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AGENCY FOR INTERNATIONAL DEVELOPMENT
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MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose VO

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 1. E-01

Attachment

2 cp Final dtd 10/31/83

2 cp Final Expanded Revision dtd 11/15/84

Project File 15-01

1E-01

College of Forestry, Wildlife and Range Sciences

Forest, Wildlife and Range Experiment Station

University of Idaho

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52961

FOOD FROM WOOD: A NEW ENZYMATIC SYSTEM FOR DEGRADATION OF LIGNOCELLULOSE

Grant No. DPE-5542-SS-1037-00

Final Report

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SUMMARY

The objectives of the project Food from Wood, Phase I, were designed to:

1. Evaluate a lignin degrading biosystem for potential industrial exploitation, primarily in regard to its efficiency of lignin conversion both in terms of total amount of degraded lignin and the speed of lignin conversion.

2. Reproduce lignin degradation under laboratory conditions *in vitro*. In the process, to isolate and propagate individual microbial strains involved in lignin degradation process, and to identify basic changes in chemical composition of degraded plant materials.

In experiments *in vivo*, the evaluated biosystem was found to be efficient and fast; an average of 50-65% of lignin was degraded in less than 48 hours. This rate of degradation significantly exceeds lignin degradation rates found in the most efficient biosystem known--that of white rot fungus, which removes 30 to 60% of lignin after incubation of more than 2-3 months.

Biodegradation of lignin *in vitro* produced encouraging results. In the first series of fermentations *in vitro*, using a mixed bacterial inoculum, fermentation process was not always adequately controlled. Successful fermentations in these experiments produced 10 to 30% of lignin removal during a 5- to 7-day incubation. However, all fermentations in this phase were not successful, probably due to the uncontrolled heavy growth of contaminating organisms.

In an effort to separate and identify lignin-degrading organisms, we have isolated and tested for lignin-degrading potential several hundred individual isolates. Several isolated cultures repeatedly degraded 10 to 25%

of wood lignin during a 7-day incubation period. In addition, some other cultures were marked as potential candidates for further testing.

Isolation and identification of the lignin-degrading organisms was accomplished during the last 2 months of the study. With additional funding, we believe the efficiency of our biosystem can be rapidly increased to match or exceed lignin degradation removal rates of 65% in less than 48 hours originally found in the studies *in vivo*. Once this is accomplished, the system will be ready for development of technological process for conversion of lignified biomass to feed.

OBJECTIVES

The main objective of the research project "Food from Wood: A New Enzymatic System for Degradation of Lignocellulose," Grant No. DPE-5542-SS-1037-00, was to identify principal mechanisms by which one animal degrades and uses for food lignified wood fiber, and to evaluate usefulness of this process for potential large-scale conversion of lignified waste products to feed.

Specific objectives were:

1. To measure the rate and the efficiency of lignin degradation by the animal system;

2. To separate and identify main bacterial and/or enzymatic groups responsible for lignin degradation;

3. To identify digestive organ(s) in which lignin [illegible]

4. To reproduce the lignin degradation system *in vitro*;

5. To identify basic chemical and physical components and the yield and quality of end-products involved in the lignin degradation process;

6. To evaluate this new enzymatic system for possible future development of a technical process for degradation and use of lignin in human and livestock feeds.

All major objectives of this research have been successfully accomplished. The most significant results are summarized and discussed on the following pages. Objective No. 2 proved to be more difficult than we had expected. It was not until 2 months before the end of this project that our work was completely successful. We think that separation of this biosystem from the animal, resulting in 10 to 25% of degradation of lignin under

controlled laboratory conditions *in vitro*, opened the road to full technical exploitation of this biosystem. We also think that the efficiency of this system could be improved with relative ease by manipulating the conditions during fermentation to match or exceed efficiency found in the system *in vivo*.

RESULTS AND DISCUSSION

1. The Rate and Efficiency of Lignin Degradation and Removal by the Animal's Biosystem

Animal Experiments: A total of 28 different *in vivo* digestibility studies were designed to evaluate natural potential of the tested animal system to break down and remove lignin from various materials. Results generated in these experiments were used to evaluate actual and potential degradation of lignin in fermentations *in vitro*.

Animals: Experimental animals were kept individually in rabbit cages. After arrival in the laboratory, all animals were allowed a minimum of 3 weeks to adjust to the laboratory conditions. A colony of 20 to 60 animals was maintained in the laboratory to provide an adequate supply of animals for lignin degradation studies *in vivo*, and to supply inoculum for fermentations *in vitro*. When not used in experiments, the animals were fed selected fibrous plant materials supplemented by a commercial rabbit diet. Specially prepared diets were fed during experiments.

Pelleted Diets: In our earlier work, degradation of lignin by animals in studies *in vivo* was established from digestibility studies of various native feeds. The animals were fed fresh plant parts such as branches, leaves, or roots. From this feed, the animals ate only the most palatable parts. The amount and quality of eaten feed was calculated from the difference between offered and refused feed. While this method was reasonably accurate in estimating digestibility of ingested natural feeds, adjustment and manipulation of feed intake and quality was impossible. Experimental animals selected plant parts which varied greatly in content of all tested nutrients and fiber and could not be forced to eat materials such as sawdust or straw.

To evaluate digestibility of important post-harvest waste products such as wood chips and straw, it was necessary to develop a palatable feed pellet from otherwise unpalatable tested materials.

Earlier attempts to feed pelletized diets of uniform quality failed, even though the animals readily consumed both green and dry plants that were used to make such pellets. After several months of testing we have developed feed pellets that are readily accepted by all experimental animals. While some of the pellet ingredients must remain unchanged, up to 60% of pelleted feed can be made of tested materials. Pellets are of uniform quality during the entire digestibility study and can be easily handled.

Digestibility of Lignin *In Vivo*: Efficiency and Rate Degradation: A total of 28 *in vivo* digestibility studies were conducted in the early stages of the study. The animal experiments were designed to test the lignin-degradi biosystem in its natural (animal) form to determine whether the system has sufficient potential for technical exploitation to justify extensive studies *in vitro*.

The digestibility studies and calculations of apparent digestibility of total lignin and several lignin fractions were performed according to the standard procedures described in greater detail in the proposal. Tested feed was offered daily *ad libitum* to 8 individually housed animals during a 7-day adaptation and stabilization period, and during a 6- to 10-day collection period. All refused feed and fecal pellets were dried, the amount of feed actually eaten was calculated, and samples of feed and fecal pellets were prepared for chemical analyses.

Chemical Analyses for content of lignin in plant tissues had been standardized. Since lignin is an amorphous material which does not have a

predetermined molecule, chemical analysis for lignin is not simple. Many laboratories work with synthetic lignin, which is somewhat, but not completely, similar to natural lignins, and easier to analyze. Pre-treated natural lignins are used by other workers, but such molecules, and possibly also degradation rates, are different than those of natural untreated lignins. Since the overall objective of our work is degradation of lignified waste products and post-harvest biomass, we had to work with natural unaltered lignins. Thus, in our laboratory, we have chosen a combination of several methods rather than a single one; we think that a combination of methods identifies more accurately total amount of natural lignin in each analyzed sample, especially in regards to lignin of different plant origin. The following analytical methods were used in our laboratory:

1. Direct lignin, analyzed according to Armitage et al. (1948);
2. Acid detergent fiber lignin, analyzed by Van Soest's (1963) method;
3. Acid insoluble lignin by Effland's (1977) method; and
4. Acid soluble lignin method No. 250, Tappi.

A spectrophotometric method used by Van Zyl (1978) and described by Johnson et al. (1961) had very poor accuracy and repeatability in our laboratory and, as a result, was not used.

Results and Discussion: Four major post-harvest waste products, tested extensively for degradation and removal of lignin *in vivo*, were:

Tested Plant Material	Average % of Lignin
Douglas-fir sawdust	27.7
Alder sawdust	25.5
Wheat straw	22.2
Barley straw	20.8

Several other plant materials, such as sword and bracken fern, and salal, were also tested. The tests were performed according to the standard

procedures (Church, 1969). Apparent digestibility of lignin was calculated after completion of chemical analyses of collected samples.

Tested plant materials were fed to the animals in several separate groups, each group receiving a different amount of tested straw or sawdust in the feed pellets. Douglas-fir sawdust was tested at levels ranging from 20 to 60%, alder sawdust from 24 to 60%, and straw at several levels of up to 60% of a total diet.

Degradation of lignin was found to vary:

- among animals, ranging from 0 to 86% of lignin removed;
- among tested plant materials--wheat and barley straw had lower digestibility of lignin than wood chips;
- according to the adjustment period--animals adjusted to the feed for longer periods of time increased their efficiency of lignin digestion.

Differences observed among individual animals in their ability to digest lignin were observed in several consecutive tests. Some animals appeared unable to degrade large amounts of lignin. However, even those animals that appeared unable to digest any lignin at the beginning of digestibility tests were capable of degrading significant amounts (20-30% or more) of lignin after a longer adjustment period. Overall lower rates of lignin removal appeared to be associated more with individual animals than with adaptation period.

Tested feeds contained gradually increasing amounts of sawdust or straw materials. With increase of these lignified materials in the feed, content of lignin also increased until, in the last tests, Douglas-fir lignin comprised 25% of the pelleted feed. This level approached 27% of lignin found in pure Douglas-fir wood. Since pure wood cannot be fed to the animals due

to its unpalatability, we have to assume that a rate of lignin degradation in pure wood would be similar to a rate found in feed containing 25% of Douglas-fir lignin.

The rate of lignin degradation by the animal's biosystem averaged from 50 to 65% for wood, and from 10 to 20% for straw lignins. In some experiments, more than 80% of lignin was removed, but this high rate was not achieved repeatedly in subsequent experiments. Table 1 summarizes degradation rates of Douglas-fir and wheat straw lignins.

Table 1. Degradation of Douglas-fir and wheat straw lignins.

				% of Lignin Digested *In Vivo*		
	Acid Insoluble Lignin	Acid Soluble Lignin	Total Lignin (insoluble + soluble)	Acid Insoluble Lignin	Acid Soluble Lignin	Total Lignin (insoluble + soluble)
Pure Douglas-fir wood	27.1	0.6	27.7			
Douglas-fir sawdust feed	14.0	4.7	18.7	29.3	77.1	40.7
	14.0	4.7	18.7	33.6	82.2	45.2
	14.0	4.7	18.7	8.2	80.1	25.3
	14.0	4.7	18.7	20.5	79.2	34.5
	14.0	4.7	18.7	47.5	83.9	56.2
	19.2	6.2	25.4	45.2	86.3	55.2
	19.2	6.2	25.4	47.2	90.7	57.9
	19.2	6.2	25.4	57.5	89.4	65.3
	19.2	6.2	25.4	57.6	89.8	65.5
	19.2	6.2	25.4	42.1	86.9	53.1
	18.3	5.3	23.6	83.3	96.3	86.2
	18.3	5.3	23.6	65.2	91.7	71.1
Pure wheat straw	22.1	2.6	24.7			
Wheat straw feed	12.8	5.4	18.2	7.3	62.7	23.7
	12.8	5.4	18.2	0.0	55.3	13.1
	12.8	5.4	18.2	0.0	51.8	8.2
	12.8	5.4	18.2	0.0	58.0	17.1
	12.8	5.4	18.2	22.6	71.1	36.9
	12.8	5.4	18.2	0.0	0.0	0.0

To measure the speed of lignin degradation *in vivo*, five separate tests were designed to measure the passage rate of ingesta by animals. Undigestible chromic oxide (Cr_2O_3) was mixed with feed or drinking water, and the samples of fecal material were collected at regular intervals and analyzed. Results show that 90 to 98% of ingested feed is excreted in less than 48 hours after ingestion; with well over 50% of dyed feed excreted in 24 hours. From this we can conclude that lignin degradation *in vivo* is completed in 24 to 48 hours.

Summary of Results:

- The animal system is capable of degrading economically important lignins.

- Approximately 50 to 65% of wood lignin is degraded by this biosystem.

- The system degrades lignin in 24 to 48 hours; compared to other efficient biosystems, which degrade similar amounts of lignin in more than 3 months, this biosystem works very fast.

2. Separation and Identification of Main Bacterial and/or Enzymatic Groups Responsible for Lignin Degradation

Degradation of lignin *in vitro* represented the single most important objective of this study; successful mastering of this process will enable us to develop a large-scale technological process for conversion of lignified wastes to feed. The work on this objective started shortly after the initiation of the project and continued through October, 1983. During this time we have inoculated, transferred, and analyzed for lignin-degrading capability several thousand microbial cultures.

While most similar fermentations have been traditionally performed in closed fermentation tubes filled with CO_2, this system was abandoned in our

laboratory for lack of results. Aerobic and anaerobic (in anaerobic chamber) fermentations, both in closed and semi-open drip-in systems, were used through most of the study. While anaerobic cultures produced slight to moderate growth of tested cultures, aerobic cultures grew faster in much shorter periods of time.

Cecal fluid was used most frequently as a source of bacterial inoculum. Other parts of the digestive system, such as small and large intestine, were also explored as possible sources of inoculum. Use of fresh fecal pellets would have eliminated a need to sacrifice animals, but proved less successful than use of fresh cecal fluid. Small intestine, on the other hand, provided very thick, mucousy fluid, which was difficult to filter to remove solid particles. Since bacterial cultures from the small intestine were similar to those found in the cecum and large intestine, use of cecal fluid was found most practical.

In the first series of fermentations *in vitro* we have used mixed microbial inoculum obtained directly from experimental animals. Successful fermentations produced 10 to 30% of lignin removal during a 5- to 7-day incubation. However, many fermentations with mixed microbial inoculum were unsuccessful in regard to degradation of lignin, probably due to the uncontrolled heavy growth of contaminating organisms. These organisms multiplied very rapidly, contaminated the environment, and inhibited the lignin-degrading organisms. Various methods were tried to minimize this effect. These included timed release of nutrients, gradual dilutions of growth media, and periodic removal and replacement of media during fermentation.

Separation of individual organisms from animal inoculum produced several hundred isolates. All isolated organisms were grown in various

liquid media prior to their transfer to a sample of Douglas-fir and alder sawdust to determine their lignin-degrading capability. All samples were tested in duplicate to prevent possible analytical error. A variety of conventional as well as enriched media had been used to provide best possible conditions for target organisms. Sheep blood, rumen fluid, and cecal fluid had been used extensively in preparation of propagation and sawdust test media.

Extensive testing of isolated microorganisms finally met with success just two months before the end of this study. One of the isolated organisms produced strong and relatively rapid degradation of Douglas-fir lignin *in vitro*. Before the end of the study we managed to test lignin-degrading properties of this organism in four separate tests with the following results:

	Average % of Lignin Digested		
Test Number	Insoluble Lignin	Soluble Lignin	Total Lignin
1	10.9	0.0	10.7
2	17.7	0.0	17.4
3	18.1	0.0	17.7
4	24.1	0.0	23.6

Since soluble lignin represents only 2% of total Douglas-fir lignin, and insoluble lignin is normally the hardest lignin fraction to degrade, removal of 24% of insoluble lignin during a 7-day incubation is exceptional. It must be emphasized that we have not had enough time after isolation of these organisms to identify optimal physical and chemical conditions required for maximum lignin-degrading capability of these organisms. By simple manipulation of these conditions we think we can increase lignin degradation to match or exceed degradation rate found in the animal system, where 65% of lignin is degraded in less than 48 hours.

3. Identification of Digestive Organs in Which Lignin Biodegradation Takes Place

Undigestible dyes such as chromic oxide (Cr_2O_3) were mixed with tested feed containing Douglas-fir or alder lignins, and fed to the experimental animals. The animals were killed and samples of ingested feed taken from different parts of the digestive system were analyzed for contents of dye and lignin. The disappearance of lignin in individual segments of the digestive tract was evaluated.

Several attempts had been made to fistulate cecum and a small intestine of some animals to gain an access to these parts of the digestive tract. This method, however, proved to be impractical and was discontinued. The animals fitted with canulae of physiologically tolerable diameter recovered well from the surgery, but a high fiber test diet produced very dry digesta which could not be extracted from the animal through the small diameter of the tube. As an alternative, we have chosen to kill the animals that had been fed tested diet for at least a week, and which had a known overall digestibility of lignin.

All tests completed to this day show that large amounts of lignin are degraded in the lower part of the small intestine and in the cecum. This finding is surprising, since degradation of fiber and cellulose in non-ruminant herbivores takes place primarily in the cecum. Removal of some lignin from the fiber before it reaches cecum thus may be of great significance to this wood-eating animal. Liberating cellulose from the lignin envelope before it reaches cellulose-degrading sites would significantly increase speed of fiber digestion.

4. Reproduction of the Lignin Degradation Process *In Vitro*

To degrade lignin under laboratory conditions, we had to identify system(s) responsible for lignin breakdown, separate all important aspects of such systems from animals' other biochemical and physiological functions, and to create in the laboratory conditions favorable to continued degradation of lignin *in vitro*. A systematic approach was developed to test and evaluate for lignin-degrading capability various organisms and enzymes present in the animal biosystem.

Culture, separation, and testing of microorganisms, discussed under #2, represent one phase of these efforts. As a result of 33 large microbiological studies, we have isolated and tested for lignin-degrading potential several hundred pure cultures of organisms. Additional tests were performed using mixed microbial inoculum and combinations of isolated organisms.

Possible involvement of lignin-degrading enzymes excreted directly by the animal was explored in 14 separate experiments. Even though lignin degradation was not observed in these tests, addition of animal's digestive fluids to the bacterial media produced better growth and also increased degradation of lignin by isolated organisms. More time is needed to clarify the role of digestive juices and/or enzymes in degradation of lignin by recently isolated lignin-degrading organisms. The degradation of lignin was reproduced repeatedly *in vitro* using mixed bacterial inoculum taken directly from the animal, or pure culture of recently isolated lignin-degrading organisms. The maximum efficiency of lignin removal in a successful incubation reached 25 to 30% of lignin removed in 5 to 7 days. Both alder and Douglas-fir wood lignins were degraded.

First degradation of wood lignin *in vitro* resulted from incubation of tested sawdust with mixed animal inoculum. Results of these incubations were

unpredictable and difficult to control. From the mixed inoculum, organisms without lignin-degrading capabilities appeared to grow, use up available nutrients, and pollute the media, very rapidly. Lignin-degrading organisms appeared unable to grow and/or degrade lignin under such limiting conditions. From hundreds of inoculated, incubated, and analyzed sawdust samples, only relatively few showed a significant degree of lignin degradation.

In an effort to isolate and propagate lignin-degrading organisms, we have separated from animal inoculum, and tested for lignin-degrading capability, several hundred microorganisms. Lignin-degrading organisms were isolated and identified 2 months before the end of this project. A few tests performed during these 2 months established that a lignin degradation rate under currently practiced laboratory conditions is 10 to 24% after a 7-day incubation. When compared to other lignin-degrading biosystems, this ratio of lignin conversion is impressive. To justify a large-scale industrial exploitation, however, the rate of lignin removal should be higher. Based on the results of our brief tests, we think that by relatively simple manipulation of conditions under which fermentation takes place we can increase the rate of lignin breakdown. We know that the system is capable of degrading 65% of wood lignin in less than 48 hours. We believe we can match or exceed this naturally occurring degradation rate in our new controlled biosystem *in vitro*.

5. Identification of Basic Chemical and Physical Components and the Yield and Quality of End-Products Involved in the Lignin Degradation Process

As a last part of this project, chemical evaluation of lignin degradation end-products could be started only after a successful degradation had been established *in vitro*. Many graphs resulting from spectrophotometric scanning of liquid portions of samples before and after fermentation had been

evaluated. Sizes and shapes of individual peaks were compared and differences recorded. Four basic patterns emerged as typical in association with lignin breakdown. Identification of chemicals produced from degraded lignin was not attempted due to a great variety of products resulting from mixed culture inoculations. Work on identification of chemicals produced by recently isolated lignin-degrading organisms will be less complex and is proposed for the next phase of this work.

6. Evaluation of the Biosystem for Future Technical Exploitation

Evaluation of the tested lignin-degrading biosystem for potential large-scale technical exploitation was carried out in two steps.

a. The system was evaluated in controlled experiments *in vivo*. In these tests the biosystem worked under natural conditions shaped by the evolution, showing an efficient, even though perhaps not maximum, attainable rate of lignin conversion.

b. In the laboratory work *in vitro*, where the system was separated from the animal, and, eventually, from other contaminating organisms.

The evaluated biosystem was found to be:

Efficient--an averag of 50-65% of wood lignin was degraded *in vivo*;

Fast--lignin degradation *in vivo* was accomplished in less than 48 hours;

Potentially useful--economically important types of lignin from lignified biomass and waste products were degraded;

Reproducible *in vitro*--isolated organisms repeatedly degraded 10 to 25% of wood lignin during a 7-day fermentation.

The system has a high potential for large-scale degradation of lignin *in vitro*. We think we can increase in the laboratory lignin degradation rate to match or exceed the 65% degradation found in the live animal biosystem.

The system then will be ready to use in development of a highly efficient technological process for conversion of lignified biomass to feed.

The tested system already produced higher degree of lignin degradation *in vitro* than any other known biosystem. By increasing the efficiency of controlled fermentations to the level found in the animal system, this biosystem will be a prime candidate for large-scale technical exploitation in the worldwide effort to relieve hunger through utilization of lignified waste biomass.

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