1, 5 and 10 g/l and initial nitrogen concentrations of 2.6, 17.4 and 34.8 mM nitrogen were employed in an experiment designed to study the effect of C/N ratio on lignin metabolism. The different nitrogen concentrations did not have any effect on lignolytic activity by C. versicolor at either of the glucose concentrations (Figs. 39, 40 and 41). However, the initial glucose concentration of 1 g/l supported higher lignolytic activity than did the initial glucose concentration of 10 g/l. No relation between C/N ratio and lignolytic activity was perceptible (Table 10).

C. versicolor demonstrated slightly higher lignolytic activities in the presence of recalcitrant carbohydrates and low initial glucose concentrations than in the presence of easily metabolized carbohydrates and high initial glucose concentrations, respectively. The lack of sensitivity to nitrogen concentration on the part of C. versicolor may lie in its inherently low efficiency of lignin metabolism. Since the ultimate potential of lignin metabolism by

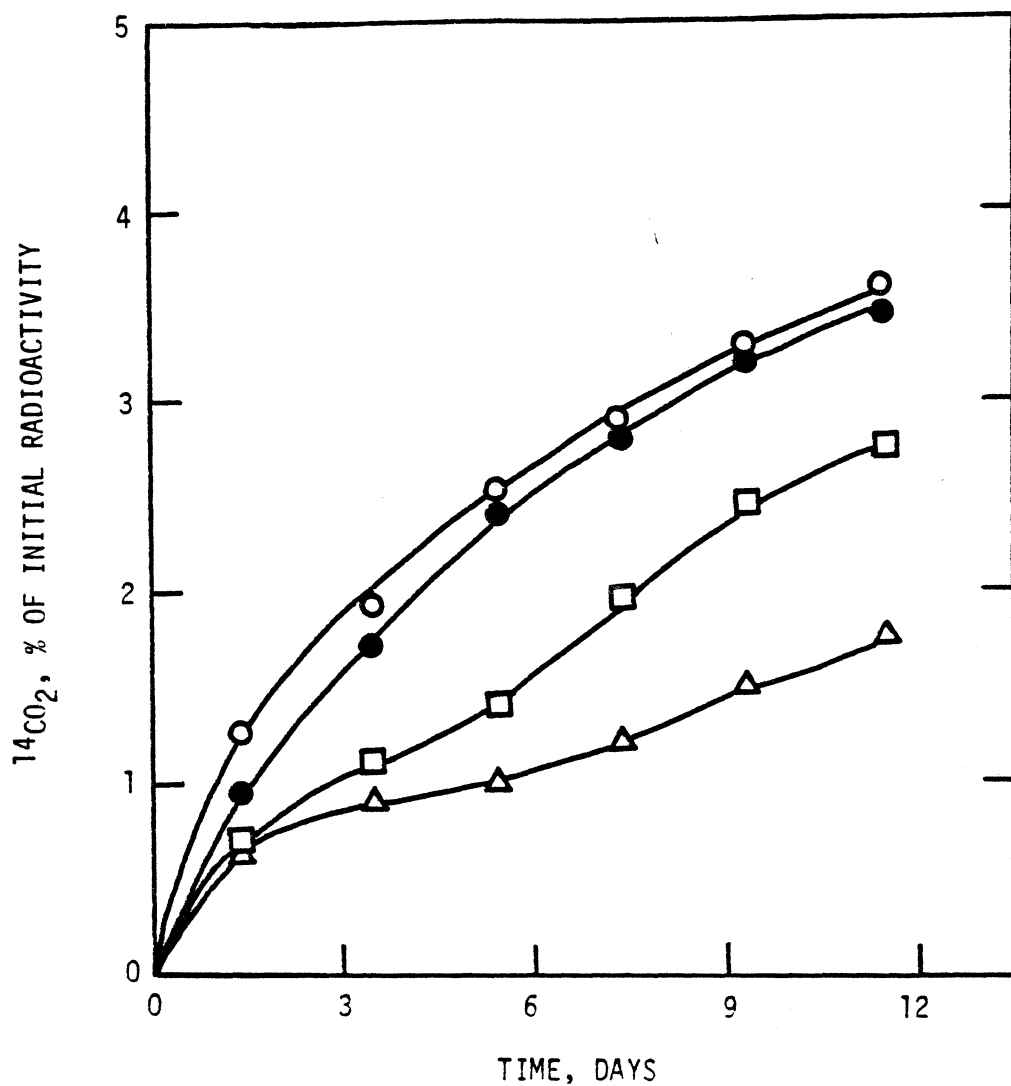


Figure 38. Effect of carbohydrates on lignin metabolism by *C. versicolor*. II.

○—○ cellulose acetate, ●—● Avicel,
□—□ soluble starch, △—△ glucose.

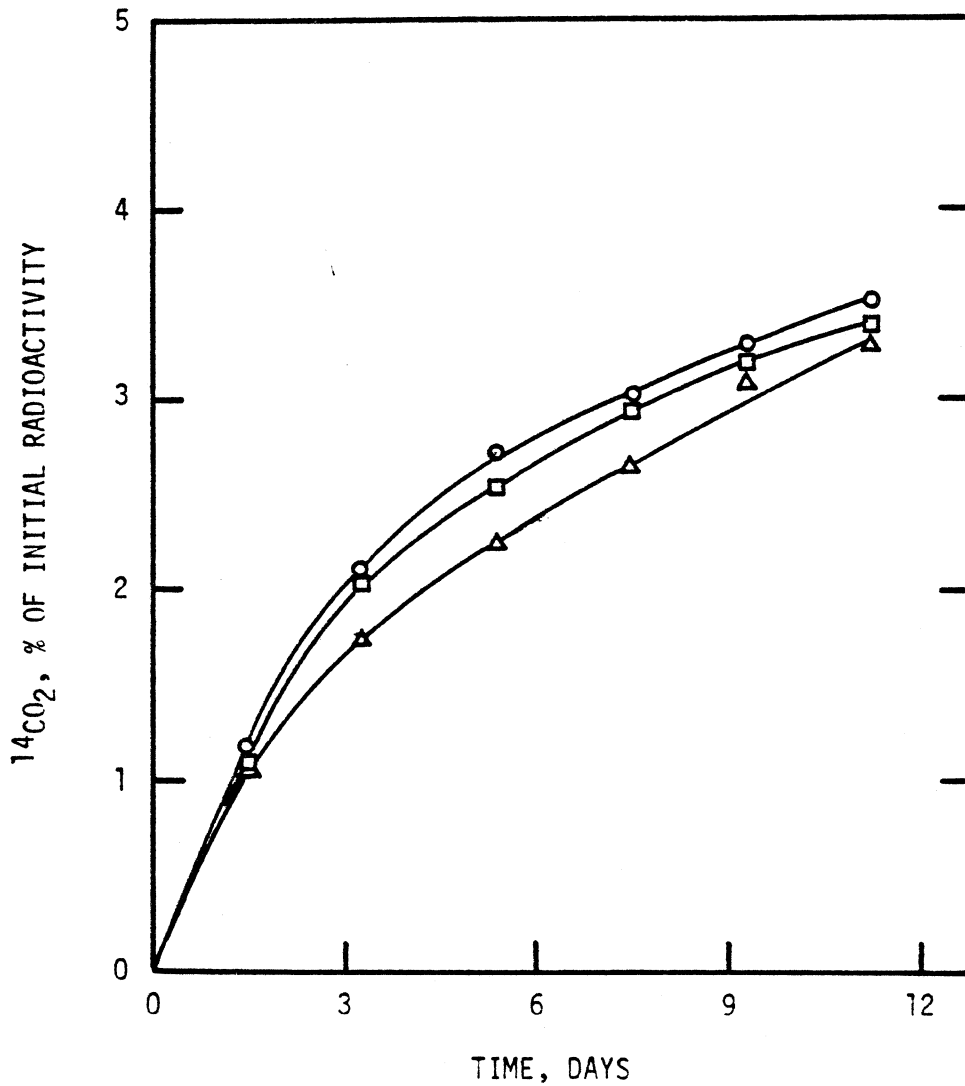


Figure 39. Effect of C/N ratio on lignin metabolism by C. versicolor: Initial glucose concentration of 1 g/l.

○—○ 2.6 mM nitrogen, □—□ 17.4 mM nitrogen,
▲—▲ 34.8 mM nitrogen.

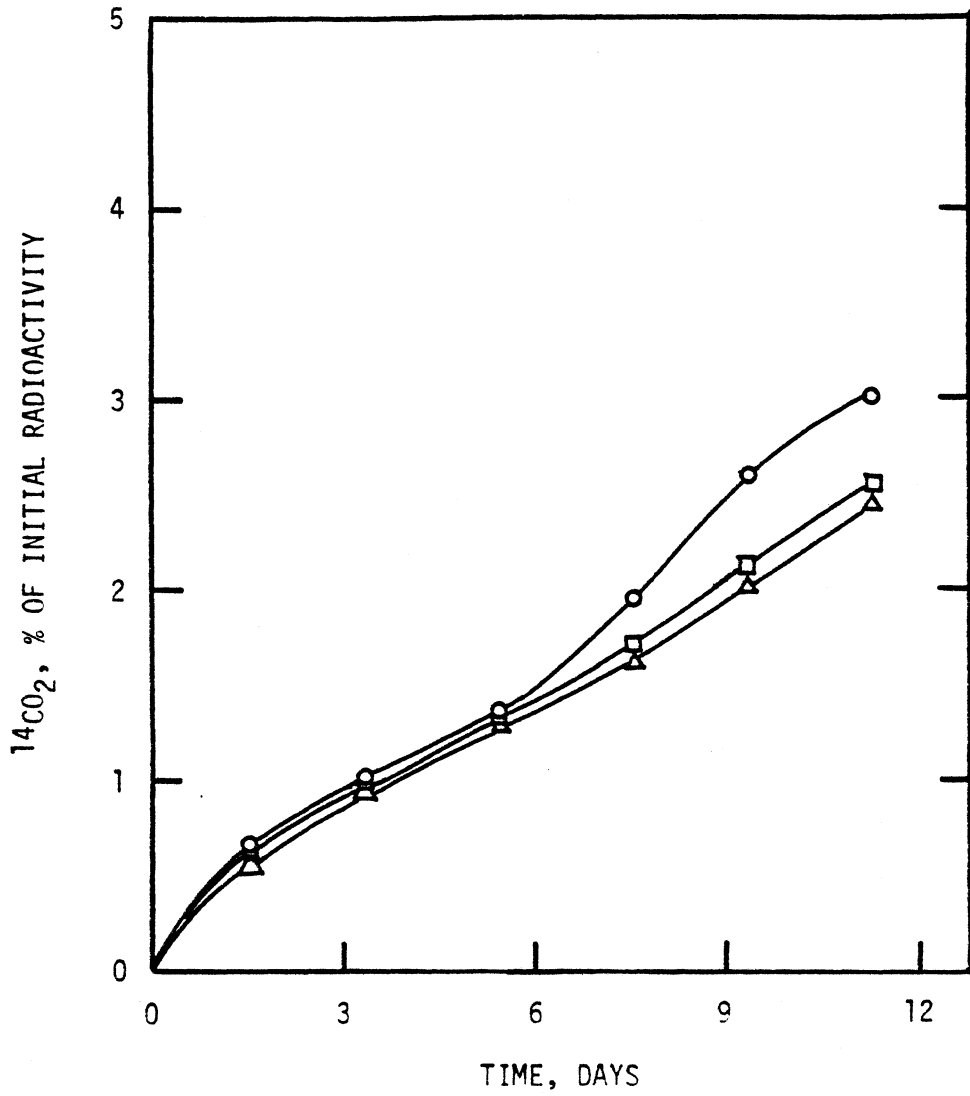


Figure 40. Effect of C/N ratio on lignin metabolism by *C. versicolor*: Initial glucose concentration of 5 g/l.

○—○ 2.6 mM nitrogen, □—□ 17.4 mM nitrogen,
▲—▲ 34.8 mM nitrogen.

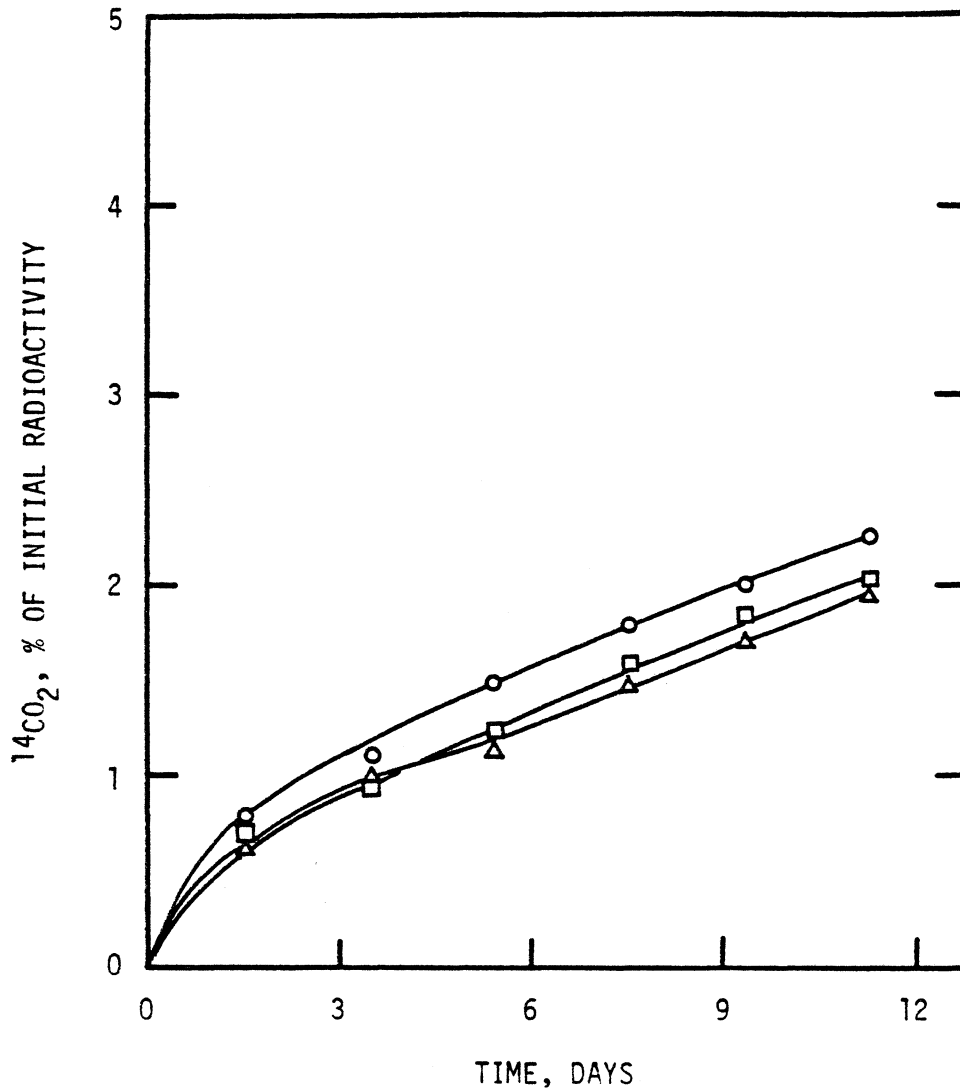


Figure 41. Effect of C/N ratio on lignin metabolism by *C. versicolor*: Initial glucose concentration of 10 g/l.

○—○ 2.6 mM nitrogen, □—□ 17.4 mM nitrogen,
△—△ 34.8 mM nitrogen.

Table 10

C/N Ratio^a and Lignin Metabolism by C. versicolor

[Glucose] ₀ = 1 g/l ^b		[Glucose] ₀ = 5 g/l ^b		[Glucose] ₀ = 10 g/l ^b	
C/N, $\frac{\text{g}}{\text{1 mM}}$	Lignolytic Activity ^c	C/N, $\frac{\text{g}}{\text{1 mM}}$	Lignolytic Activity ^c	C/N, $\frac{\text{g}}{\text{1 mM}}$	Lignolytic Activity ^c
0.0287	1.07	0.1437	0.544	0.287	0.708
0.0575	1.13	0.287	0.62	0.575	0.683
0.3846	1.17	1.923	0.632	3.846	0.794

^aC/N = $\frac{\text{initial glucose concentration, g/l}}{\text{initial nitrogen concentration, mM}}$

^b[glucose]₀ = initial glucose concentration

^cafter 1.5 days of incubation

C. versicolor is probably minuscule to begin with, changes brought about in levels of lignin metabolism by nitrogen concentration variation etc. are expected to be vanishingly small, too. In other words, lignolytic activities measured at varying experimental conditions are about the same since the lignolytic capacity of C. versicolor has an exceedingly small upper limit.

6. Glucose Catabolite Repression: Low initial glucose concentrations previously supported slightly higher lignolytic activities than high initial glucose concentrations. Though the difference between the above two was not astounding, the investigation of a possible glucose catabolite repression phenomenon was considered worthwhile. C. versicolor was grown in media containing ^{14}C -labeled lignin and glucose. The nitrogen concentration was 2.6 mM and initial glucose concentrations were varied from 0.1 g/l to 10 g/l in six increments. At low initial glucose concentrations, the organism may become energy limited in the latter part of the fermentation due to exhaustion of glucose supply. For this reason the initial rate of $^{14}\text{CO}_2$ evolution was used as the criterion to study glucose catabolite repression.

0.1 g glucose/l, the lowest initial glucose concentration used, supported the highest initial $^{14}\text{CO}_2$ evolution rate and 10 g glucose/l, the highest initial glucose concentration used, supported the lowest initial $^{14}\text{CO}_2$ evolution rate (Figs. 42 and 43). The

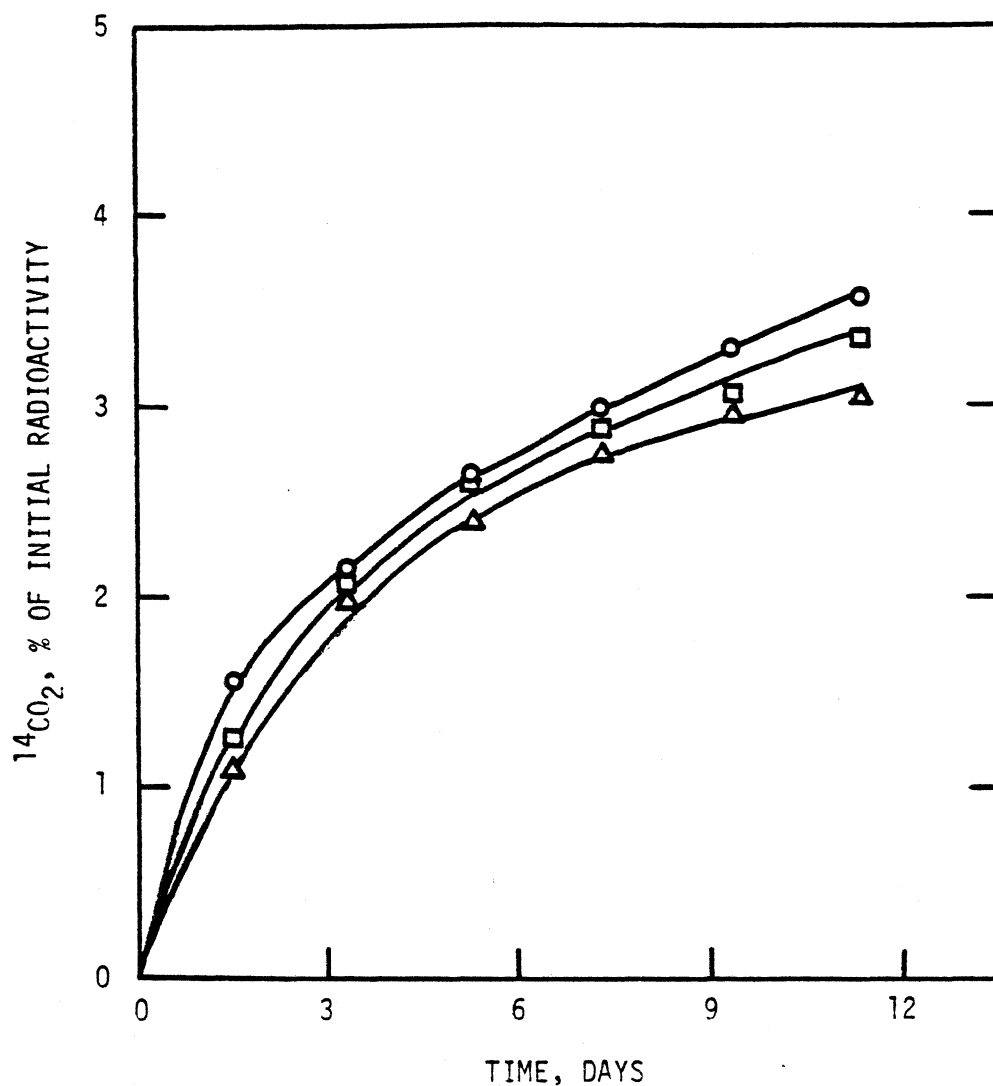


Figure 42. Investigation of glucose catabolite repression in lignin metabolism by *C. versicolor*. I.

Initial glucose concentrations: ○—○ 0.1 g/l,

□—□ 0.25 g/l, ▲—▲ 0.5 g/l.

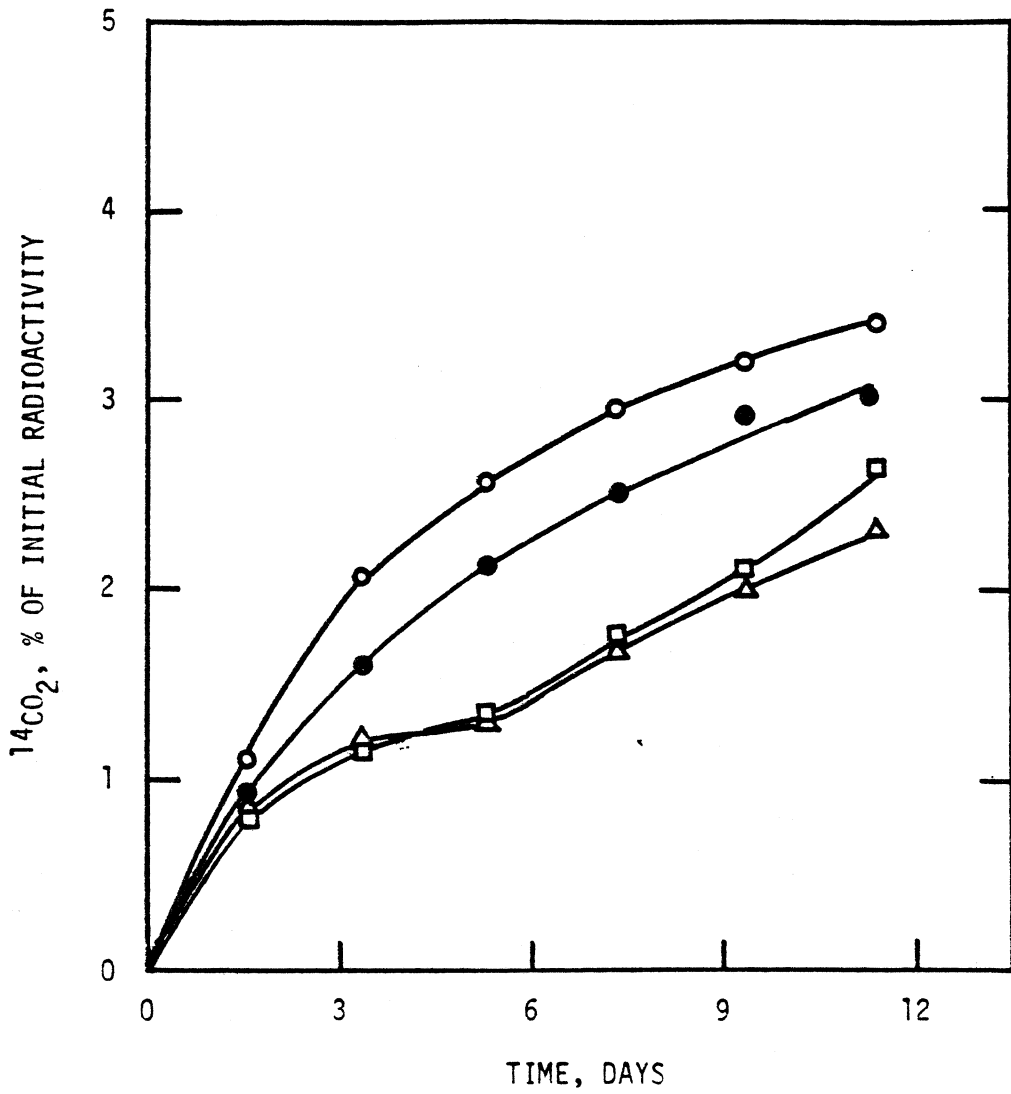


Figure 43. Investigation of glucose catabolite repression in lignin metabolism by *C. versicolor*. II.

Initial glucose concentration : ○—○ 0.75 g/l,

●—● 1 g/l, □—□ 5 g/l, ▲—▲ 10 g/l.

differences among initial rates of $^{14}\text{CO}_2$ evolution at various initial glucose concentrations were, however, either statistically insignificant or not too great.

Fig. 44 depicts the variation in $^{14}\text{CO}_2$ evolution rates as a function of time for different initial glucose concentrations. These rates indicate instantaneous values and were computed by drawing a tangent to the smoothened curves of $^{14}\text{CO}_2$ vs. time. At 0.5 and 1 day intervals, we see a notable decrease in the rates of $^{14}\text{CO}_2$ evolution as we go from low to high glucose concentrations. The slightly anomalous behavior of the plot at 0.5 day can be attributed to the fact that the $^{14}\text{CO}_2$ evolution rates plotted are instantaneous and the first $^{14}\text{CO}_2$ flushing was not carried out until 1.5 days after inoculation. As seen in earlier fermentations, the rate of $^{14}\text{CO}_2$ evolution always decreased as the fermentation progressed.

As the glucose concentration decreases through consumption, the flasks with originally high glucose concentrations should show a substantial increase in lignolytic activity since glucose catabolite repression, if it existed, would then have been circumvented. No such increase in $^{14}\text{CO}_2$ evolution during the latter part of the fermentation was observed from flasks containing initially high glucose concentrations. Hence, it can be concluded that no true catabolite repression by glucose exists in lignin metabolism by C. versicolor.

How do we then account for the fact that we see higher lignolytic activities at low initial glucose concentrations and with recalcitrant carbohydrates than at high initial glucose concentrations and with

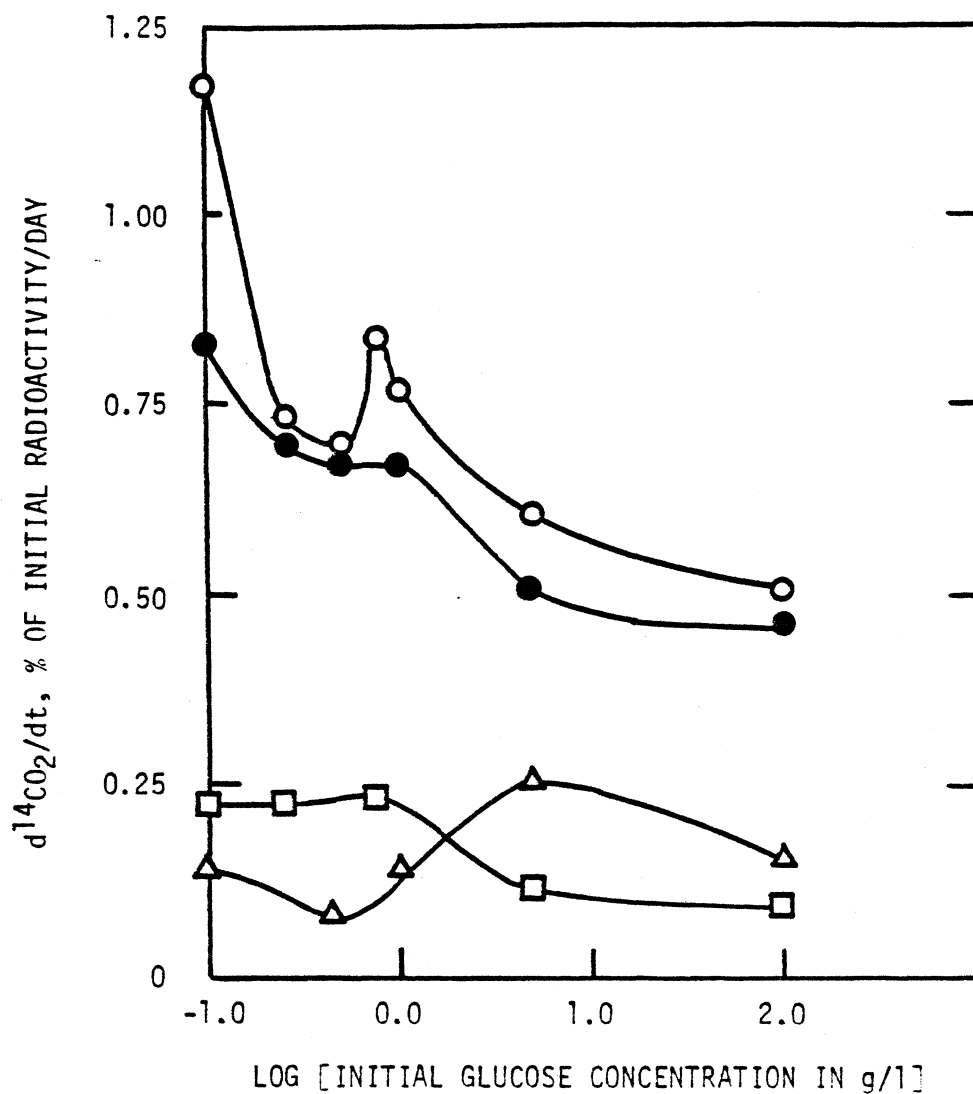


Figure 44. Rates of $^{14}\text{CO}_2$ evolution during lignin fermentations having different initial glucose concentrations.

○—○ 0.5 day, ●—● 1 day, □—□ 5 days,
 △—△ 8 days.

easily metabolized carbohydrates, respectively? The history of the organism may be the answer. Whence the low free carbohydrate concentration is approached may be important. If such is the case, the reason that flasks containing initially high glucose concentrations do not show high lignolytic activity even when the glucose concentration has dropped to a low level may be the "direction" in which the low glucose concentration is approached. When we consolidate the above observations, a phenomenon similar to hysteresis seems to emerge. Fig. 45 pictorially explicates the above hypothesis claiming the history of the organism to be important.

Since low initial glucose concentrations support higher lignolytic activity than high initial glucose concentrations, we can envisage a critically high initial glucose concentration at which lignolytic activity will be infinitesimal. On the other hand, since no lignin metabolism takes place in the absence of carbohydrates, we can also envision a threshold concentration of free carbohydrate or glucose below which lignolytic activity will be vanishingly small. These two critical, albeit hypothetical, glucose concentrations are shown in Fig. 46. The operating glucose concentration then will be somewhere in between the two extremes.

Glucose and nitrogen concentrations were also followed during this investigation of a possible phenomenon of glucose catabolite repression. Problems encountered during the iodine-starch assay also plagued the glucose oxidase assay for glucose. Hence, the glucose concentration vs. time plots represent only the relative and not

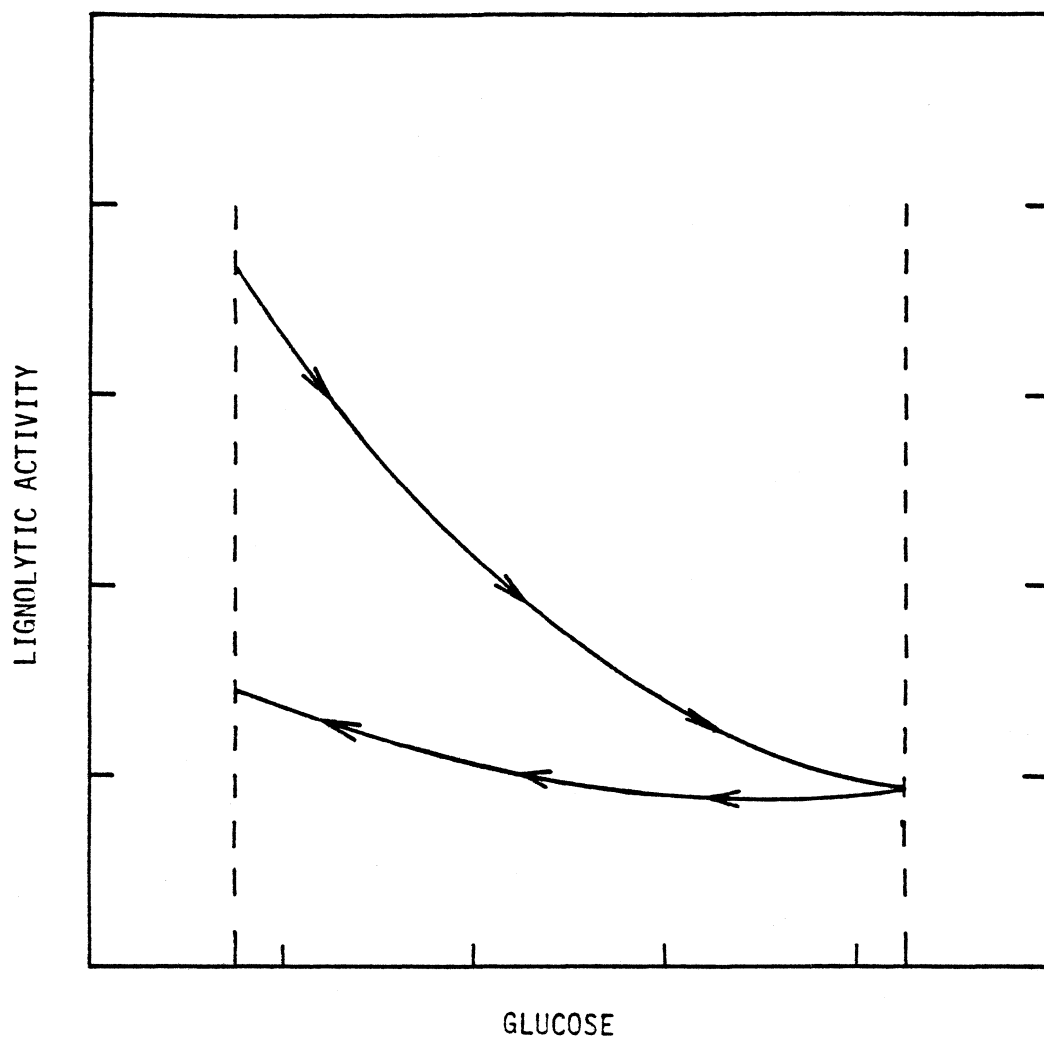


Figure 45. History of organism and lignin metabolism. Arrows denote the direction in which a certain glucose concentration is approached.

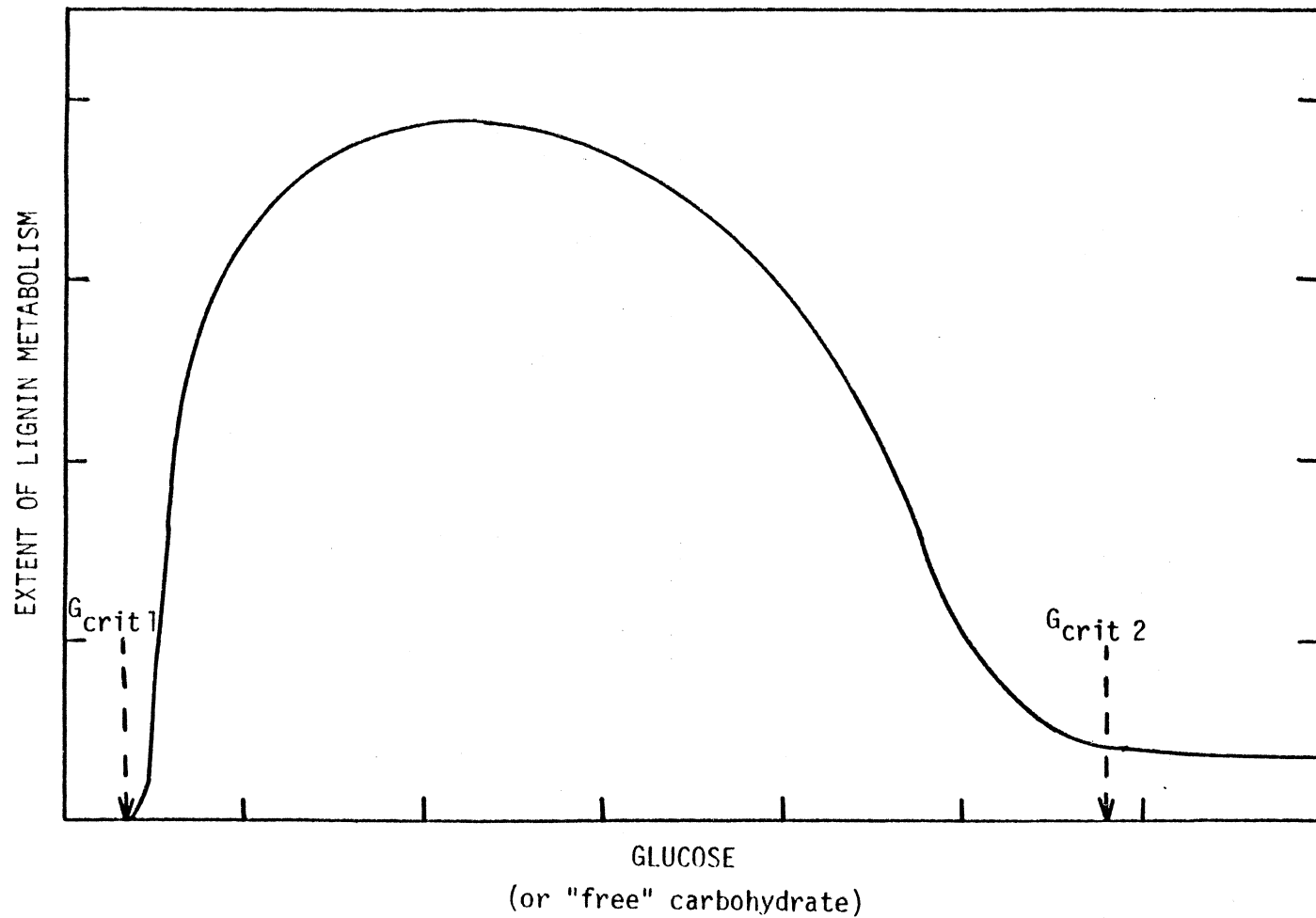


Figure 46. Lignin metabolism and free carbohydrate concentration: A hypothetical case depicting critical glucose concentrations.
 $G_{crit\ 1}$: lower critical concentration, $G_{crit\ 2}$: upper critical concentration.

absolute values of glucose availability. Nitrogen concentrations were followed by using an ammonia electrode. Glucose and nitrogen depletion data were obtained from corresponding flasks containing unlabeled lignin instead of ^{14}C -labeled lignin. The concentrations of all species in corresponding flasks were exactly the same. Nitrogen depletion and glucose depletion curves followed the same pattern for a given condition (Figs. 47, 48 and 49). C/N ratio curves also behaved in a fashion similar to nitrogen and glucose depletion curves except for the case where the initial glucose concentration was 10 g/l. In the last mentioned case, a maximum in the C/N ratio vs. time curve was seen at 4.5 days and an increase in $^{14}\text{CO}_2$ evolution rate was also noticed at the same time (Fig. 49). However, in the case where the initial glucose concentration was 5 g/l, no change in C/N ratio was apparent at 4.5 days even though $^{14}\text{CO}_2$ evolution rate increased at the same time. Hence, the lack of a correlation between C/N ratio and lignolytic activity was confirmed.

L. Mixed and Successive Fermentations

Lignin is degraded in nature by several wood-inhabiting and other microorganisms acting in conjunction and succession. It is, therefore, sound engineering to design lignin fermentations which emulate nature, at least in part. Brown-rot and white-rot fungi comprise a significant portion of the microbial population responsible for wood decay. Intuitively, we have every reason to

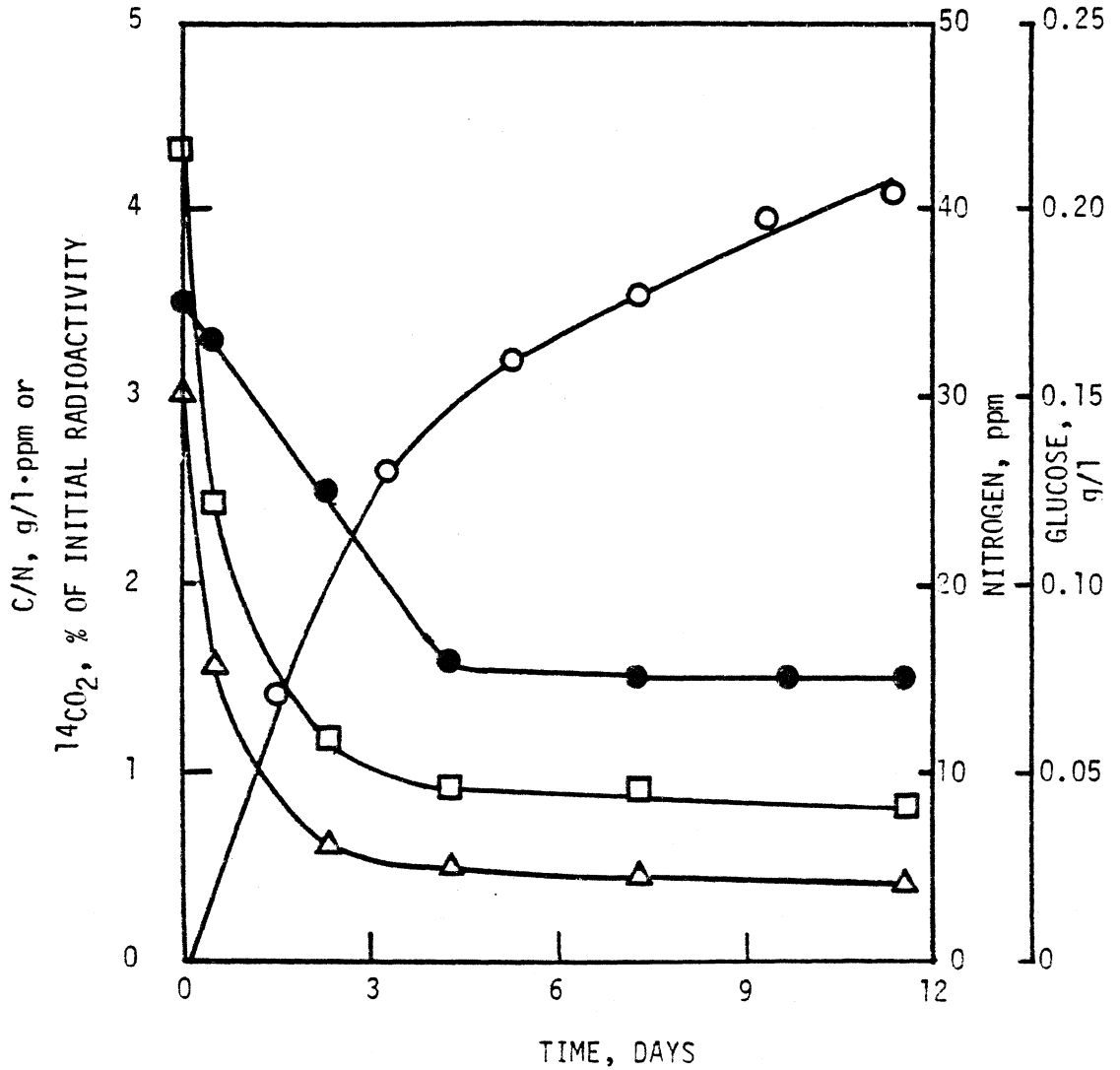


Figure 47. Parameters of a lignin fermentation by *C. versicolor*: Initial glucose concentration of 1 g/l.

○—○ $^{14}\text{CO}_2$, ●—● nutrient nitrogen,
 □—□ C/N ratio $\times 10^3$, △—△ glucose.

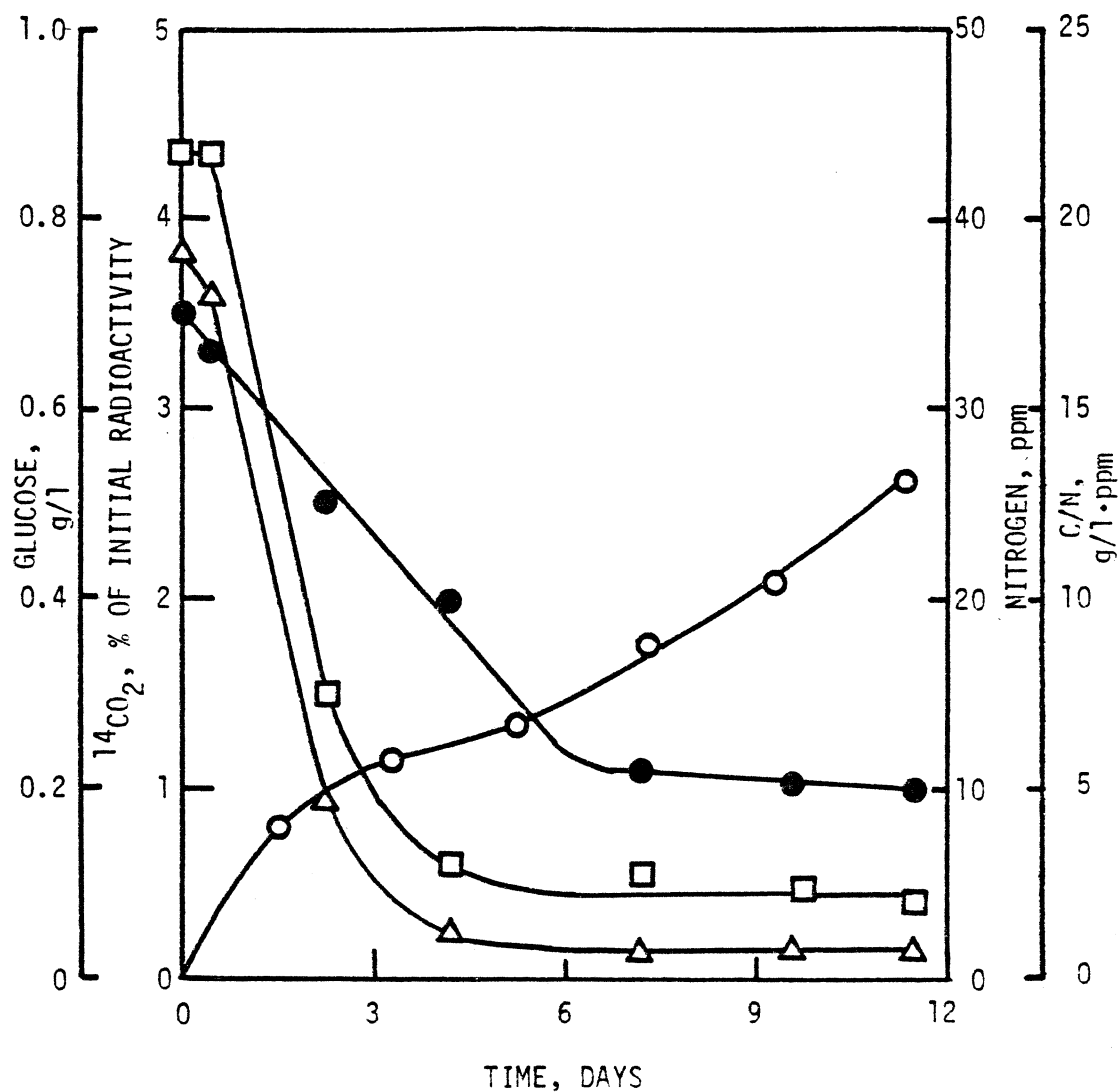


Figure 48. Parameters of a lignin fermentation by *C. versicolor* : Initial glucose concentration

○—○ $^{14}\text{CO}_2$, ●—● nutrient nitrogen,
 □—□ C/N ratio $\times 10^3$, △—△ glucose.

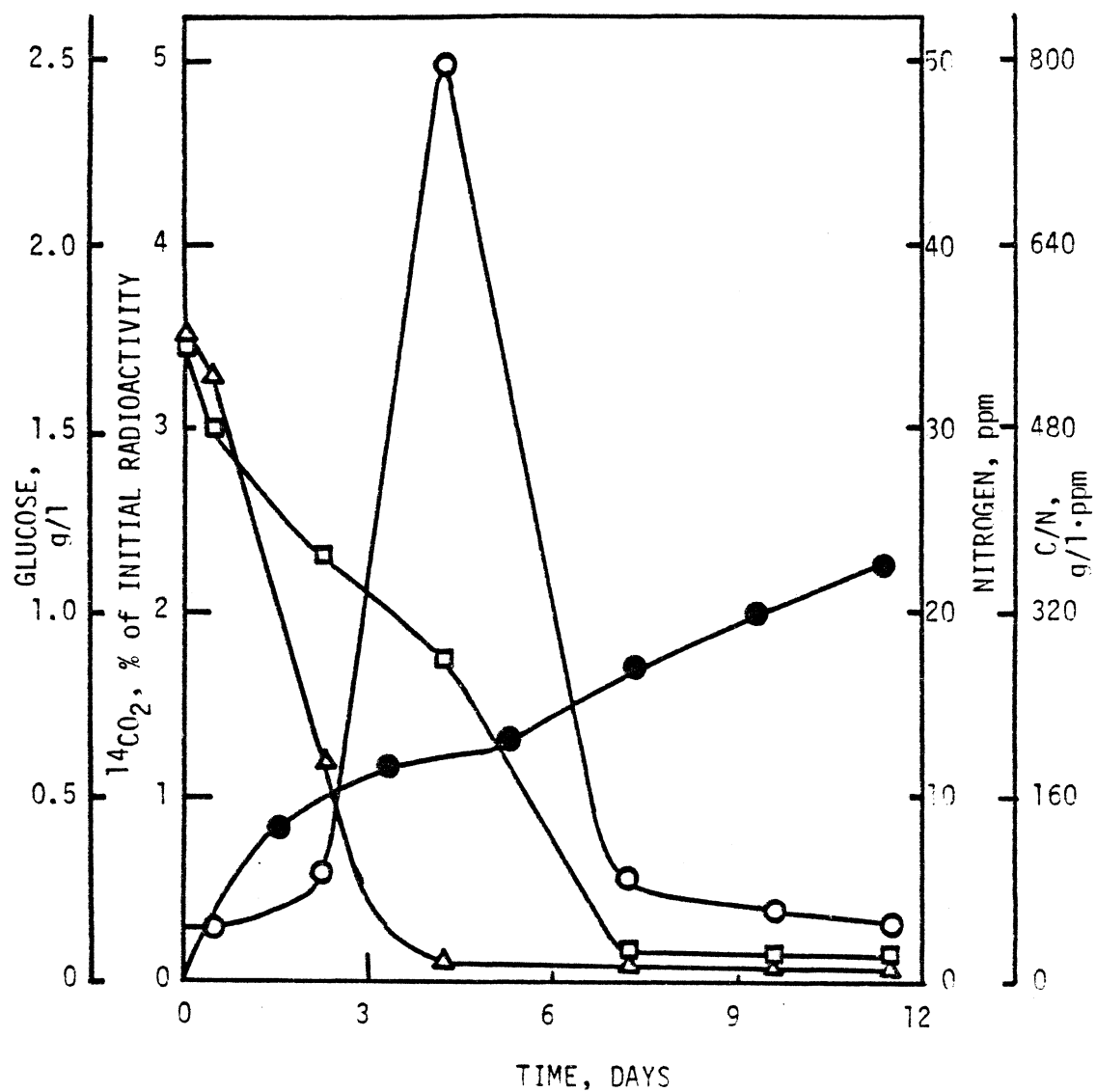


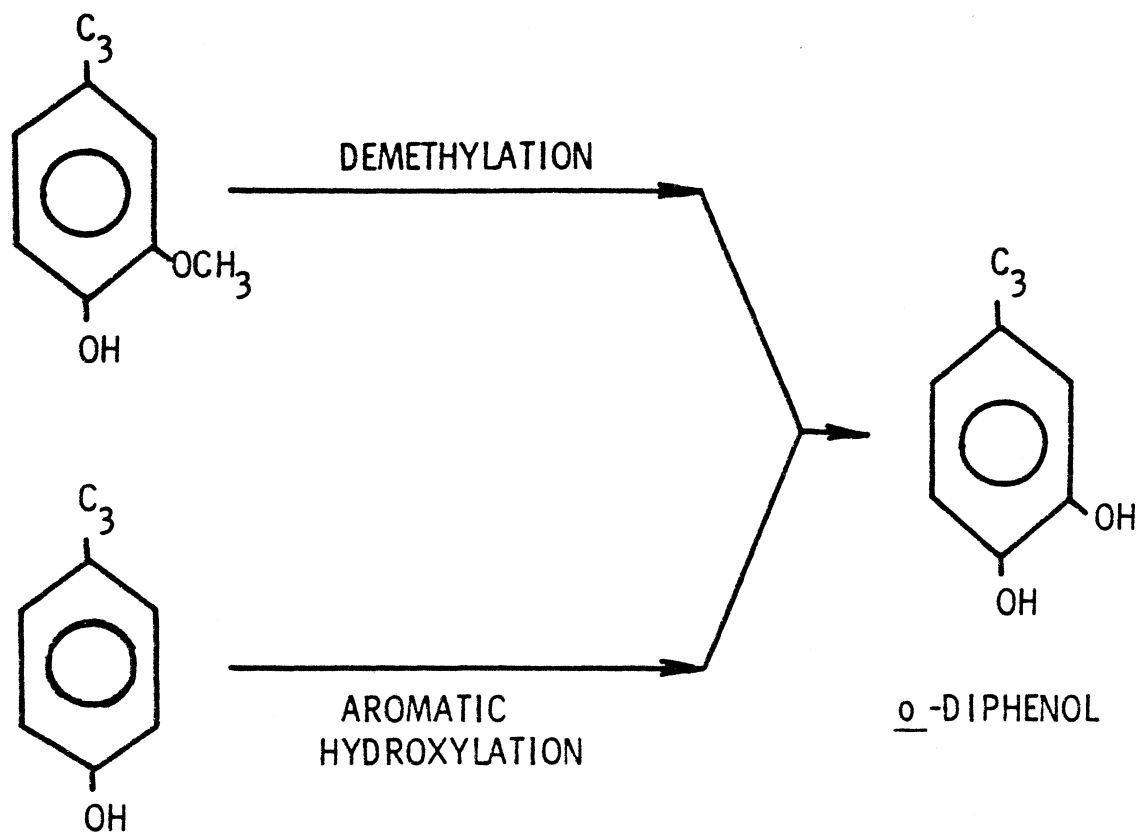
Figure 49. Parameters of a lignin fermentation by *C. versicolor*: Initial glucose concentration of 10 g/l.

○—○ C/N ratio $\times 10^3$, ●—● $^{14}\text{CO}_2$,
 □—□ glucose, △—△ nutrient nitrogen.

suspect an interdependence or a symbiotic relation between the above two types of fungi as they exist in the natural habitat.

1. Rationale: Kirk (1975) has postulated that the rate-limiting step in lignin metabolism is the formation of o-diphenol structures. Monooxygenases are the enzymes which create o-diphenol structures in the lignin polymer (Fig. 50) which are subsequently cleaved by dioxygenases (Fig. 5). The o-diphenol structures can be formed by either demethylation of methoxyl groups or by aromatic hydroxylation as shown in Fig. 50. It should be pointed out that though the aromatic rings shown in Fig. 50 possess a C₃ side chain, some monooxygenases can be quite persnickety about the side chain length. Thus, if necessary, the side chain will have to be chopped off to an appropriate length before a monooxygenase can act.

Brown-rot fungi, while attacking wood, completely metabolize cellulose but leave almost all of the lignin behind only in a slightly modified form. The major change brought about by brown-rot fungi is the demethylation of lignin. Hence, one can expect a preponderance of o-diphenolic units in lignin which has been acted upon by brown-rot fungi. Kirk (1975a) reported that lignin degradation by Lenzites trabea, a brown-rot fungus, was largely oxidative, and demethylation of both phenolic and non-phenolic units was the major degradative action. He further stated that about 35% of the total C₉-units in the brown-rotted lignin were demethylated and since brown-rot fungi lack a ring-cleaving enzyme system, some o-diphenol structures persisted.



ENZYME : MONOOXYGENASES

Figure 50. Formation of o-diphenol structures during lignin metabolism.

Through the confluence of Kirk's (1975) hypothesis and the knowledge of modus operandi of brown-rot fungi, we can devise a two-stage fermentation involving a brown-rot and a white-rot fungus. In the pretreatment stage, lignin can be acted upon only by a brown-rot fungus and the hypothetically rate-limiting step of o-diphenol formation can be carried out. In the following stage, lignin rich in o-diphenolic structures can be treated by a white-rot fungus which should metabolize pretreated lignin at a much faster rate than it would metabolize untreated lignin.

2. P. placenta and C. versicolor Fermentations: The concept of pretreating lignin with a brown-rot fungus was tested by carrying out mixed and successive fermentations with Poria placenta, a brown-rot fungus, and C. versicolor. In the mixed fermentation, P. placenta and C. versicolor were cultivated together in a medium containing soluble starch and ^{14}C -labeled lignin. In the successive fermentation, ^{14}C -labeled lignin was acted upon by P. placenta alone for the first 5.6 days followed by the addition of C. versicolor alone or C. versicolor with supplemental starch and nitrogen source. Flasks containing C. versicolor alone and P. placenta alone were included as controls.

No stratling results were seen either in the mixed or the successive fermentation (Fig. 51). The mixed fermentation, which was not statistically different from the pure culture fermentation with C. versicolor in overall performance, demonstrated the highest initial

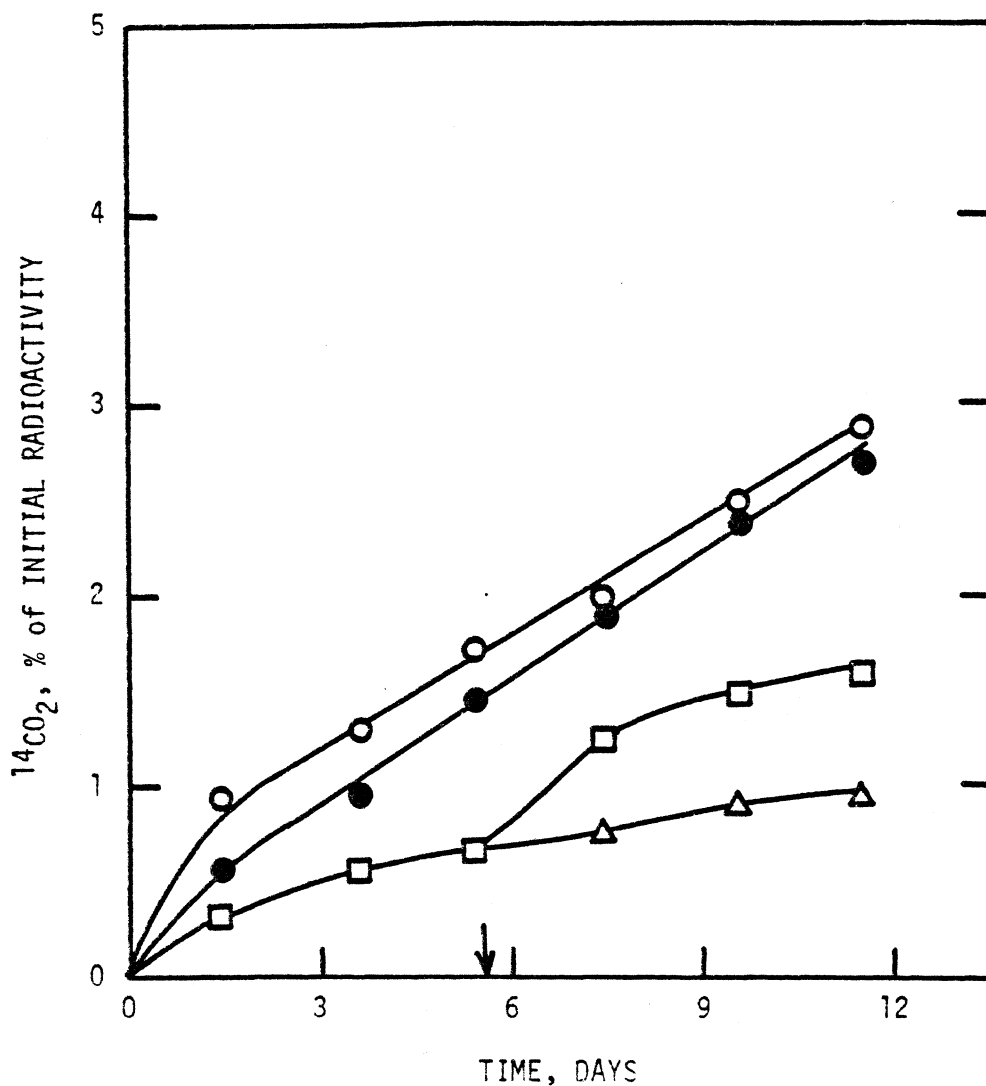


Figure 51. Mixed and successive fermentations with *P. placenta* and *C. versicolor*.

- *P. placenta* + *C. versicolor* (mixed fermentation),
- *C. versicolor* alone, □—□ *P. placenta*
- followed by *C. versicolor* (successive fermentation),
- △—△ *P. placenta* alone, ↓ indicates addition of
- C. versicolor* in a successive fermentation.

rate of $^{14}\text{CO}_2$ evolution. The successive fermentation was not affected by the addition of supplemental nutrients or lack thereof. Hence, a single curve represents both cases in Fig. 51. The successive fermentation showed much lower $^{14}\text{CO}_2$ evolution rate at the beginning of the second stage (see $\downarrow$ in Fig. 51) than the initial $^{14}\text{CO}_2$ evolution rates seen in the mixed or C. versicolor fermentation. The ineffectiveness of mixed and successive fermentations is probably due to the fact that the rate of demethylation by brown-rot is a very slow process. Thus, 5.6 days of lignin pretreatment by P. placenta may not amount to much demethylation. Furthermore, C. versicolor, which is a rather inefficient lignin-degrader, may not be able to effectively capitalize on whatever little amount of o-diphenols formed by P. placenta. If a more potent lignin-degrader than C. versicolor is used along with a longer period of pretreatment by P. placenta or an even more efficacious brown-rotter, the endeavors of developing lignin fermentations to mimick nature should prove fruitful.

V. CONCLUSIONS

This investigation of lignin biotransformation was different from studies in the literature on the same topic insofar as it looked at the attendant problem from a process engineering point of view. Though the primary interest was in the applied aspects of lignin biodegradation, some basic questions were also raised and tackled. The development of a kinetic model for lignin catalysis was the first attempt of its kind. The model, with its obvious limitations, gives us a mathematical picture of events in lignin fermentation and may inspire more sophisticated models.

The following are the general conclusions of this study:

- i. There is no net accumulation of lignin-derived low molecular weight metabolites in lignin fermentation. This severely affects the economic feasibility of lignin bioprocessing.
- ii. Recalcitrant carbohydrates and low initial glucose levels support higher lignolytic activities than do easily metabolized carbohydrates and high initial glucose levels, respectively.
- iii. There is no glucose catabolite repression, in the true sense, during lignin fermentations.
- iv. Nitrogen levels affect lignin metabolism but there is no correlation between C/N ratio per se and lignin metabolism.
- v. The ratio of lignin derived CO_2 /carbohydrate derived CO_2

varies during a lignin fermentation. Thus, lignin metabolism may not be stoichiometrically linked with carbohydrate metabolism.

- vi. There is a good correlation between lignin derived CO_2 /total CO_2 and the rate of $^{14}\text{CO}_2$ evolution from ^{14}C -labeled lignin.
- vii. Cell-lignin contact may be important in lignin degradation. A kinetic model based on the above assumption was developed and the consequences of the model are in general agreement with real observations.
- viii. The response to temperature and nitrogen levels is different during A. fumigatus fermentations than during P. chrysosporium fermentations. A. fumigatus, a soft-rot fungus, may have a different type of regulatory control than P. chrysosporium, a white-rot fungus.
- ix. A. fumigatus brings about drastic changes in the lignin structure whereas C. versicolor does very little to the lignin macromolecule.
- x. The OCH_3 loss, OH loss and $^{14}\text{CO}_2$ from ^{14}C -labeled lignin data are compatible with each other.
- xi. A batch or semibatch lignin fermentation with live cells is the most practical mode of operation for studying a relatively large system.
- xii. Lignolytic efficiency and normalized lignolytic efficiency are indices of the performance of a large-scale fermentation and can be used to study standardized fermentations.

- xiii. Lignin pretreatment by brown-rot fungi may be an important process innovation though mixed and successive fermentations with P. placenta and C. versicolor were not successful. The effectiveness of the above pretreatment may be improved by identifying a potent brown-rot fungus which is optimal in demethylating lignin.
- xiv. A. fumigatus can be used to decolorize the black liquor (which contains lignin) from paper mills.
- xv. Lignin utilization via biotransformation as it stands is not economically feasible since no valuable products in quantity are produced. Genetic engineering is necessary to create mutants which will accumulate the desired products.

VI. RECOMMENDATIONS

Since lignin biodegradation is a fledgling field there is a wide scope for improvement over the present state of the knowledge. Analytical difficulties and the absence of basic information regarding lignin metabolism are the two major obstacles in the development of lignin bioprocessing systems. Nevertheless, the following recommendations should help pave the way toward an efficient bioprocess for lignin utilization.

- i. A. fumigatus can be possibly restored to its once prodigious lignin degradation capacity. This may be achieved by subjecting the current culture to UV light and monitoring lignin degradation visually by an agar-plate assay described by Sundman and Näse (1971). Cultures suspected of high lignolytic activity can then be tested with ^{14}C -labeled lignin.
- ii. An organism efficient in $^{14}\text{CO}_2$ production from ^{14}C -labeled lignin may be genetically manipulated to yield a mutant which will accumulate low molecular weight compounds derived from lignin. This may be a difficult task since there is no simple assay for detecting the accumulation of such compounds. Mutants can be selected by visually ascertaining their lignin degradation capacity as described in i. Such mutants can then be tested in a dialysis fermentation for their ability to accumulate lignin-derived low molecular weight compounds.

- iii. If a low molecular weight pool of lignin-derived compound exists, its nature as to static or dynamic can be determined using a dialysis fermentation with ^{14}C -labeled lignin as a substrate. If the dialysis bags are changed every hour or so, the nature of the low molecular weight pool may become evident. If the low molecular weight compounds derived from lignin are present in the fermentation broth for more than an hour and the equilibration of such compounds between the broth and the dialysis bag takes place in a time period considerably shorter than an hour then the above approach should yield an answer. If the half-life of such compounds is of the order of seconds or minutes, the above approach will not work.
- iv. Low initial glucose concentrations and recalcitrant carbohydrates seem to support relatively high lignolytic activity. A deep-tank fermentation can be operated in a semibatch mode where glucose is continuously added to maintain a low glucose concentration. The effectiveness of this system can be judged by lignolytic and normalized lignolytic efficiencies. Degraded lignin can be analyzed for OCH_3 loss and other structural details to further study the efficiency of the above fermentation.
- v. $^{14}\text{CO}_2$ evolution and the possible production of low molecular weight ^{14}C -labeled compounds from ^{14}C -labeled lignin is a sound measure of lignin degradation. This criterion, however, can be improved by simultaneously following typical monooxygenase and dioxygenase activities. Since these oxygenases are thought to

be mostly membrane-bound both intracellular as well as extracellular activities should be followed. Flasks containing ^{14}C -labeled lignin can be simultaneously incubated with corresponding flasks containing unlabeled lignin and the latter can be used to prepare extracellular broths and cell extracts required for the enzyme assays. It can also be advantageous to follow activities of carbohydrate degrading enzymes such as cellulases when cellulose is used as the cosubstrate. These enzyme activities may shed some light on variations in $^{14}\text{CO}_2$ evolution rates and ratios of lignin derived CO_2 /carbohydrate derived CO_2 .

- vi. The development of a mutant not requiring or requiring only trace amounts of a growth substrate for lignin degradation will certainly be a breakthrough. Again, the visual agar-plate assay can be used for this purpose but the probability of creating such a mutant may be very slim.
- vii. The idea of mixed or successive fermentations sounds very promising and should prove practical with an active brown-rot fungus, possibly Lenzites trabea, and a potent lignin-degrader. The duration of treatment by the brown-rot fungus can be optimized for maximum demethylation. Using several brown-rot and white-rot fungi may further ameliorate lignin metabolism.
- viii. The practicability of treating black liquor with A. fumigatus should be further explored.

The previous recommendations focus mainly on microbial treatment of lignin. Since chemical or microbial treatment of lignin does not sound promising individually, a combined treatment may be beneficial. Stern and Gassner (1974) have studied lignin treatment by a combination of ozonation and microbial attack. A thorough search for a suitable chemical-pretreatment of lignin will be worthwhile. Such a chemical pretreatment may eliminate the apparent necessity for genetic manipulation of lignin-degrading microorganisms for the production of low molecular weight products.

VII. SUMMARY

Though the potential of renewable resources for providing essential chemicals has been recognized for some time, the research toward developing a viable technology for utilization of lignocellulosics has gathered momentum only recently. Enzymatic hydrolysis of cellulose for the production of glucose and ethanol has been proven feasible on pilot-plant scales. Lignin biodegradation, on the other hand, is poorly understood and lignin utilization through enzymatic treatment is in the developmental stage. This work has approached the concept of lignin bioprocessing from a biochemical engineering viewpoint.

The economics of a future process for lignin biotransformation would depend on products of the process and their marketability. During modified dialysis fermentations, none of the lignin-degrading fungi tested gave rise to any net accumulation of lignin-derived products of low molecular weight. The A. fumigatus strain with a low activity also failed to accumulate any lignin-derived metabolites. Thus lignin biodegradation is different from cellulose biodegradation which yields glucose in appreciable quantities. These observations seriously affect the viability of lignin bioprocessing. Genetic manipulation of lignin-degrading fungi is necessary to engineer the overproduction of desired intermediates.

The absolute requirement for carbohydrates during lignin metabolism by white-rot and other lignin-degrading fungi has a severe impact on

the operation and economics of lignin fermentations. Recalcitrant carbohydrates such as cellulose supported higher lignolytic activity than did easily metabolized carbohydrates like cellobiose and glucose. Though low initial glucose levels fostered higher lignolytic activity than did high initial glucose levels, no increase in lignolytic activities was seen when the glucose concentration dropped to a low level in C. versicolor cultures which originally had a high glucose concentration. Thus, glucose catabolite repression does not seem to exist in lignin metabolism by C. versicolor. It was hypothesized that the effect of carbohydrate on lignin metabolism may depend on the history of the organism. Through the amalgamation of the absolute requirement for carbohydrates during lignin metabolism and the ability of low initial glucose concentrations to sustain more efficient lignin metabolism than high initial glucose concentrations, the existence of two critical, upper and lower, glucose concentrations in lignin metabolism was postulated.

Lignin metabolism in relation to carbohydrate metabolism was studied by following the ratio of lignin derived CO_2 /total CO_2 . Contrary to intuition, the above ratio was variable during lignin fermentations by A. fumigatus and C. versicolor, thus making it possible for lignin metabolism not to be stoichiometrically linked with carbohydrate metabolism. The plot of lignin derived CO_2 /total CO_2 vs. time showed a maximum for the A. fumigatus fermentation. Since lignin derived CO_2 /total CO_2 is an index of efficiency of gross lignin metabolism, the ratio can be used in determining optimal harvest time of a standardized batch fermentation.

A batch fermentation with viable cells appears to be the most practical mode of lignin biodegradation. A multi-stage fermentation consisting of growth, induction and biotransformation stages was devised to study lignin degradation in a large-scale system. Two new parameters, lignolytic efficiency and normalized lignolytic efficiency, were defined to evaluate the performance of large fermentations. Lignolytic efficiency corresponded well with the biomass during an A. fumigatus fermentation. Normalized lignolytic efficiency, which can be looked upon as an intrinsic lignolytic capacity of the organism, was found to decrease continually during the fermentation. This was reminiscent of continually decreasing $^{14}\text{CO}_2$ evolution rates during ^{14}C -labeled lignin fermentations. Lignolytic and normalized lignolytic efficiencies can be employed in studying the characteristics of large-scale lignin fermentations.

Structural analyses of degraded lignins revealed that A. fumigatus brought about corporeal changes in the lignin macromolecule whereas C. versicolor was responsible for only cursory changes. The OCH_3 loss, OH loss, $^{14}\text{CO}_2$ evolution and permanganate oxidation data were in concurrence within experimental limits.

A. fumigatus responded to nitrogen concentration variations differently than did P. chrysosporium. The relation, or lack thereof, between C/N ratio and lignin metabolism by A. fumigatus could not be studied since the organism lost its originally high lignolytic capacity. The loss of lignolytic activity was believed to be due to strain degeneration. C. versicolor fermentations were grossly insensitive to such parameters as nitrogen concentration and C/N ratio.

The study of hypha-lignin contact in natural wood decay (Kirk, 1975) and analyses of cell-associated lignin (Drew et al., 1978) tend to suggest that cell-lignin contact may be essential in lignin degradation. Based on the above conjecture, a kinetic model for lignin biodegradation was developed. The model, which stems from traditional heterogeneous catalysis, is qualitatively in accordance with the real situation.

In an attempt to capitalize on the interplay that may exist between white-rot and brown-rot fungi in their natural habitat, mixed and successive fermentations involving the above types of fungi were designed. Though the particular fermentations with P. placenta and C. versicolor proved unsuccessful, the idea of devising lignin fermentations to simulate, at least in part, the natural situation is worth pursuing.

Though the treatment of black liquor by A. fumigatus to remove lignin seems practical, the microbial treatment of lignin in general is economically unattractive at present. This picture may change as strides are made toward unravelling the lignin degradation pathways and as chemicals from fossil fuels become increasingly expensive.

VIII. BIBLIOGRAPHY

- Ander, P. and K.-E. Eriksson. Influence of carbohydrates on lignin degradation by the white-rot fungus Sporotrichum pulverulentum. Sven. Papperstidn., 18, 643-652, 1975.
- Ander, P. and K.-E. Eriksson. The importance of phenol oxidase activity in lignin degradation by the white-rot fungus Sporotrichum pulverulentum. Arch. Microbiol., 109, 1-8, 1976.
- Ander, P. and K.-E. Eriksson. Lignin degradation and utilization by microorganisms. In: Progress in Industrial Microbiology, vol. 14, ed. M. J. Bull. Elsevier Scientific Publishing Company, Amsterdam, The Netherlands, 1978.
- Blaich, R. and K. Esser. Function of enzymes in wood destroying fungi. II. Multiple forms of laccase in white rot fungi. Arch. Microbiol., 103, 271-277, 1975.
- Cain, R. B., R. F. Bilton and J. A. Darrah. The metabolism of aromatic acids by microorganisms. Biochem. J., 108, 797-828, 1968.
- Carberry, J. J. Chemical and Catalytic Reaction Engineering. McGraw-Hill Book Co., New York, 1976.
- Cartwright, N. J. and K. S. Holdom. Enzymic lignin, its release and utilization by bacteria. Microbios, 8, 7-14, 1973.
- Christman, R. F. and R. T. Oglesby. Microbiological degradation and the formation of humus. In: Lignins: Occurrence, Formation, Structure and Reactions, ed. K. V. Sarkanen and C. H. Ludwig. Wiley-Interscience, New York, 1971.
- Cochran, T. W. and J. R. Vercellotti. Hexosamine biosynthesis and accumulation by fungi in liquid and solid media. Carbohydrate Res., 61, 529-543, 1978.
- Cowling, E. B. Comparative biochemistry of the decay of sweetgum sapwood by white-rot and brown-rot fungi. USDA Tech. Bull., 1258, 79, 1961.
- Crawford, D. L. Lignocellulose decomposition by selected Streptomyces strains. Appl. Environ. Microbiol., 35, 1041-1045, 1978.

- Crawford, D. L. and R. L. Crawford. Microbiol degradation of ligno-cellulose: The lignin component. Appl. Environ. Microbiol., 31, 714-717, 1976.
- Crawford, R. L. and P. E. Olson. Microbiol catabolism of vanillate: Decarboxylation to guaiacol. Appl. Environ. Microbiol., 36, 539-543, 1978.
- Dagley, S. Catabolism of aromatic compounds by microorganisms. Advan. Microbiol. Physiol., 6, 1-46, 1971.
- Drew, S. W., K. L. Kadam, S. P. Shoemaker, W. G. Glasser and P. Hall. Chemical feedstocks and fuels from lignin. AIChE Symp. Ser., 74, 21-25, 1978.
- Elias, H.-G. Raw materials for the polymer industry. Part II: The unconventional situation. Chemtech, 6, 244-249, 1976.
- Eriksson, K.-E. Private communication, 1976.
- Eriksson, K.-E. and E. W. Goodell. Pleiotropic mutants of the wood-rotting fungus Polyporus adustus lacking cellulase, mannanase, and xylanase. Can. J. Microbiol., 20, 371-378, 1974.
- Eslyn, W. E., T. K. Kirk and M. J. Effland. Changes in the chemical composition of wood caused by six soft-rot fungi. Phytopath., 65, 473-476, 1975.
- Fahraeus, G. and G. Lindeberg. Influence of tyrosinase and some other substances on the laccase formation in Polyporus spec. Physiol. Plant., 6, 150-158, 1953.
- Falkehag, S. I. Lignin in materials. Appl. Polymer Symp., 28, 247-257, 1975.
- Flaig, W. and K. Haider. Utilization of phenolic compounds by white-rot fungi. Arch. Mikrobiol., 40, 212-223, 1961.
- Forney, L. J. and C. A. Reddy. Degradation of high molecular weight kraft lignin by strains of Pseudomonas. ASM Annual Meeting Abstracts, Q6, 1978.
- Fukuzumi, T. Enzymatic degradation of lignin. I. Paper-chromatographical separation of intermediate degradation products of lignin by the wood rotting fungus Poria subacida (Peck) Sacc. Bull. Agr. Chem. Soc. Jap., 24, 728-36, 1960.
- Gadd, O. Wood decay resulting from rot fungi. Pap. Puu., 39, 363-374, 1957.

- Gibson, D. T. Microbial degradation of aromatic compounds. Metabolic pathways to reveal a general formula for the degradation of several aromatic compounds. Science, 161, 1093-1097, 1968.
- Glasser, W. G. Private communication, 1978.
- Glasser, W. G. and H. R. Glasser. Private communication, 1978.
- Goheen, D. W. Low molecular weight chemicals. In: Lignins: Occurrence, Formation, Structure and Reactions, ed. K. V. Sarkanen and C. H. Ludwig. Wiley-Interscience, New York, 1971.
- Goring, D. A. I. Polymer properties of lignin and lignin derivatives. In: Lignins: Occurrence, Formation, Structure and Reactions, ed. K. V. Sarkanen and C. H. Ludwig. Wiley-Interscience, New York, 1971.
- Grandziel, K., K. Haider, J. Kochmanska, E. Malarczyk and J. Trojanowski. Bacterial decomposition of synthetic ^{14}C -labeled lignin and lignin monomer derivatives. Acta Microbiol. Pol., 27, 103-109, 1978.
- Green, T. R. Significance of glucose oxidase in lignin degradation. ASM Annual Meeting Abstracts, K130, 1977.
- Haider, K. and J. Trojanowski. Decomposition of specifically ^{14}C -labelled phenols and dehydropolymers of coniferyl alcohol as models for lignin degradation by soft and white rot fungi. Arch. Microbiol., 105, 33-41, 1975.
- Haider, K., J. Trojanowski and V. Sundman. Screening for lignin degrading bacteria by means of ^{14}C -labelled lignins. Arch. Microbiol., 119, 103-106, 1978.
- Harkin, J. M. and J. R. Obst. Demethylation of 2,4,6-trimethoxyphenol by phenol oxidase. A model for chromophore formation in wood and pulp. TAPPI, 57, 118-121, 1974.
- Hata, K. Investigations on lignins and lignification. XXXIII. Studies on lignins isolated from spruce wood decayed by Poria subacida Bll. Holzforschung, 20, 142-147, 1966.
- Henderson, M. E. K. Release of aromatic compounds from birch and spruce sawdusts during decomposition by white-rot fungi. Nature, 175, 634-635, 1955.
- Higuchi, T. Biochemical study of wood-rotting fungi. I. Studies on the enzymes which cause Bavendamm's reaction. J. Jap. Forest. Soc., 35, 77-85, 1953.

- Higuchi, T. Formation and biological degradation of lignins. In: Advances in Enzymology, vol. 34, ed. F. F. Nord. Wiley-Interscience, New York, 1971.
- Hurst, H. M. and N. A. Burges. Lignin and humic acids. In: Soil Biochemistry, ed. A. D. McLaren and G. H. Peterson. Marcel Dekker, Inc., New York, 1967.
- Hsu, O. H.-H. and W. G. Glasser. Polyurethane foams from carboxylated lignins. Appl. Polymer Symp., 28, 297-307, 1975.
- Ishihara, I. and M. Miyazaki. Demethylation of lignin and lignin models by fungal laccase. J. Jap. Wood Res. Soc., 20, 39-41, 1974.
- Ishikawa, H., W. J. Schubert and F. F. Nord. Investigations on lignin and lignification. XXVII. The enzymic degradation of softwood lignin by white-rot fungi. Arch. Biochem. Biophys., 100, 131-139, 1963.
- Ishikawa, H., W. J. Schubert and F. F. Nord. Lignins and lignification. XXX. Enzymic degradation of guaiacyl glycerol and related compounds by white-rot fungi. Biochem. Z., 338, 153-163, 1963a.
- Kawakami, H. Bacterial degradation of lignin. 1. Degradation of MWL by Pseudomonas ovalis. Mokuzai Gakkaishi, 22, 252-257, 1976.
- Keyser, P., T. T. Kirk and J. G. Zeikus. Ligninolytic enzyme system of Phanerochaete chrysosporium: Synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol., 135, 790-797, 1978.
- Kirk, T. K. Effects of microorganisms on lignin. Ann. Rev. Phytopath., 9, 185-210, 1971.
- Kirk, T. K. Lignin-degrading enzyme system. Biotechnol. Bioeng. Symp., 5, 139-150, 1975.
- Kirk, T. K. Effects of a brown-rot fungus, Lenzites trabea, on lignin in spruce wood. Holzforschung, 29, 99-107, 1975a.
- Kirk, T. K. Physiological studies of lignin metabolism by white-rot fungi. In: Lignin Biodegradation: Microbiology, Chemistry and Potential Applications, ed. T. K. Kirk. CRC Press, Cleveland, Ohio, 1979.
- Kirk, T. K. and E. Adler. Methoxyl-deficient structural elements in lignin of sweetgum decayed by a brown-rot fungus. Acta Chem. Scand., 24, 3379-3390, 1970.

- Kirk, T. K. and H.-M. Chang. Decomposition of lignin by white-rot fungi. I. Isolation of heavily degraded lignins from decayed spruce. Holzforschung, 28, 217-222, 1974.
- Kirk, T. K. and H.-M. Chang. Decomposition of lignin by white-rot fungi. II. Characterization of heavily degraded lignin from decayed spruce. Holzforschung, 29, 56-64, 1975.
- Kirk, T. K., W. J. Connors, R. D. Bleam, W. F. Hackett and J. G. Zeikus. Preparation and microbial decomposition of synthetic [^{14}C] lignins. Proc. Nat. Acad. Sci. USA, 72, 2515-2519, 1975.
- Kirk, T. K., W. J. Connors and J. G. Zeikus. Requirement for a growth substrate during lignin decomposition by two wood-rotting fungi. Appl. Environ. Microbiol., 32, 192-194, 1976.
- Kirk, T. K., W. J. Connors and J. G. Zeikus. Advances in understanding the microbiological degradation of lignin. In: Recent Advances in Phytochemistry, vol. 11: Structure, Biosynthesis and Degradation of Wood, ed. F. A. Loewus and V. C. Runeckles. Plenum Press, New York, 1977.
- Kirk, T. K. and A. Kelman. Lignin degradation as related to the phenoloxidases of selected wood-decaying Basidiomycetes. Phytopath., 55, 739-745, 1965.
- Kirk, T. K., S. Larsson and G. E. Miksche. Aromatic hydroxylation resulting from attack of lignin by a brown-rot fungus. Acta Chem. Scand., 24, 3379-3390, 1970.
- Kirk, T. K. and L. F. Lorenz. Oxygenation of 4-alkoxyl groups in alkoxybenzoic acids by Polyporus dichrous. Appl. Microbiol., 26, 173-175, 1973.
- Kirk, T. K. and K. Lundquist. Comparison of sound and white-rotted sapwood of sweetgum with respect to properties of the lignin and composition of extractives. Svensk Papperstidn., 73, 294-306, 1970.
- Lai, Y. Z. and K. V. Sarkanen. Isolation and structural studies. In: Lignins: Occurrence, Formation, Structure and Reactions, ed. K. V. Sarkanen and C. H. Ludwig. Wiley-Interscience, New York, 1971.
- Larsson, S. and G. E. Miksche. Gas chromatographic analysis of lignin oxidation products. IV. The structure of birch lignin. Acta Chem. Scand., 25, 647-662, 1971.
- Lundquist, K. and T. K. Kirk. De novo synthesis and decomposition of veratryl alcohol by a lignin-degrading Basidiomycete. Phytochem., 17, 1676, 1978.

- Lyr, H. Detoxification of heartwood toxins and chlorophenols by higher fungi. Nature, 195, 289-299, 1962.
- Marton, J. Reactions in alkaline pulping. In: Lignins: Occurrence, Formation, Structure and Reactions, ed. K. V. Sarkanen and C. H. Ludwig. Wiley-Interscience, New York, 1971.
- Mason, H. S. Oxidases. Ann Rev. Biochem., 34, 595-634, 1965.
- Odier, E. and B. Monties. Biodégradation de la lignine de blé par Xanthomonas 23. Ann Microbiol. (Inst. Pasteur), 129A, 361-377, 1978.
- Robinson, L. E. and R. L. Crawford. Degradation of ^{14}C -labeled lignins by Bacillus megaterium. FEMS Microbiol. Letters, 4, 301-302, 1978.
- Sarkanen, K. V. and H. L. Hergert. Classification and distribution. In: Lignins: Occurrence, Formation, Structure and Reactions, ed. K. V. Sarkanen and C. H. Ludwig. Wiley-Interscience, New York, 1971.
- Sarkanen, K. V. and C. H. Ludwig. Definition and nomenclature. In: Lignins: Occurrence, Formation, Structure and Reactions, ed. K. V. Sarkanen and C. H. Ludwig. Wiley-Interscience, New York, 1971.
- Schubert, W. J. Lignin Biochemistry. Academic Press, New York, 1965.
- Selin, J.-F., V. Sundman and M. Räihä. Utilization and polymerization of lignosulfonates by wood-rotting fungi. Arch. Microbiol., 103, 63-70, 1975.
- Skoog, D. A. and D. M. West. Fundamentals of Analytical Chemistry. Holt, Rinehart and Winston, Inc., New York, 1963.
- Sopko, R. Ligninolytic activity of the white-rot fungus Pleurotus ostreatus. I. The influence of some nutrient solutions on the decomposition of lignin. Drev. Vysk., 2, 81-91, 1967.
- Stern, A. M. and L. L. Gasner. Degradation of lignin by combined chemical and biological treatment. Biotechnol. Bioeng., XVI, 789-805, 1974.
- Sundman, V. and L. Näse. A simple plate test for direct visualization of biological lignin degradation. Pap. Puu, 2, 67-71, 1971.
- Tichý, V. Decomposition of lignocellulose during successive action of the fungi Ganoderma applanatum and Serpula lacrymans. Folia Fac. Sci. Nat. Univ. Purkynianae Brunensis, Biologia 27, 11, 81-94, 1970.

- Tichý, V. An experimental study of fungus succession in wood. Ceska Mykologie, 29, 90-96, 1975.
- Tricart, G., S. N. Lo, J. J. Garceau and A. Cholette. Épuration biologique de la liquer au bisulfite dans un réacteur à deux stages en serie. Pulp. Paper Canada, 79, 84-89, 1978.
- Trojanowski, J., K. Haider and V. Sundman. Decomposition of ^{14}C -labelled lignin and phenols by a Nocardia sp. Arch. Microbiol., 114, 149-153, 1977.
- Trojanowski, J. and A. Leonowicz. The biodeterioration of lignin by fungi. Microbios, 3, 247-251, 1969.
- Walseth, C. S. The influence of fine structure of cellulose on the action of cellulases. TAPPI, 35, 233-238, 1952.
- Westermarck, U. and K.-E. Eriksson. Cellobiose : quinone oxidoreductase, a new wood-degrading enzyme from white-rot fungi. Acta Chem. Scand., B28, 209-214, 1974.
- Wilke, C. R. and G. Mitra. Process development studies on the enzymatic hydrolysis of cellulose. Biotechnol. Bioeng. Symp., 5, 253-274, 1975.
- Wood, J. M., R. L. Crawford, E. Münck, R. Zimmerman, J. D. Lipscomb, R. S. Stephens, J. W. Bromley, L. Que, J. B. Howard and W. H. Orme-Johnson. Structure and function of dioxygenases. One approach to lignin degradation. J. Agri. Food Chem., 25, 698-704, 1977.

**The two page vita has been
removed from the scanned
document. Page 1 of 2**

**The two page vita has been
removed from the scanned
document. Page 2 of 2**

MICROBIAL TRANSFORMATION OF LIGNIN

by

Kiran L. Kadam

(ABSTRACT)

Petroleum reserves are forecast to dwindle during the next century. The imminent shortage of chemicals derived from fossil fuels has given an impetus to research on utilization of renewable resources through microbial treatment. This work looks at the concept of lignin bioprocessing from the vantage point of a biochemical engineer.

Coriolus versicolor, Phanerochaete chrysosporium and Sporotrichum pulverulentum, which being white-rot fungi are traditional lignin degraders, were found to be much less efficient in lignin metabolism than Aspergillus fumigatus, a soft-rot fungus isolated in our laboratory. Lignin fermentations with white-rot fungi failed to show any net accumulation of low molecular weight products derived from lignin. Genetic manipulation is necessary to contrive the accumulation of desired intermediates during lignin fermentations.

Though recalcitrant carbohydrates and low initial glucose concentrations supported higher lignolytic activities than easily metabolized carbohydrates and high initial glucose concentrations, respectively, glucose catabolite repression was not evident during lignin metabolism by C. versicolor. It was surmised that the effect of carbohydrates on lignin metabolism may depend on the history of the organism. Through

the concretion of above observations and the absolute requirement for carbohydrates during lignin metabolism, the existence of upper and lower critical glucose concentrations in lignin metabolism was postulated.

Lignin metabolism relative to carbohydrate metabolism was studied by monitoring the ratio of lignin derived CO_2 /total CO_2 during A. fumigatus and C. versicolor fermentations. The variability of the above ratio during lignin fermentations suggested that lignin metabolism may not be stoichiometrically linked with carbohydrate metabolism. The rate of $^{14}\text{CO}_2$ evolution from ^{14}C -labeled kraft lignin and the ratio of lignin derived CO_2 /total CO_2 during A. fumigatus fermentation were concordant and each showed a maximum with respect to time.

Two new parameters, lignolytic efficiency and normalized lignolytic efficiency, were defined to evaluate the performance of large-scale lignin fermentations. During a deep-tank fermentation with A. fumigatus, lignolytic efficiency correlated well with biomass whereas normalized lignolytic efficiency decreased continually.

Analyses of degraded lignins divulged that A. fumigatus brought about corporeal changes in the lignin macromolecule whereas C. versicolor was responsible for only cursory changes. Data obtained from degraded lignins such as OCH_3 loss, OH loss and yield of monomers and dimers upon permanganate oxidation were consonant with $^{14}\text{CO}_2$ evolution data from ^{14}C -labeled lignin.

The effect of nitrogen concentration and C/N ratio on lignin metabolism by A. fumigatus could not be adequately studied since the

organism lost its once prodigious lignolytic capacity. C. versicolor fermentations were grossly insensitive to variations in nitrogen concentration and C/N ratio.

A kinetic model was developed on the premise that cell-lignin contact is imperative for lignin metabolism. The model is, in general, concordant with real observations. Though particular mixed and successive fermentations with Poria placenta and C. versicolor were not successful, the idea of such fermentations involving brown-rot, white-rot and other lignin-degrading fungi is worth investigating.

Though the treatment of black liquor to remove lignin and production of carboxylated lignin for copolymerization seem practical, the microbial treatment of lignin in general is economically unattractive at present.