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SEMI-ANNUAL PROGRESS REPORT

Report #6

June-December, 1951

For the Office of Naval Research
Microbiology Branch

PHYSIOLOGY OF THE WOOD-ROTTING FUNGI

Contract N6-onr-248, T. O. II,

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Syracuse University

Syracuse, New York

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GENERAL AND SPECIFIC OBJECTIVES

General Objectives

This research has as its broad objective a fundamental study of the nutrition and physiology of the wood-rotting fungi. These organisms -- classified as Basidiomycetes -- include the so-called "brown rots" and the "white rots" which primarily attack, respectively, the cellulose and the lignin of the cellulose-lignin complex of wood. It is hoped that these studies may contribute to the practical problems of the prevention of wood decay and of the fermentative utilization of carbohydrate materials (including waste cellulose), as well as extending fundamental scientific knowledge of the organisms.

Specific Objectives

More specifically, this investigation involves (1), a systematic study of the nutritional requirements of representative wood-rotting fungi under controlled conditions of artificial culture; and (2), study of various aspects of the physiology of selected organisms. Some 42 species of wood rots -- representative of different types involved in the decay of wood -- are presently under investigation.

Study of the nutritional requirements (1) involves, among other things, development of chemically-defined (synthetic) culture media for growth. This includes the qualitative needs of all the organisms for "trace" elements and inorganic salts, for nitrogen compounds and carbon compounds, and for vitamins or other nutrilites, and the quantitative characterization of these nutrients for the optimal growth of the organisms.

Study of the physiology (2) includes investigation of the cellulytic enzymes, which are responsible for the primary breakdown of wood;

utilization of various carbohydrates by the organisms; determination of the end-products of the fermentation of carbon compounds, including waste cellulosic materials; study of oxidation-reduction changes in culture and their relation to growth and fermentation; pH and temperature optima, and related problems.

The above studies of nutrition and physiology are fundamental to a rational approach to the control of wood decay and to the practical applications of the organisms in the fermentation of carbohydrate materials to economically valuable products.

LIST OF ORGANISMS USED

Although the names of the organisms studied have been given in previous reports, they have not been identified by culture number and source. Because of the importance of such characterization, this information is given herewith.

<u>Name</u>	<u>Culture #</u>	<u>Source</u>
Brown rots--		
Coniophora cerebella	# 2	Brazil**
Daedalea quercina	FP 57076-S	U.S.D.A.*
Fomes meliae	50336-R	"
Fomes officinalis	F 1276	"
Fomes roseus	Snell 11	"
Fomes subroseus	Snell 20	"
Hydnum pulcherrimum	81027-R	"
Hymenochaete sallei	# 3	Brazil**
Lentinus lepideus	534	U.S.D.A.*
Lenzites saepiaria	537	"
Lenzites striata	# 1	Brazil**
Lenzites trabea	539	U.S.D.A.*
Merulius lacrymans	FP 94365	"
Polyporus betulinus	53514-S	"
Polyporus immitus	FP 71384	"
Polyporus palustris	91452	"
Polyporus schweinitzii	71356-S	"
Polyporus spraguei	14857-S	"
Polyporus sulphureus	48603-S	"
Poria cocos	71051	"

List of organisms used (cont.)

<u>Name</u>	<u>Culture #</u>	<u>Source</u>
Brown rots (cont.)--		
Poria incrassata	563	U.S.D.A.*
Poria luteofibrata	FP 94373-	"
Poria monticola (originally microspora)	575	"
Poria nigra	71118	"
Poria oleraceae	198	"
Poria vaillantii	90877	"
Poria xantha	192-S	"
Ptychogaster rubescens	UIFP 716	"
Trametes malicola	71956	"
Trametes serialis	11977	"
White rots--		
Armillaria mellea	FP 46700	"
Fomes annosus	90898-R	"
Fomes fomentarius	59009-S	"
Fomes geotropus	# 9	Univ. of Md.***
Fomes pini	71757	U.S.D.A.*
Lentinus tigrinus	# 11	Univ. of Md.***
Peniophora gigantea	56475-S	U.S.D.A.*
Polyporus abietinus	# 13	Univ. of Md.***
Polyporus anceps	58526	U.S.D.A.*
Polyporus fumosus*	# 4	Brazil**
Polyporus tulipiferus	Mad. 517	U.S.D.A.*
Polyporus versicolor	57034-R	"
Poria subacida	71955	"

* Dr. Ross Davidson, U.S.D.A., Division of Forest Pathology, Beltsville, Md.

** Dr. Armando Russo, Instituto de Pesquisas Tecnologicas, Sao Paulo, Brazil.

*** Dr. Sidney Gottlieb, University of Maryland. Cultures originally from
Dr. Ross Davidson.

The methods used are described in detail in Summary Technical Report #2, January-December, 1949. Briefly, the basic routine method for growing the wood rots was that of aerated liquid culture (submerged culture), using small Erlenmeyer flasks (250-ml.) on a reciprocating shaking machine (shake culture) or, for certain studies, large bottles with forced aeration. Standard inocula and other standard conditions were used throughout. In the nutrition studies, in order to rule out the carry-over of nutrients from one medium to another of different composition, an organism was always serially subcultured in a test nutrient at least three times before growth response was determined quantitatively. Growth was quantitated by the dry weight of mycelial pellets produced in the 70 ml. of culture fluid per flask, at 28°C.

The basal synthetic medium used routinely for qualitatively determining nutrient requirements (particularly nitrogen) is as follows:

BASAL SYNTHETIC MEDIUM

Glucose	10.0 gm./liter
KH_2PO_4 (K=430, P=342 mg./l.)	1.5 gm./liter
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Mg=49.5 mg./l.)	0.5 gm./liter
Thiamine monohydrochloride	1.00 mg./liter
<u>Trace elements</u>	
B (as H_3BO_3)	0.10 mg./liter
Mn(as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$)	0.01 " "
Zn(as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)	0.07 " "
Cu(as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	0.01 " "
Mo(as $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$)	0.01 " "
Fe(as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.05 " "

To this basal medium the known nitrogen compound under study was added, usually in a concentration of 120 mg./liter total nitrogen.

This basal medium, with an added nitrogen source, supports growth of all of the organisms under study. It was therefore also used as the basis for the development of quantitatively optimal media, varying all constituents in numerous combinations.

BRIEF SUMMARY OF WORK TO DATE

The basic nutritional requirements of 42 representative basidiomycetous wood-rotting fungi have been studied qualitatively and quantitatively in submerged (shake) culture. This work involved approximately 40,000 shake cultures. Most species will produce good to abundant growth in continued serial subculture in a single "standard" synthetic medium containing six "trace" elements, a carbon source (various carbohydrates), a source of nitrogen (a variety of inorganic or organic compounds), thiamine, potassium, phosphorous and magnesium. A few species have additional minimum requirements for other vitamins, for purine or pyrimidine bases, or for special control of Eh or pH. Several of the organisms can utilize one or the other component of the thiamine molecule, instead of requiring the whole molecule. In the nutrition of many of these fungi, biotin can be substituted for thiamine. "Optimal" media, producing up to a few thousandfold increase in growth over the "standard" synthetic medium, have been developed for several of the fungi. The basic studies on nutrition, as originally conceived, are now completed.

Several aspects of the physiology of the wood-rotting fungi have been investigated to a limited extent, some with special reference to their application to the practical problems of the control of wood decay and

to the utilization of carbohydrate materials by fermentation. These include studies on optimum temperature; on pH and Eh in relation to growth and its inhibition; pigment formation; and the production, in shake culture, of fungal polysaccharides and organic acids from carbohydrates. A cellulolytic enzyme has been concentrated from the culture fluid of several of the wood rots. Bark and other celluloses have been shown to be degraded by these organisms in submerged culture.

Specific studies to date may be grouped in the following categories.

1. Growth of wood rots in non-synthetic culture media
2. Development of purely synthetic (chemically-defined) media for growth and nutrition studies
3. Utilization of different forms of organic and inorganic nitrogen
4. Utilization of different carbon compounds
5. Growth curves of the wood rots
6. Vitamin requirements, substitutions, components and synthesis
7. Optimum temperature for growth
8. Optimum pH for growth
9. Oxidation-reduction potential (Eh) in relation to growth
10. Development of synthetic media optimal for growth
11. Separation and concentration of cellulolytic enzymes
12. Development of "cellulose assay tube" for rapid determination of cellulase activity.
13. Production of organic acids by wood-rotting fungi
14. Pigment production
15. Production of fungal polysaccharides

16. Method for determining cellulose breakdown in sawdust
17. Large-scale growth of wood rots in aerated liquid culture
18. Fermentation of sawdust, bark, and other complex carbohydrate materials
19. Respiration studies

PROGRESS

Results since June, 1951

Since June, 1951, about 1400 shake cultures have been run, making a total to date of approximately 40,100 such cultures. Because the nutritional studies as such are now virtually completed, and the progress of these studies could be measured roughly by the number of shake cultures run, the figures for such cultures are no longer of significance as regards the investigations as a whole.

1. Nutrition studies.

The nutrition studies as such and as originally conceived, are completed. Various "loose ends" in the studies of optimal media have been taken care of, and we now have good "optimal" media for 8 of the fungi under study. Actually, these media contain only the minimum essential constituents, i. e., only those present in the "standard" basal medium (p. 4), but they are optimal concentrations in the "optimal" media. Certain constituents not present in the basal medium, e. g., purine and pyrimidine bases, are known to enhance growth, but have not been added to the "optimal" media under discussion. The present optimal media produce an increase in growth over the "standard" basal medium of from 300 to 3700%, depending upon the organism.

Thirty-six of our 42 cultures of wood-rotting fungi have been found to grow with biotin substituted for thiamin in the basal medium. Microbiological assays for thiamin in the medium and in the mycelium show that with biotin as the only vitamin there is no synthesis of thiamin by these organisms, indicating that they have at least two metabolic pathways in their over-all metabolism. With these same organisms studies also are being made of the substitution of inositol for thiamin.

2. Cellulase investigations.

In Report #4 we described the production and concentration of a "cellulase" which attacked the soluble cellulose derivative, carboxymethyl cellulose. The present discussion concerns a true cellulase which attacks insoluble cellulose. The greatest production of this enzyme is in a casein hydrolysate medium. Our "standard" basal medium, with glutamic acid or ammonium carbonate as the nitrogen source, was considerably less satisfactory. Cellulase was determined by the "cellulase assay tube." The concentration of carbohydrate, rather than the type of compound, is more important, at least so far as glucose, maltose, soluble starch, lactose, galactose, sucrose, cellobiose and carboxymethyl cellulose are concerned. The data will be reported in detail when this study is completed.

Continuation of work on concentration of cellulase from the culture fluid shows that organic solvents are not good precipitants. Best results in concentration have been obtained with a combination of ion exchange resins followed by vacuum distillation. This work is continuing.

3. Studies of Oxidative Metabolism

Work is under way on the oxidative metabolism of selected wood rots, including the use of postulated intermediate compounds as substrates. The manometric technique, employing the Warburg respirometer, is being used to study the respiration of the organisms. Much difficulty has been experienced in standardizing conditions for these determinations, particularly as regards the degree of fragmentation of the fungal pellets. Preliminary data on oxygen uptake in 1% glucose show, however, that the endogenous respiration of P. palustris and L. trabea is high compared with exogenous respiration.

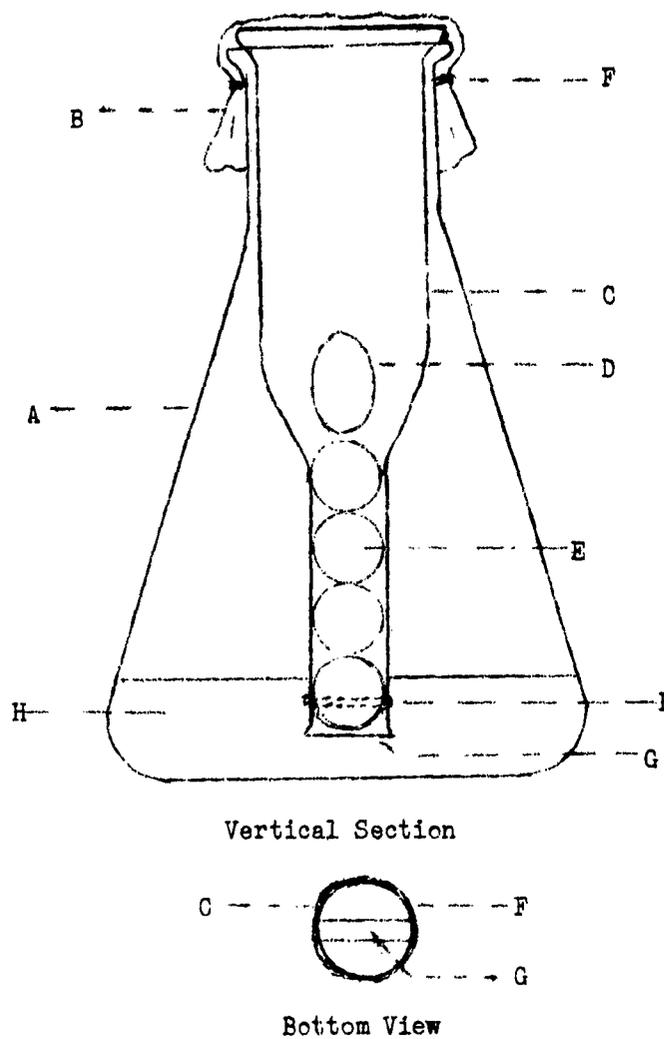
4. Pigment Production

It was previously reported that several of the wood-rotting fungi produce yellow-red, water-soluble pigments in shake culture in our standard basal medium containing a source of nitrogen. The organisms which are being studied in this regard are Lenzites trabea, Polyporus schweinitzii, Poria oleracea, Poria luteofibrata, Merulius lacrymans, Fomes pini, Lenzites striata and Lentinus tigrinus. Maximum pigment is brought about by proper concentrations of the "trace" elements in the medium. Iron and zinc, and to a lesser degree boron and molybdenum, are critical in pigment production. For iron, the optimum concentration for maximum pigment production is 0.1-0.15 mg./l.; for zinc, 0.14-0.21 mg./l.; for molybdenum, 0.02-0.03 mg./l.; and for boron, 0.02-0.03 mg./l.

The pigment of L. striata is a carotinoid, probably a xanthophyll, as shown by chemical tests and absorption spectra. The composition of the other pigments has not been investigated.

5. The "cellulase assay tube."

In Report #4 an apparatus for determining cellulase activity was described briefly. It can be used either for a growing liquid culture or for a filtrate. This consists of a glass tube open at both ends, smaller at one end than the other, and with both ends slightly flared. The tube fits into the neck of our standard shake flask (250 ml. Erlenmeyer). The lower end of the tube dips below the surface of the liquid (culture or filtrate) in the flask. This lower end has a narrow strip of cellophane across it, and on the cellophane the tip of a glass rod rests. Production or presence of cellulase in the liquid weakens the strip, allowing the glass rod to fall to the bottom of the flask. The time required for the strip to weaken sufficiently for the rod to fall is a measure of cellulase concentration or activity. Modification of this method ("drop weight method") uses stainless steel balls in place of the glass rod. The greater weight decreases the time required for the strip to break. Average cellulase activity allows the weight to drop in 72 to 96 hours. (See Figure 1, below, for the construction and assembly of the apparatus).



- A 250 ml Erlenmeyer flask
- B cellophane cap
- C cellulase assay tube
- D inoculation port
- E stainless steel balls
- F rubber collar
- G cellophane strip
- H culture medium or filtrate

FIGURE 1: CELLULASE ASSAY TUBE ASSEMBLY

6. Fermentation of cellulose

Work has continued on fermentation, by the wood rots in submerged culture, of cellulosic materials. In addition to bark, previously reported as being directly hydrolyzed by certain organisms, we have also found that reducing sugars can be formed from corn cobs as the cellulose substrate. This work is in the preliminary stages, and will be reported in detail later, but offers important practical possibilities for the utilization of waste cellulose.

7. Special nutrition studies

a. Utilization of amino acids as carbon sources by two fungi.

P. palustris (brown rot) and P. tulipiferus (white rot) were tested for growth in shake culture in the basal medium minus glucose, but with various single amino acids as the sole source of nitrogen and carbon. Growth in continued subculture did not occur in any of the amino acids tested, viz: alpha alanine, beta alanine, l-arginine, l-asparagine, dl-aspartic acid, l-cystine, l-glutamic acid, glycine, l-histidine, dl-isoleucine, dl-leucine, l-lysine, dl-methionine, d-norleucine, d-ornithine, dl-phenylalanine, dl-serine, l-tryptophane, dl-valine.

b. Utilization of l-glutamic acid as a nitrogen and carbon source by all fungi.

All of our cultures (see List of Organisms used, p. 2) were grown in shake culture in the basal medium minus glucose, but with l-glutamic acid as the sole source of carbon and nitrogen. None of the species grew in continued subculture in this medium.

c. Utilization of various carbon compounds by two fungi.

P. palustris and P. tulipiferus were used as representative species to determine utilization of various carbohydrates and organic acids in place of glucose in the basal medium, with l-glutamic and as the source of nitrogen.

Qualitatively, both organisms grew in sucrose, lactose, maltose, fructose, galactose, glycerol and starch. Glycerol, for both organisms, produced more growth than in the glucose control; all of the other carbohydrates produced less growth than glucose. No growth was obtained with acetic acid, citric acid, tartaric acid or maleic acid, while succinic acid, pyruvic acid and lactic acids were utilized.

CONCLUSIONS

A variety of fundamental data have been obtained on the growth, nutrition and certain aspects of the physiology of the wood-rotting Basidiomycetes. These data give a good basis for the understanding of their growth characteristics, their nutritional pattern, and some of their physiological potentialities as regards the application of the studies to practical problems of possible economic importance. Certain of the important practical applications which justify intensive and immediate study, some of which we have investigated in a preliminary way, are as follows.

1. Quantitative study of synthesis of vitamins by the wood-rotting fungi. Possible use of these organisms as a commercial source of vitamins, as certain other organisms are now used, has been started.

2. Further study of the separation and concentration of cellulolytic enzymes. In addition to the fundamental importance of these enzymes, they may be of practical use in a manner similar to the present uses of microbial amylases. For example, it is known that in paper-making, wood which has been slightly attacked by wood-rotting fungi is much more easily pulped.

3. Production of sterols. This work has been started.

4. Investigation of the application of some of the above fundamental studies to the control of wood decay. For example, we have found that nearly all of the wood-rotting fungi require a high oxidation-reduction potential for growth. The best chemical wood preservatives then, should be those with a low Eh. This appears to be a new finding and a new concept, and its importance warrants intensive study. Also, a good wood preservative should be alkaline (the fungi grow best in acid solution), and might well be able to inactivate thiamine (required by wood-rots). Possible development of new chemical wood preservatives having the desirable Eh, pH, etc. characteristics in addition to their other toxic properties.

5. Investigation of protein synthesis by the wood-rots, using waste cellulose or other cheap carbohydrate material. High protein synthesis by these organisms, particularly if they also synthesize large amounts of vitamins, might make them useful as animal-feed supplements. Possible development of high-protein and high-vitamin synthesizing strains of wood rots, as regards their use as animal feed supplements or commercial sources of vitamins.

6. Intensive study of the fermentative utilization of carbohydrates, including waste cellulose, by wood-rotting fungi. The organisms grow rapidly and profusely in aerated liquid culture and on a large scale.

We know that large amounts of organic acids, and of other compounds, are produced. Various forms of waste cellulose (bark, sawdust, etc.) are fermented directly, or may be hydrolyzed by the wood-rots, then fermented by other organisms. Possible development of large-scale fermentation of waste cellulosic materials to economically useful products.

7. Continuation of study of production and characteristics of fungal polysaccharides. Polysaccharides from certain other microbes have been suggested as substitutes for blood plasma, and for other uses.

BIBLIOGRAPHY

No papers have been published during the period June, 1951 -- December, 1951. Various aspects of the whole project are in process of being written up for publication.

OTHER ASPECTS OF THE STUDIES

a. Changes in emphasis or orientation.

With the virtual completion of the fundamental studies on nutrition, more stress is being put on practical applications and extensions of these studies. Studies of vitamin synthesis by the wood rots are under way, as well as fundamental studies on thiamin-biotin-inositol substitution. Work on the cellulolytic enzymes is continuing, as are studies on oxidative metabolism. Work also continues on the fermentation of waste cellulose by the wood rots, as this is an important practical aspect and also time-consuming as regards results.

b. Personnel changes

Dr. Richard Henderson, biochemist on these investigations, leaves the University as of January 31, 1952. Miss Melva Derrick, who has done most of the work on nutrition, receives her Doctor's degree in

January, 1952, and will therefore finish her work on this project. Richard O'Neill received his Doctor's degree for work on this project in June, 1951, and continues at the University as an Assistant Professor and consultant without pay on these investigations. Misses Philomena Moretti and Irene Zuck obtained their Master's degrees in June, 1951, and have left the University. Miss Josephine D'Agostino and Mr. David Cooman are half-time Navy technicians, and Mr. Paul Borick is working on pigments of the wood rots without remuneration from the Navy. As of February 1, 1952, Mr. Chester Koda will replace Mr. Cooman. Mr. Harvey Newcomb continues working on oxidative metabolism without financial aid under this project.

c. Graduate students on contract.

Miss D'Agostino, Mr. Cooman and Mr. Koda, mentioned above, are graduate students on this contract.

d. Other research support.

The Department of Plant Sciences continues to furnish secretarial assistance, space, and certain capital equipment. The university Institute of Industrial Research has purchased certain pieces of apparatus used in these investigations, and has furnished a full-tuition fellowship for a graduate student (Miss D'Agostino) on this contract for the academic year September, 1951 - June, 1952.

e. Difficulties encountered.

Because of the unfortunate lack of funds for the continuation of this project beyond June, 1952, and the small amount available between now and June (enough for two graduate students, half-time), relatively little new work can be started, and work already under way cannot be finished. This is particularly regrettable at this time, as a great

deal of fundamental investigation has been carried out which forms a basis for important practical applications. These applications are considered above under "Conclusions." The importance of the practical applications of these studies warrants intensive continuation of the project.

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