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Using Thermophilic Bacteria to Produce Enzymes for Wood Pulping

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USING THERMOPHILIC BACTERIA TO PRODUCE ENZYMES FOR WOOD PULPING

by

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ABSTRACT

The objective of this Senior Thesis Project was to determine if thermophilic (heat loving) bacteria are capable of producing enzymes to break down lignin. Currently known ligninases produced by white-rot fungi are destroyed by high temperatures, and are only useful for pre-treatments in refiner mechanical pulping. Thermophilic lignin-degrading enzymes could potentially be useful in a high temperature pulping process.

Milled wood lignin was isolated from birch to be used in bacterial cultures. The lignin would serve as the only source of carbon for the bacteria, so their growth would depend on their ability to utilize the lignin for food. Standard cultures containing glucose as a carbon source were used to determine if the bacteria could grow in our apparatus.

After several attempts, it became clear that our methods could not determine conclusively if the bacteria were growing or not. If more sophisticated techniques and equipment are used to grow thermophilic bacteria, a better understanding of their enzymatic activity can be obtained.

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INTRODUCTION

The objective of this Senior Thesis project was to determine if thermophilic bacteria are capable of producing enzymes to degrade lignin. Thermophilic bacteria are microorganisms that can live and even thrive at temperatures near the normal boiling point of water. There are some organisms, such as white rot fungi, which produce lignin-degrading enzymes at lower temperatures. White rot fungi have been successfully used as a pretreatment to soften wood chips before refiner mechanical pulping (1). However, their enzymes are unsuitable for use as a complete pulping process, because they react too slowly at low temperatures and are unstable at higher temperatures. If thermophillic bacteria are capable of breaking down lignin, their enzymes could possibly be used in a fast, high-temperature, high-yield pulping process.

BACKGROUND AND THEORETICAL

Two species of thermophilic bacteria were selected for this project: *Pyrococcus furiosus* and *Thermotoga maritima*. Both are strictly anaerobic heterotrophs which use simple and complex carbohydrates for growth, producing organic acids, carbon dioxide, and hydrogen gas (2,3,4). *P. furiosus* grows optimally at 100°C, while *T. maritima* thrives at 80°C. Most thermophilic bacteria are sulfur-dependent, obtaining energy for growth by the reduction or oxidation of elemental sulfur, but *P. furiosus* and *T. maritima* can grow equally well with or without sulfur (3).

To determine whether these bacteria can utilize lignin as a carbon source, pure lignin had to be isolated and used in the culture media. The Björkman process is the best method to iso-

late lignin from wood without significantly altering its structure (5). If the carbohydrates normally used in the culture media are replaced with lignin, the bacteria would have to use lignin as a food source in order to grow. If growth occurs, it is an indication that the bacteria have adapted to the new food source.

EXPERIMENTAL PROCEDURE

Isolation of Lignin. Birch lignin was isolated using the Björkman process (6). Birch lumber was first ground into fine sawdust using a table saw. This sawdust was further reduced in size by grinding it in a Wiley mill. The Wiley-milled sawdust was then ball-milled for one hour. The ball mill consisted of a 3-liter plastic jar containing twenty-eight 3/4" x 3/4" and nine 1 1/4" x 1" ceramic cylinders, with 100 grams of sawdust in each run. Ball milling serves to open up the cellular structure of the wood, for better liquid penetration during the extraction step.

The lignin was extracted from the milled wood using a 98 percent dioxane/water mixture to minimize carbohydrate content. The ratio of liquid to wood was 5 to 1 by weight. The slurry of dioxane, water, and wood meal was stirred for one hour in a stoppered Erlenmeyer flask.

After extraction, the slurry was filtered through a Buchner funnel to separate the dioxane-lignin solution from the wood meal. The dixoane was then evaporated off by placing the solution on watch glasses and heating it on a steam bath. The remaining lignin residue was allowed to dry, scraped off the watch

glasses, and washed with distilled water.

Preparation of Growth Media. A glucose-containing growth medium was prepared as directed by Adams and co-workers (2). If the bacteria grew in this media, a second growth medium would be prepared with lignin substituting for glucose. The growth medium had the following composition: NaCl (0.4 M), $MgSO_4$ (7 mM), $MgCl_2$ (7 mM), NaBr (0.2 mM), H_3BO_3 (0.12 mM), $SrCl_2$ (0.14 mM), KCl (2.1 mM), KI (0.15 mM), $CaCl_2$ (3 mM), KH_2PO_4 (3.7 mM), $NiCl_2$ (10 μM), $Fe(NH_4)_2(SO_4)_2$ (40 μM), $FeSO_4$ (10 μM), Na_2WO_4 (10 μM), $CaCO_3$ (10 mM), Na_2S (0.5 g/L), yeast extract (0.5% w/v), glucose (0.4% w/v), and trace minerals (15 mL/L-see reference 7). The medium was stored under refrigeration in a 1-liter volumetric flask.

Growth of the Bacteria. Three different types of apparatus were used in the attempts to grow the bacteria Thermotoga maritima. In each apparatus, the vessel containing the growth media was autoclaved at 121°C to sterilize it and drive off oxygen before inoculating it with bacteria.

The first apparatus consisted of a three-neck boiling flask with rubber stoppers in each neck. A glass tube led from one neck to a beaker of distilled water. The center neck held a thermometer, and the third neck held a solid rubber stopper through which bacteria could be introduced and samples withdrawn with a hypodermic needle. The flask was placed on a heating mantle adjusted to 80°C. During the first night of operation, the heating mantle apparently lost power temporarily. The flask cooled down, and the resulting vacuum drew water from the beaker into the flask.

The second apparatus was similar to the first, but with an Erlenmeyer flask placed as a trap between the boiling flask and the exit beaker of water. The setup appeared to work well. The culture media gradually changed from a pale yellow to a very dark brown. Adams and co-workers reported using absorbance measurements at 600 nm to monitor bacterial growth (3). Periodic absorbance measurements of the culture at 600 nm did indeed show increasing absorbance. However, when a blank culture with no bacteria was run for comparison, it also turned dark, with a corresponding increase in absorbance. Microscopic analysis of the inoculated culture did show the presence of bacteria, but it was impossible to determine if growth had actually occurred.

The third and final attempt to grow the bacteria tried to ensure anaerobic conditions. An Erlenmeyer flask containing the culture was placed in a Gas-Pak^{tm} anaerobic jar along with reactants to create and anaerobic atmosphere and an anaerobic indicator strip. The entire system was placed in a drying oven at 82°C. Two cultures and one blank were run using this system. One culture used the ordinary growth media, while the other had both glucose and lignin in the media. It was hoped that visible bacterial colonies might grow on the solid lignin particles.

Microscopic Analysis. Samples were withdrawn from the culture flasks using a hypodermic syringe. The samples were dried on microscope slides, heat-set over a Bunsen burner, and Gram-stained. The samples were then examined under a microscope at 100x magnification.

RESULTS

Absorbance readings for the second culture attempt are shown in Table 1. Absorbance readings for the third attempt could not be made, because it was impossible to take samples without losing the anaerobic atmosphere in the Gas-Pak jar.

Table 1

Approximate Time (hrs)	Absorbance at 600 nm
19	0.11
24	0.22
47	0.28
53	0.37
66	0.46
72	0.50
93	0.62

DISCUSSION

The stated objective of this thesis was to determine if thermophilic bacteria could utilize lignin for growth. The experiment failed to meet that objective, however, because we were unable to determine whether the bacteria were growing or not. Increasing absorbance measurements were inconclusive because the absorbance of the media increased when heated even with no bacteria present. Examination of the culture under a microscope required the sample to be dried and heat-set, effectively killing any living cells present. Thus, even though bacteria were visible, it was impossible to tell if they had been active and growing.

The most difficult part of culturing these thermophillic bacteria is producing and maintaining an anaerobic atmosphere. The Gas-Pak system doesn't work well at high temperatures, and the sealed jar makes it impossible to take samples and still maintain an anaerobic atmosphere. With the three-neck boiling

flask, there is no way of guaranteeing an anaerobic atmosphere at all.

CONCLUSIONS

There was no conclusive evidence of bacterial growth in any of the cultures, nor was there certainty that they were not growing. Even if they did grow in the glucose media, we never got a chance to prepare a culture with lignin as the only carbon source. It's likely that the equipment and techniques available were not suitable for the growth and monitoring of these strict anaerobic bacteria, and a researcher with more sophisticated equipment could get more conclusive results.

RECOMMENDATIONS

In spite of the difficulties in growing anaerobic thermophilic bacteria, the possible industrial uses for their enzymes make further study of them worthwhile. Many processes in the paper industry, including pulping, bleaching, and starch conversion, could potentially benefit from a greater understanding of these bacteria.

Researchers interested in working with thermophiles should acquire a sealed anaerobic chamber that can hold all the necessary equipment. A three-neck boiling flask apparatus might work well if nitrogen or argon is constantly bled through the system to maintain an anaerobic atmosphere.

To detect and monitor growth, perhaps the best method would be to check for production of the carbon dioxide, hydrogen, and organic acids released by the bacteria as byproducts.

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